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BIOSYNTHESIS OF STEROIDS BY HUMAN FETAL ADRENAL PERFUSED *IN VITRO*

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Synopsis Formation of steroids in fetal tissue has been studied mostly by incubation technique. In the present experiment, human fetal adrenal obtained from two fetuses aborted at midtrimester were perfused *in vitro* with labeled precursors including ^{3}H-pregnenolone-sulfate and ^{14}C-dehydroepiandrosterone (DHA). Both adrenal and kidney wer perfused at the same time in this study. As perfusate, 100 ml of oxygenated type O blood diluted with saline was used. Perfusion was performed without recirculation. Radioactive metabolites extracted after the termination of perfusion from the perfusate and the tissue were hydrolysed, separated and isolated by Hyflo-Supercel partition column chromatography followed by thin layer chromatography. Radiochemical purity of the metabolites was achieved by recrystallization to constant specific activity. Perfusion of the fetal adrenal resulted in the formation of both labeled DHA and 16αOH-DHA, tritiated 16αOH-pregnenolone and 17αOH-pregnenolone. Since those metabolites were mainly found in the perfusate, it is suggested that the steroids formed in the tissue were easily released to the circulatory system.

Formation of 16α-hydroxylated steroids in the fetal adrenal and the role of those compounds on the formation of estriol during pregnancy are discussed.

Introduction

It has been recognized that a large amount of estrogen arises during pregnancy, and dehydroepiandrosterone (DHA) or/ and DHA-sulfate (DHA-S) has been reported as major precursors of estriol found in maternal urine during pregnancy. DHA or/and DHA-S secreted from fetal adrenal were 16α-hydroxylated in the liver and adrenal before aromatized in the placenta[10].

The presence of a series of enzymatic activities related to *de novo* steroidogenesis of neutral steroids, has been shown in the human fetal adrenals. Bloch and Benirschke[2)3)] demonstrated the formation of cholesterol, pregnenolone, DHA, androstenedione, 11β-hydroxyandrostenedione and cortisol by using ^{14}C-acetate as precursor in fetal adrenal. Among the steroids formed, DHA was found as a main metabolite. *De novo* synthesis of steroid sulfates such as DHA-S, pregnenolone-S and 17αhydroxy-pregnenolone-S (17α-OH-pregnenolone-S) has also been reported in *in vitro* study of fetal adrenal tissue[7)20)]. Those studies, however, have been carried out by incubation technique. Perfusion studies and injection of the steroid precursors into the vessels may be more representative of the biosynthetic pathways in the intact cell, while slices, minces and homogenates reflect the biochemical conversion of the cells whose architecture has been disrupted. And another merit of the perfusion study may be explained the distribution of the metabolites which is synthesized in the cells and transferred into the vessels.

In this study adrenal glands and kidneys were perfused with the precursors including tritium labeled pregnenolone-S and carbon 14 labeled DHA *in vitro*.

The radioactive metabolites isolated both

in the perfusate and in the tissue were studied. The role of the fetal adrenal gland regarding the steroid formation during the pregnancy was also discussed.

Materials and Methods

1. *Clinical Material and Perfusion Procedure*

Adrenals were obtained from human fetuses of 22 and 23 weeks gestation which were removed at therapeutic termination of pregnancy by cesarian section.

Following midline incision of the abdomen, arteries not essential to adrenal and kidney circulation were ligated. And then polyethylen tubes were cannulated into abdominal aorta and inferior vena cava and aortic arteries were ligated caudal and cranial of the bifurcation to the renal arteries in order to perfuse adrenal glands, but technically it was difficult to perfuse without kidney. Therefore, adrenal and kidney were prefused at the same time in this study.

Heparinized saline solution was immediately infused into abdominal aorta to wash out blood and including intrinsic steroids, and to prevent blood coagulation. The following surgical procedure was performed within 30 minutes after abortion.

As the perfusate, oxygenated type O blood diluted by heparinized saline was used. Fluid volume was 150 ml in this study. Perfusion was performed without recirculation with the condition of 37°C for 45 minutes. The perfusate was collected via vena cava.

2. *Steroids*

The following radioactive steroids were used; Pregnenolone-S (NET 167 Pregnenolone-7α-^{3}H-S, Ammonium salt, 20 Ci/mmol) and DHA (NEC-206 DHA-4-^{14}C, 58.8 mCi/mmol) obtained from New England Nuclear Co.. The radioactive steroids were purified by thin layer chromatography (TLC) prior to use and dissolved in 0.5 ml of ethanol and 0.5 ml of saline.

