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Metabolism of Pregnenolone-4-^{14}C and Pregnenolone-7α-^{3}H Sulfate by the *Macaca mulatta* Fetal Adrenal *in Vitro*[1]

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These studies were initiated to ascertain the feasibility of utilizing the fetal monkey adrenal as a model for further studies on comparative steroid metabolism at various periods of gestation. Homogenates of midtrimester fetal monkey (*Macaca mulatta*) adrenals were incubated simultaneously with pregnenolone-4-^{14}C and pregnenolone-7α-^{3}H sulfate. Conversion of both substrates to 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, 11β-hydroxyandrostenedione, and cortisol as well at 17α-hydroxypregnenolone sulfate and dehydroepiandrosterone sulfate was demonstrated. Conversion of pregnenolone to progesterone was also shown. Neither free nor conjugated 16α-hydroxypregnenolone or 16α-hydroxydehydroepiandrosterone were found.

These studies demonstrate the capacity of the midtrimester fetal monkey adrenal to convert these free and sulfurylated steroid substrates to other Δ^5-3β-hydroxysteroids and Δ^4-3-ketosteroids, as well as to other Δ^5-3β-hydroxysteroid sulfates.

The human fetal adrenal has the capacity to sulfurylate neutral (Bolté *et al.*, 1966; Pion *et al.*, 1967; Solomon *et al.*, 1967; Mancusco *et al.*, 1967) and phenolic (Diczfalusy *et al.*, 1961) steroids, and to directly metabolize steroid sulfates to other steroid sulfates (Pérez-Palacios *et al.*, 1968). A relative lack of human fetal adrenal 3β-hydroxysteroid dehydrogenase activity with pregnenolone (P)[4] as substrate has also been reported (Villee *et al.*, 1962; Solomon *et al.*, 1967). To study

[1] Portions of this study were presented at a meeting of the Society for Gynecologic Investigation, Denver, March, 1969.

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[4] The following trivial names and abbreviations have been used: pregnenolone (P), 3β-hydroxy-5-pregnen-20-one; pregnenolone sulfate (PS), 20-oxo-5-pregnen-3β-yl sulfate; dehydroepiandrosterone (DHA), 3β-hydroxy-5-androsten-17-one; androstenedione (Δ^4A), 4 androstene-3,17-dione: dehydroepiandrosterone sulfate (DHAS), 17-oxo-5-androsten-3β-yl sulfate; 17α-hydroxypregnenolone (17 OHP), 3β,17α-dihydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone sulfate (17 OHPS), 17α-hydroxy-20-oxo-5-pregnen-3β-yl sulfate; progesterone (Prog), 4-pregnene-3,20-dione; 17α-hydroxyprogesterone (17 OHProg), 17α-hydroxy-4-pregnene-3,20-dione; cortisol (F), 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; 16α-hydroxypregnenolone (16 OHP), 3β,16α-dihydroxy-4-pregnen-20-one; 16α-hydroxydehydroepiandrosterone (16 OHDHA), 3β,16α-dihydroxy-5-androsten-17-one; 16-ketoandrostenediol, 16-oxo-5-androstene-3β,17β-diol; 11β-hydroxyandrostenedione (11β OHA), 11β-hydroxy-4-androstene-3,17-dione; cortisone, 17α,21-dihydroxy-4-pregnene-3,11,20-trione; pregnanolone, 3β-hydroxy-5β-pregnan-20-one; specific activity (SA); column partition chromatography (CPC); paper chromatography (PC); holdback volume (HBV); dichloromethane ($MeCl_2$). Unless otherwise indicated the steroid sulfates were as ammonium salts.

steroid metabolism in the monkey fetus, and as part of a study to ascertain whether the monkey fetus might serve as a useful model in which to study possible changes in steroidogenesis with advancing gestational age, adrenal glands from midtrimester monkeys were simultaneously incubated with pregnenolone-4-^{14}C and pregnenolone-7α-^{3}H sulfate.