Non radioactive free steroids were obtained from IKAPHARM LTD., Israel. There purity was checked by TLC.

Acetylated compounds were also supplied from commercial source.

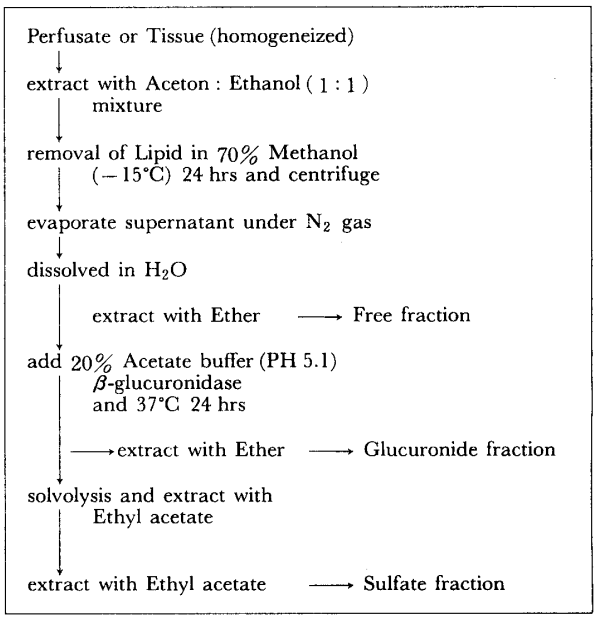
Beta gluculonidase was purchased from TOKYO ZOKI Chemical Corp.

All solvents were redistilled before use.

3. *Extraction and Hydrolysis*

As shown in Table 1, the extraction and hydrolysis of the perfusate and tissue were carried out according to the method described elsewhere[15]. Perfusate and tissue (homogenized)were extracted with mixture of acetone: ethanol (1:1) and evapolated. Extracted radioactive materials were dissolved in 70% ethanol and kept in −20°C overnight and centrifused 7,000 rpm, for 1 hour to remove lipid. Supernatant was evaporated to dryness under N_2 gas and the residue was dissolved in 100 ml of cold water.

Table 1. Extraction and Hydrolysis of Radioactive Metabolites in Perfusate and Tissue



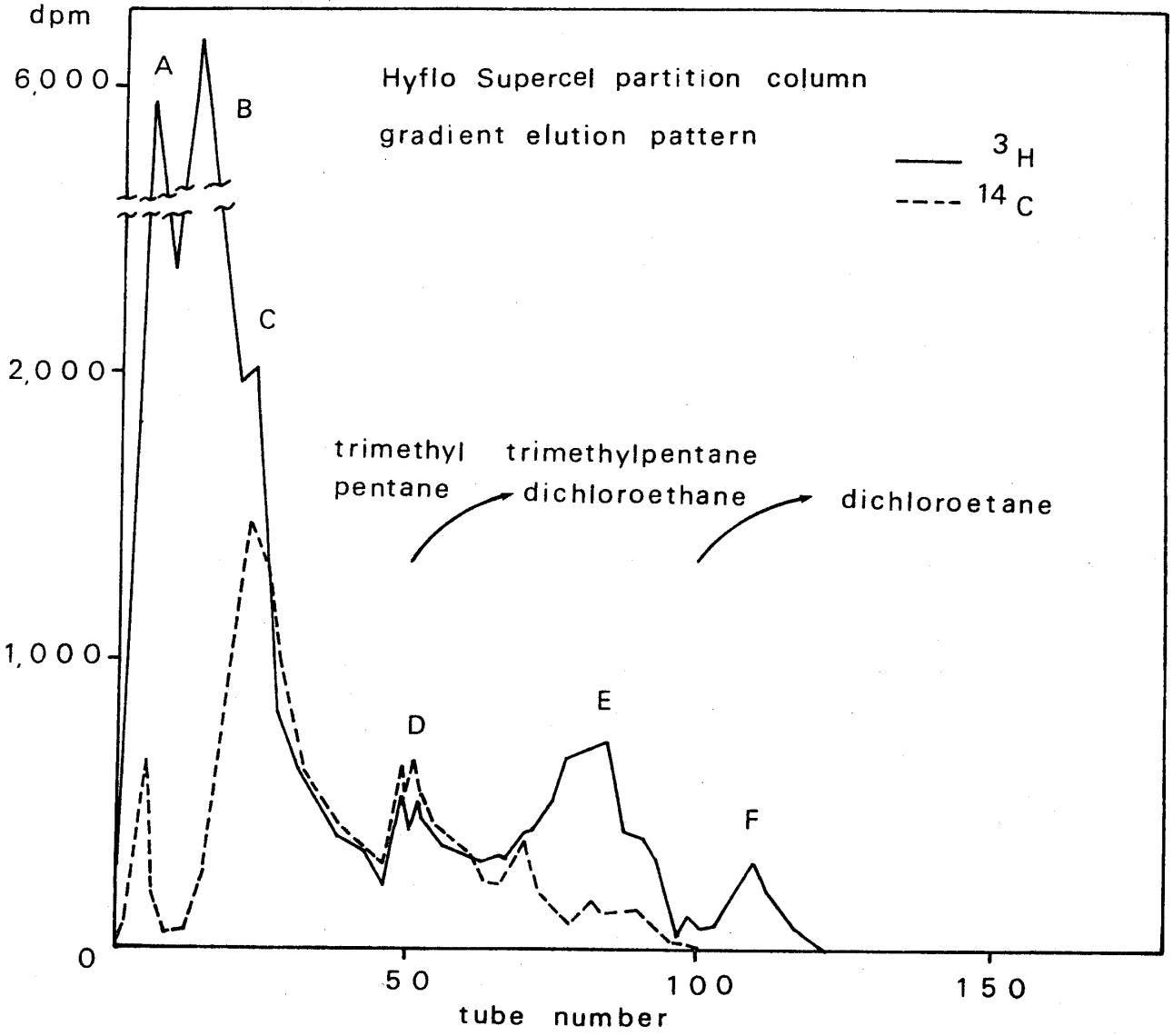
Free fraction was extracted with 300 ml (100×3) of ether. Conjugated materials were

hydrolysed by β-gluculonidase in acetate buffer (pH 5.1), and chloroform (30 ml) and extracted with ether ("gluculonide fraction"). The aqueous phase was then solvolyzed according to the method of Burstein and Lieberman and extracted with same volume of ethyl acetate ("sulfate fraction").

4. *Chromatography*

Each fraction was collected in one pool and was chromatographed on a Hyflo Supercel[4] was blended with 3 ml of lower (stationary) phase of the system: 2,2,4-trimethylpentane/ 90% methanol: 10% water. Using upper (mobile) phase the Hyflo Supercel was made into slurry and manually packed into a glass column. The sample absorbed on Hyflo Supercel was transfered to the column. Mobile phase was allowed to run through the column and 2 ml of each fraction was collected. After 50 fractions were collected, gradient elution was begun by introducing 2,2,4-trimethylpentane: dichloroehtane (1:1).

Fig. 1



After 100 fractions were collected, upper phase was changed to dichloroethane. Gradient elution was continued until additional 50 fractions. Six hundred μg of pregnenolone, DHA, 17α-OH-pregnenolone, Δ^5-androstenediol, 16α-hydroxy-pregnenolone (16αOH-pregnenolone) Δ^5-androstenetriol were added as standard carriers (Fig. 1).

The effluent fractions were allowed to evapolate in N_2 gas at room temperature. The residue was dissolved in 4 ml of absolute ethanol and aliquots were taken for counting and for measurement of carried steroids and metabolites.

Measurement of the "free" form of Δ^5-3β-hydroxysteroids was carried out by the method of Oertel and Eik-Nes[13], and estrogens were measured by fluorometer (TURNER Corp. Model 110).

The tubes from the gradient elution column containing carrier and its associate radioactive material were separated and purified by TLC at room temperature. The following systems were used;

No. 1 ethyl acetate: benzen (5: 1) 3 runs
No. 2 benzen : chloroform : cyclohexane : ethyl acetate: methanol (10: 5: 5: 2: 2: 1) 3 runs
No. 3 benzen: ethyl acetate (1: 1) 3 runs
No. 4 ethyl actate: cyclohexane: ethanol (5: 4: 1) 3 runs

Radioactive peaks on chromatogram were detected by Aloka Thin-Layer Chromatogram Scanner (Model JTC 226).

5. *Acetylation*

Steroids were acetylated with acetic anhydride in pyridin (1:1).