MATERIALS AND METHODS

Materials. Two male *Macaca mulatta* fetuses were obtained at hysterotomy[5] 98 days after insemination (late second trimester). The adrenals were removed, weighed, and immediately incubated. One fetus (experiment 1) weighed 118.4 g, the left and right adrenals weighing 77.3 and 62.5 mg, respectively. The other fetus (experiment 2) weighed 129 g, left and right adrenal tissue weighing 62.4 mg and 26 mg (incompletely removed), respectively. In both fetuses, cardiac activity was present at the time of adrenalectomy.

Pregnenolone-7α-^{3}H-sulfate, specific activity 16 Ci/mmole, obtained from New England Nuclear Corporation, was purified by CPC in system 1 (Table 1) and paper chromatography (PC) in system 5. Pregnenolone-4-^{14}C, specific activity 52.4 μCi/mmole, was purified in PC systems 6, 7, and 10.

All organic solvents were redistilled prior to use. Crystallization steroid standards were recrystallized and their chromatographic behavior was assessed prior to use. Cofactors were purchased from Sigma Chemical Company.

Incubation procedure. The adrenals of each fetus were homogenized separately in a Potter-Elvehjem all-glass homogenizer in 0.5 ml of a solution containing 21.2 ml 0.1 *M* phosphate buffer at pH 7.4, 20 ml 0.13 *M* NaCl, 0.8 ml 0.15 *M* KCl, 0.2 ml 0.15 *M* KH_2PO_4, 0.2 ml 0.7 *M* $MgSO_4 \cdot 7H_2O$, 0.46 ml 0.1 *M* sodium fumarate, and 9.0 ml 0.04 *M* nicotinamide.

The homogenates were incubated with 14 μCi pregnenolone-7α-^{3}H sulfate and 2 μCi pregnenolone-4-^{14}C as substrates. To each incubation was added ATP 37.5 mg/g, DL-isocitric acid, 38 mg/g, and isocitric dehydrogenase, 10 mg/g (1 mg converts 5.7 moles). Each cofactor was dissolved in phosphate buffer. The ratio of tissue to buffer solution was 1.2:1 (w:v) in the first experiment and 0.94:1 in the second.

The incubations were performed in a Dubnoff metabolic incubator for 3 hours at 37° using air as the gas phase. The reaction was terminated with 80% ethanol. Simultaneous incubation of a tissueless control was performed.

Extraction and partition. For each incubation, three extractions with 80% ethanol followed by two with absolute ethanol were performed. In experiment 2, 300 μg each of DHA, Δ^4A, P and F as well as 3 mg each of PS, DHAS, and 17 OHPS were added to allow correction for losses. Separation of "free" from "conjugated" steroids was then carried out in a countercurrent fashion using a dichloromethane–water partition with 3 separatory funnels and 6 lower phase transfers.

Chromatography. CPC was performed on Johns-Manville Celite No. 545 previously washed as described by Kelly *et al.* (1962) using the dry packing technique described by Siiteri (1963). The flow rate of the mobile phase through the column was maintained at approximately 1 HBV per hour. Fractions of 0.1–0.2 HBV were collected.

Descending PC was performed on Whatman No. 1 paper. All chromatography was carried out at 24 ± 2°. The chromatographic systems listed in Table 1 were used and will be referred to by number. Their utilization in experiment 2 is depicted in Figs. 1 and 2.

Estimation of steroids. The sulfate esters of the Δ^5-3β-hydroxysteroids were located and quantitated following CPC as described by Crépy and Rulleau-Meslin (1960).

On PC, methylene blue reagent (Crépy and Judas, 1960) was used to detect steroid sulfates, alkaline *m*-dinitrobenzene reagent (Savard, 1953) was used to detect 17-ketosteroids, and ultraviolet light (Dusza *et al.*, 1963) was employed for Δ^4-3-ketosteroids. The Δ^5-3β-hydroxysteroids were detected on paper with phosphomolybdic acid (Kritchevsky and Kirk, 1952). Quantification of unconjugated steroid was carried out using ultraviolet absorption for Δ^4A and F, using the method of Oertel and Eik-Nes (1959) for P, and *m*-dinitrobenzene for DHA. Radioactive samples were counted in an automated 3-channel Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3375). Each sample was counted in 10 ml of toluene containing 3 g/l PPO (2,5-diphenyloxazole) and 100 mg/l dimethyl POPOP (1 4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene) for a sufficient time to assure a counting error no greater than 3%. Conjugated steroids were first dissolved in 1 ml of methanol prior to the addition of the phosphor solution. One-tenth milliliter of NCS solubilizer (Nuclear-Chicago Corporation) was added to each of the vials used in the recrystallization of 17 OHPS. Appropriate

[5] Kindly performed by Dr. Kenneth Kirton, Upjohn Laboratories, Kalamazoo, Michigan.