6. *Recrystallization*

Steroids purified by TLC were mixed with carrier steroids and recrystallized to constant specific activity using the solvent, ethanol, methanol, ethylacetate and methanol: chloroform (5: 1). An allowable limit for radiochemical purity was 5% of difference in the specific activity of the last two set of crystals and mother liquors. These criteria are in accordance with those used by Axelrod *et al.*[1].

7. *Measurement of Radioactivity*

Radioactive samples were counted in Aloka Tri-Carb Liquid Scintillation Spectrometer (Model Aloka LSC 653). Each sample was counted in 10 ml toluen containing 4 g/l PPO (2, 5-diphenyl-oxazole) and 100 ml/l dimethyl POPOP [1,4-bis-2-(4-methyl-5-phenyl-oxazole) benzen].

Results

1. *Recovery and Distribution of the Radioactivity*

Table 2 shows the precursors used in this

Table 2. Recovery of Radioactivities

Case		Initial count	Perfusate	Adrenal	Kidney	Total
I	^3H	4.95 μCi	34.7	1.3	5.2	41.2
	^{14}C	1.72 μCi	34.5	1.1	4.7	40.3
II	^3H	3.20 μCi	32.1	1.0	2.7	35.8
	^{14}C	1.40 μCi	23.5	0.7	1.6	25.8

Figures are expressed as percent of initial radioactivity

Table 3. Distribution of recovered Radioactivity after Hydrolysis

^3H-Pregnenolone-Sulfate

	Case	"Free"	"Glucuronide"	"Sulfate"	Total
Perfusate	I	25.0	3.9	71.0	100
	II	22.3	6.0	71.7	100
Adrenal	I	49.4	15.7	34.9	100
	II	39.6	36.3	24.1	100
Kidney	I	38.3	8.4	53.3	100
	II	42.7	11.1	46.2	100

^{14}C-DHA

	Case	"Free"	"Glucuronide"	"Sulfate"	Total
Perfusate	I	77.1	1.5	21.2	100
	II	52.5	9.3	38.2	100
Adrenal	I	52.1	3.3	44.6	100
	II	62.3	21.6	16.2	100
Kidney	I	25.7	6.8	67.6	100
	II	19.5	8.1	72.4	100

Figures are expressed as percent of recovered radioactivity

study and recovery of the radioactivity. Three to 5 μCi of tritiated pregnenolone-S and 1.4 to 1.7 μCi of carbon 14 labeled DHA were used in this perfusion study of fetal adrenal. The recovery found in the perfusate and tissues were 36–41% of tritieted material and 25–40% of carbon labeled material administered. Most of these radioactivities were found in the perfusate. In the tissues, 0.7–1.3% of radioactivities were found in the adrenal and 1.6–5.6% were in the kidney, respectively. More radioactivities were found in the kidney than in the adrenal.

Table 3 shows the distribution of recovered radioactivities after hydrolysis.

In the perfusate, it is interesting to note that 22–25% of the tritiated materials administered as sulfate form was recovered in the "free fraction", and 21–38% of the recovered carbon 14 material which was administered as free form was found in the "sulfate fraction". Relatively high tritiated radioactivities were in the "free fraction" recovered from the tissue and 39.4–49.4% from the adrenal.

In the tissue, though the total radioactivities were lower than the perfusate, relatively high percentage of recovered tritiated materials was in the "conjugate fraction". On the other hand, approximately 40% and more than 70% of the recovered carbon 14 materials were found in the "conjugate fraction" in the adrenal and in the kidney. These results suggest that the adrenal and kidney have both sulfatase and sulfokinase activities.

2. *Identification of the Metabolites*

Fig. 1. shows the elution pattern of the radioactivities extracted from perfusate on Hyflo Supercel.

Five radioactive peaks were observed. Peak B and C were collected and further separated by TLC. These radioactive peaks corresponded to the authentic pregnenolone and DHA in the using system No. 1. Peak E was collected and developed on TLC using the system No. 2 and 3. The radioactive peaks corresponded to the authenitc steroids.

Table 4 and 5 shows the conversion

Table 4. Conversion of ^{3}H-Pregnenolone-sulfate and ^{14}C-DHA in fetal adrenal

Case I

Metabolite		Perfusate (dpm)	Adrenal (dpm)	Kidney (dpm)
Pregnenolone	^{3}H*	391,200	19,400	94,900
	^{14}C	—	—	—
17α OH-Pregnenolone	^{3}H	5,800	2,150	2,850
	^{14}C	—	—	—
16α OH-Pregnenolone	^{3}H	10,900	700	900
	^{14}C	—	—	—
DHA	^{3}H	33,400	3,000	1,000
	^{14}C*	258,800	10,400	40,500
16α OH-DHA	^{3}H	10,200	—	—
	^{14}C	5,600	—	—
Δ^{5}-Androstenediol	^{3}H	3,200**	—	500*
	^{14}C	2,400	—	5,800
Δ^{5}-Androstenetriol	^{3}H	—	—	600
	^{14}C	—	500	—

* unchanged

— negligible radioactivity

** not recrystallized

Table 5. Conversion of ^{3}H-Pregnenolone-sulfate and ^{14}C-DHA in fetal adrenal

Case II

Metabolite		Perfusate (dpm)	Adrenal (dpm)	Kidney (dpm)
Pregnenolone	^{3}H*	112,400	43,600	18,500
	^{14}C	—	—	—
17αOH-Pregnenolone	^{3}H	—	—	600
	^{14}C	—	—	—
16αOH-Pregnenolone	^{3}H	1,340	700	—
	^{14}C	—	—	—
DHA	^{3}H	—	121,200	—
	^{14}C*	208,400	1,700	5,600
16αOH-DHA	^{3}H	3,400	—	—
	^{14}C	2,260	—	—
Δ^{5}-Androstenediol	^{3}H	—	—	—
	^{14}C	—	3,600	800
Δ^{5}-Androstenetriol	^{3}H	—	—	—
	^{14}C	—	—	—

* unchanged — negligible radioactivity

of ^{3}H-pregnenolone and ^{14}C-DHA in fetal adreanl in Case 1 and 2. Tritiated DHA was isolated as a main metabolite from pregnenolone-S. Sixteen-α-hydroxy-DHA (16α-OH-DHA), 16α-OH-pregnenolone and 17α-OH-pregnenolone were also isolated and identified in the perfusate. Carbon 14 16α-OH-DHA was also isolated and identified as the only metabolite derived from DHA. In the adrenal, ^{3}H-DHA was also identified. Both labeled Δ^{5}-androstenediol was isolated, but the radioactive purity of the metabolite was not obtained. Crystalline authentic steroid was added to each of the radioactive steroids isolated, and they were successively recrystallized until constant spe-

Table 6. Radiochemical purity of metabolites in perfusion study of fetal adrenal tissue with ^{3}H-Pregnenolone-S and ^{14}C-DHA

(Case I)

Steroid		Specific activity (dpm/mg)		
		1st cryst.	2nd cryst.	3rd cryst.
Pregnenolone (S)	^{3}H*	—	—	—
	^{14}C	—	—	—
17αOH-Pregnenolone	^{3}H**	141	144	137
	^{14}C	—	—	—
16αOH-Pregnenolone	^{3}H	304	293	280
	^{14}C	—	—	—
DHA	^{3}H**	944	953	942
	^{14}C*	6,297	6,246	6,281
16αOH-DHA	^{3}H	364	354	351
	^{14}C	209	194	207

— not identified

* unchanged

** recrystallized as acetate

Table 7. Radiochemical purity of metabolites in perfusion study of fetal adrenal tissue with ^{3}H-Pregnenolone-S and ^{14}C-DHA

(Case II)

Steroid		Specific activity (dpm/mg)		
		1st cryst.	2nd cryst.	3rd cryst.
Pregnenolone (S)	^{3}H*	6,735	6,410	6,244
	^{14}C	—	—	—
17αOH-Pregnenolone	^{3}H	—	—	—
	^{14}C	—	—	—
16αOH-Pregnenolone	^{3}H	—	—	—
	^{14}C	—	—	—
DHA	^{3}H	—	—	—
	^{14}C*	15,362	15,303	15,452
16αOH-DHA	^{3}H	237	223	222
	^{14}C	146	146	150

— not identified

* unchanged

cific activity was obtained (Table 6 and 7).

Discussion

A functional role in steroidogenesis in the human fetal adrenal during pregnancy has long been favoured because of the relatively large size of these glands and the characteristic fetal zone present in this tissue. Most of the previous investigations, however, were performed using homogenized or sliced tissue preparation. In the present study, the steroid metabolism was firstly demonstrated using the perfusion method.