TABLE 1

CHROMATOGRAPHIC SYSTEMS EMPLOYED

System No.	Chromatographic system
	Column partition chromatography
1	Isooctane, *t*-butanol, NH_4OH, H_2O, 12:20:1:19 (Siiteri *et al.*, 1963)
2	Isooctane, chloroform, *n*-butanol, methanol, 0.3 *M* pyridinium sulfate, pyridine, 40:20:4:20:19:1 (Calvin *et al.*, 1966)
3	Isooctane, chloroform, methanol, 0.3 *M* pyridinium sulfate, 2:3:2:2 (Calvin *et al.*, 1966)
4	Isooctane, chloroform, *n*-butanol, methanol, 0.3 *M* pyridinium sulfate, pyridine, 40:20:8:20:19:1 (Calvin *et al.*, 1966)
	Paper chromatography
5	Isopropyl ether, *t*-butanol, NH_4OH, H_2O, 6:4:1:9 (Schneider and Lewbart, 1959)
6	Heptane, methanol, H_2O, 5:4:1 (Bush, 1952)
7	Heptane, benzene, methanol, H_2O, 33:17:40:10 (Bush, 1952)
8	Toluene, methanol, H_2O, 2:1:1 (Bush, 1952)
9	Benzene, methanol, H_2O, 2:1:1 (Bush, 1952)
10	Toluene, isooctane, methanol, H_2O, 67:33:60:40 (Eberlein and Bongiovanni, 1955)
11	Toluene, propylene glycol (Zaffaroni and Burton, 1951)
12	Benzene, propylene glycol (Neher, 1964)
13	Ligroine, propylene glycol (Savard, 1954)
14	Hexane, formamide (Zaffaroni, 1953)
15	Cyclohexane, benzene 1:1, formamide (Neher, 1963)

quench corrections were performed using an internal standard technique. Counting efficiencies were monitored using standards prepared in the same manner as the samples.

Paper chromatograms were scanned for radioactivity using a Packard radioachromatogram scanner (Model 7201).

Derivative formation. Solvolysis was carried out using the method of Segal *et al.* (1960). Acetylation was performed with pyridine and acetic anhydride (2:1) at room temperature overnight. Saponification was carried out with 2.5% Na_2CO_3 for 48 hours. Conversion of ammonium salts of steroid sulfates to the pyridinium salts was carried out by the method of McKenna and Norymberski (1960).

Crystallization. Crystalline authentic steroid (10–25 mg) was added to each of the radioactive steroids isolated. Successive recrystallizations were carried out until constant SA was achieved.

RESULTS

The recovery of incubated radioactivity following the dichloromethane–water partition exceeded 97% in both experiments and the control. The distribution of radioactivity following the dichloromethane-water partition is shown in Table 2.

TABLE 2

DISTRIBUTION OF RADIOACTIVITY RECOVERED AFTER DICHLOROMETHANE-WATER PARTITION

	^3H (%)		^{14}C (%)	
Expt.	Aqueous	Organic	Aqueous	Organic
Expt. 1	95	5	35	65
Expt. 2	91	9	56	44
Control	98	2	2	98

After this partition, the aqueous fraction from experiment 1 was submitted to CPC in system 1. Two areas of radioactivity were observed, in HBV III-V and VI-VIII. Both areas were chromatographed in PC system 5. Subsequently, after solvolysis, further analysis was carried out in PC systems 6, 7, 8, 9, 10, 12, and 13. P, 17 OHP (as the acetate) and DHA were recrystallized until constant SA was achieved for both ^{14}C and ^3H. No material with chromatographic similarity to 16α-hydroxypregnenolone, 16α-hydroxydehydroepiandrosterone, or 16-ketoandrostenediol was found. The dichloromethane fraction was studied initially in PC system 7 and subsequently in PC systems 6, 7, 9, and 10–15. P, 17 OHP, 17 OHProg, DHA, Δ^4A and F were identified and recrystallized to constant $^3H/^{14}C$ after the addition of authentic carrier steroids. In the case of material identified as Prog, constant SA for ^{14}C only could be achieved. Material which behaved similarly to 11β-hydroxyandros-

tenedione carrier was pooled with similar material from experiment 2 for recrystallization.