Two midterm human fetal adrenals were perfused *in vitro* with ^{3}H-pregnenolone and ^{14}C-DHA. Relatively high percent of the ^{14}C-metabolites were recovered in the conjugate fraction suggest the strong sulfokinase activity in this tissue[7)19)]. These results were in agreement with the earlier report. However, because of the relatively high recovery of the ^{3}H-metabolites found in the free fraction also suggests the sulfatase activity in this tissue. Sulfatase activity has been demonstrated to be virtually absent in this organ. Villee and Loring incubated fetal adrenal homoganate and found that a small portion of DHA-S was converted to free steroids[17)]. The result of this perfusion study demonstrates a significant activity of steroid sulfatase in fetal adrenal.

A recent analysis of the pattern of steroids in human fetal adrenal tissue and cord plasma[8)] is in agreement with the metabolites of the present study. The metabolites derived from pregnenolone are 17α-OH-pregnenolone, 16α-OH-pregnenolone, DHA, 16α-OH-DHA and Δ^{5}-androstenediol which are considered to be high in concentration in cord plasma. Most of those metabolites were extracted in the perfusate. It is interesting to note that only a small amount of the metabolites was found in the tissue. These results suggest that the steroids formed in the tissue was easily released to the perfusate. However, it is not clear whether the steroids are released as free or sulfate form. Those problems have to be clarified in the future. The main metabolite formed in the perfusion study was DHA or it's sulfate. Pērez-Palacios *et al.*[14)], have demonstrated the direct conversion of pregnenolone-S to DHA-S in the incubation of fetal adrenal homogenate.

The capacity of 16α-hydroxylation in fetal adrenal gland is demonstrated in this perfu-

sion study. Both labeled 16α-OH-DHA and tritiated 16α-OH-pregenolone were isolated and identified in the perfusate. Interestingly, most of these 16α-hydroxylated metabolite were found in the perfusate but not in the tissue. According to Huhtaniemi *et al.*[5], analysed the steroid content by gas liquid chromatography and gas chromatography-mass spectrometry, concentration of 16α-OH-DHA-S was somewhat higher in adrenal than in liver. This previous study supports that 16α-hydroxylation was took place in fetal adrenal. *De novo* synthesis of 16α-OH-DHA has been reported by Yanaihara *et al.*[20]. Nakayama *et al.*[11][12]reported the conversion of estradiol to estriol, DHA to 16α-OH-DHA-S. Formation of 16α-hydroxy-pregnenolone from pregnenolone in fetal adrenal *in vitro* was also reported[16]. The formation of 16α-OH-DHA in fetal adrenal may be important mechanism for providing direct precursor for the biosynthesis of estriol by the placenta. Villee *et al.*[16], Yamasaki[18] and Shimizu, have demonstrated the formation of 16α-OH-pregnenolone from pregnenolone. Pērez-Palacios *et al.*[14], identified 16α-OH-DHA-S following incubation of fetal adrenal with pregnenolone-S. The present study is the first report to demonstrate the formation of 16α-OH-pregnenolone from pregnenolone-S. Sixteen-α-hydroxy-DHA and 16α-OH-pregnenolone or those sulfate were found relatively large quantities in cord blood. Since the identification of these 16α-hydroxylated compound was achieved following solvolysis, the evidence whether the conversion was took place with or without the cleavage of steroid sulfate is not clear in the present experiment.

Identification of 17α-OH-pregnenolone from pregnenolone-S suggests the possible pathway from pregnenolone to DHA which includes 17α-OH-pregnenolone. Same result has been reported previously by *in vivo* and *in vitro* studies.

The effect of fetal kidney on steroid metabolism in the present sutdy could not be roled out, since the adrenal was perfused with this organ. Huhtaniemi and Vihko[6] have demonstrated the enzyme activity related to the steroid metabolism in fetal kidney. They observed the 16α-hydroxylase and sulfokinase activities for DHA. Their results, however, confirmed the previous study by Matsumoto *et al.*[9] that pregnenolone was not 16α-hydroxylated by the fetal kidney. The possible role of fetal kidney on steroid metabolism during pregnancy is not apparent.

Though it has been well established that the fetal adrenal gland is the main source of androgen (DHA and its sulfate), the present *in vitro* perfusion study further demonstrated the biosynthesis of DHA or its sulfate and 16α-hydroxylated C-19 steroids in the adrenal and they were readily released to the circulatory system for estrogen especially estriol formation in the placenta. The significance of the formation of 16α-OH-pregnenolone in this tissue is not clear.

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