In order to quantitate the steroid products isolated in experiment 1, known amounts of these steroids were added to the ethanol extract in experiment 2.

Aqueous Fraction

Following dichloromethane–water partition, the aqueous fraction of experiment 2 was chromatographed in CPC system 1. Two areas of radioactivity were observed in HBV's III-V and VI-VIII (Fig. 1). The radioactive material in the former area, after conversion from the ammonium salt to the pyridinium salt, was further chromatographed as outlined in Fig. 1. The radioactive material behaved in a similar manner to authentic PS in CPC system 2. After conversion to the ammonium salt, recrystallization to constant SA with additional crystalline PS was performed (Table 3). The radioactive material from HBV VI-VIII was converted to pyridinium salts and studied in CPC system 3. Two areas of radioactivity were found (HBV's I-IV and IV-VIII). The first of these, with mobility similar to carrier DHAS, was studied in CPC system 2. Here, the radioactivity was found in HBV II-V. Similar mobility was demonstrated for carrier DHAS. After adding additional DHAS, the material was recrystallized to constant SA for both ^{14}C and ^{3}H (Table 3). The radioactive material in HBV IV-VIII, having both ^{3}H and ^{14}C labels, the mobility of which was similar to carrier 17 OHPS, was placed in CPC system 4, in which it again behaved like carrier 17 OHPS (HBV IV-VII). It was reconverted to the ammonium salt, and recrystallized to constant SA (Table 3).

Dichloromethane Fraction

A detailed outline of the purification and identification procedure is presented in Fig. 2. Again, as in experiment 1, P, 17 OHP, 17 OHProg, DHA, Δ^4A and F were identified bearing both ^{14}C and ^{3}H labels. These steroids were recrystallized to constant SA with added authentic steroid car-

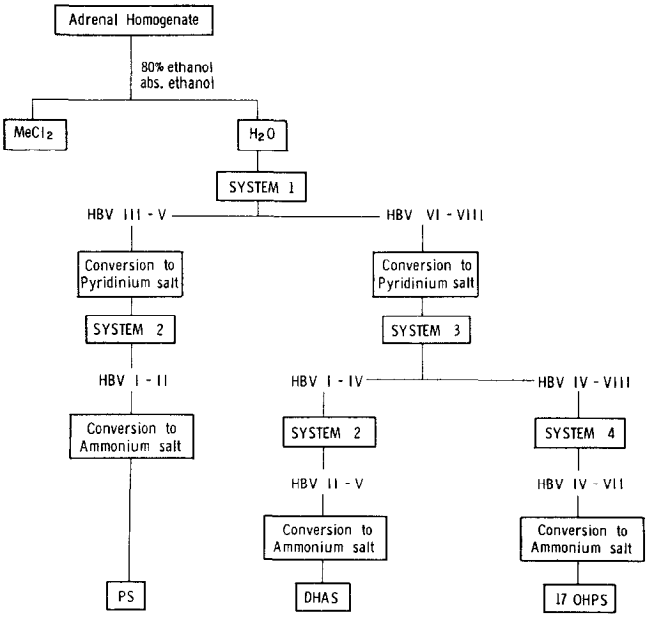

FIG. 1. Procedures employed in analysis of aqueous fraction of fetal monkey adrenal incubated with pregnenolone-4-^{14}C and pregnenolone-7α-^{3}H sulfate.

TABLE 3
RECRYSTALLIZATION DATA: CONJUGATED METABOLITES IN FETAL MONKEY ADRENAL INCUBATED WITH PREGNENOLONE-4-^{14}C AND PREGNENOLONE-7α-^{3}H SULFATE

Solvent	Successive crystallizations[a]	^{3}H (dpm/mg)	^{14}C (dpm/mg)	^{3}H/^{14}C
	Pregnenolone sulfate			
	SM	6820	294	23.2
Methanol	C_1	7144	324	22.0
Ethanol	C_2	6851	303	22.6
Methanol	C_3	6359	300	22.2
Ethanol	C_4	6918	311	21.9
	ML_4	6822	298	22.9
	17α-Hydroxypregnenolone sulfate			
	SM	4912	1137	4.3
Methanol	C_1	5576	1325	4.2
Methanol	C_2	5614	1390	4.0
Methanol	C_3	5239	1312	4.1
	ML_3	5466	1331	4.1
	Dehydroepiandrosterone sulfate			
	SM	793	354	2.2
Methanol	C_1	1010	742	1.4
Methanol	C_2	756	699	1.1
Methanol	C_3	529	653	0.8
Methanol	C_4	631	776	0.8
	ML_4	643	776	0.8

[a] SM = starting material, C = crystal; ML = mother liquor.

rier (Table 4). Since most of the radioisotopic F was ^{14}C-labeled, this may have influenced the ^{3}H counts. Constant SA could be achieved only for ^{14}C in Prog recrystallization. Constant SA for both ^{14}C and ^{3}H was achieved for the 11β-hydroxyandrostenedione identified in both experiments in small amounts and combined for recrystallization. In addition, polar radioactivity from PC system 7 which had a mobility similar to cortisone was found in PC system 9. However, with added authentic cortisone, recrystallization to constant SA could not be achieved. In the same system 9 chromatogram, the least polar area of radioactivity ($R_f = 0.8$) had similar mobility to 16 OHP when submitted to PC systems 10 and 12. In PC system 11, however, the radioactive material was less polar than added authentic 16 OHP ($R_f = 0.28$ vs $R_f = 0.43$). In PC system 10 and again in PC system 12, an area of radioactivity similar to 16 OHDHA was found. However, in PC system 11 the radioactive material was less polar than authentic 16 OHDHA ($R_t = 0.57$ vs $R_t = 0.91$). In PC system 12, a less polar area ($R_t = 0.73$) was found which had mobility similar to 16-ketoandrostenediol. The radioactivity and added authentic carrier behaved similarly ($R_f = 0.45$) in PC system 10; too few counts were found to permit further study. The least polar radioactive material in the initial PC system 7, in addition to containing radioactivity which was recrystallized with Prog to constant SA for ^{14}C, contained radioactivity which moved in PC system 14 and subsequently in PC system 6 similarly to pregnanolone. Recrystallization to constant SA with added authentic pregnanolone was attempted, but could not be achieved.

The relative incorporation of P and PS into identified metabolites in experiment 2 is shown in Fig. 3.

DISCUSSION

The studies described herein demonstrate that the midtrimester fetal monkey adrenal has the capacity to convert both the free and sulfurylated form of pregnenolone to other unconjugated Δ^5-3β-hydroxysteroids and Δ^4-3-ketosteroids, as well as to other Δ^5-3β-hydroxysteroid sulfates. Thus the conversion of P and PS to 17 OHP, 17 OHProg, DHA, Δ^4A, 11βOH A and F as well as 17α OHPS and DHAS has been shown. Additionally, conversion of P to Prog was demonstrated.

In human pregnancy, the role of steroid sulfates in the synthesis of estrogens has been demonstrated (Siiteri and MacDonald, 1966). There is accumulating evidence in other endocrine organs that steroid sulfates may be utilized both as precursors of active unconjugated steroids

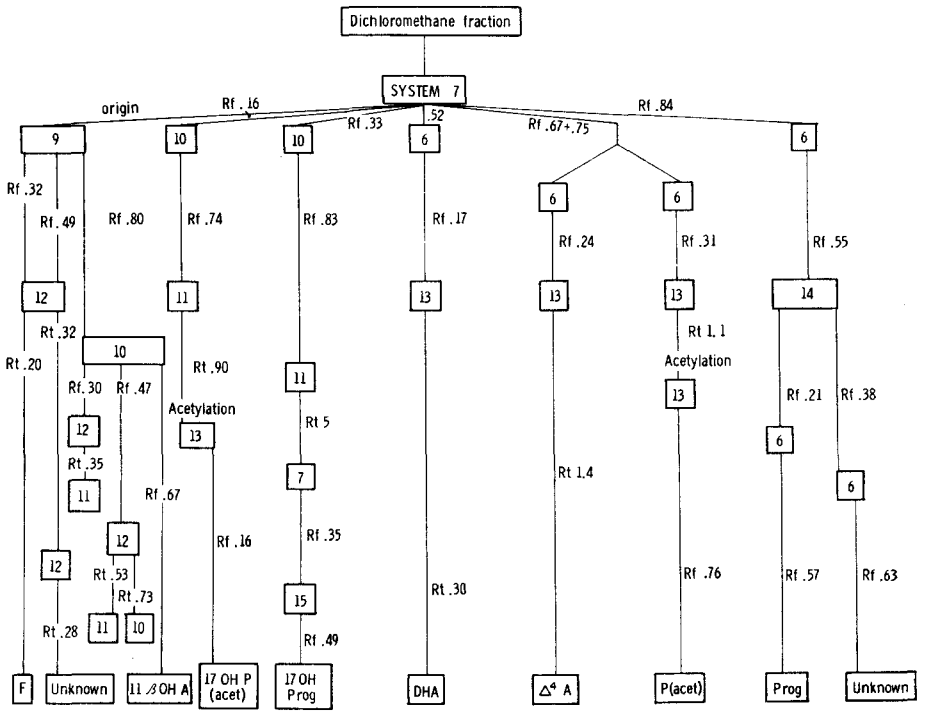

FIG. 2. Procedures employed in analysis of dichloromethane fraction of fetal monkey adrenal incubated with pregnenolone-4-^{14}C and pregnenolone-7α-^{3}H sulfate.

(Dixon *et al.*, 1965; Aakvaag *et al.*, 1964; O'Malley *et al.*, 1967; Pérez-Palacios *et al.*, 1969) and as substrates for direct metabolism to other steroid sulfates (Calvin and Lieberman, 1966). We have also described the conversion of PS to Δ^5-3β-hydroxysteroid sulfates in the human fetal adrenal (Pérez-Palacios *et al.*, 1968). The present study demonstrates that the fetal monkey adrenal had the capacity to form free and sulfurylated steroids from neutral steroid sulfate precursors. The ability to hydrolyze PS and to form free 3β-hydroxysteroids and Δ^4-3-ketosteroids in the fetal monkey adrenal is in contrast to our earlier *in vitro* (Pérez-Palacios *et al.*, 1968) and *in vivo* (unpublished) studies of the human fetal adrenal in which little sulfatase or 3β-hydroxysteroid dehydrogenase activity could be demonstrated with P and PS as substrates.

Interpretation of the relative conversion of these two substrates is limited both by the *in vitro* nature of these studies and by the relative excess of free steroid substrate. Also, the extent to which the 17 OHPS and DHAS were formed directly from PS as opposed to prior formation of the free alcohol cannot be ascertained from these experiments.

Circulating 16 hydroxylated neutral steroids have been shown to be efficient precursors of placental estriol (Dell'Acqua *et al.*, 1967) in human pregnancy. Sixteen hydroxylation of DHA has been shown to occur in the human fetal liver (Slaunwhite *et al.*, 1965) and of DHAS in the adrenal (Pérez-Palacios *et al.*, 1968). Our inability to demonstrate 16 hydroxylation in these midtrimester fetal monkey experiments is similar to the experience of Solomon *et al.* (1967), who found no 16 hydroxylation following injection of Prog into the umbilical circulation of the rhesus monkey near term. Heinrichs and Colas (1970) have been unable to demonstrate 16-hydroxylation of DHA in fetal monkey liver microsomes. This deficiency in 16-hydroxylase activity may, in part, explain the markedly lower maternal urinary estriol excretion in the

TABLE 4

RECRYSTALLIZATION DATA: UNCONJUGATED METABOLITES IN FETAL MONKEY ADRENAL INCUBATED WITH PREGNENOLONE-4-^{14}C AND PREGNENOLONE-7α-^{3}H SULFATE[a]

Solvent		^{3}H (dpm/mg)	^{14}C (dpm/mg)	^{3}H/^{14}C	Solvent		^{3}H (dpm/mg)	^{14}C (dpm/mg)	^{3}H/^{14}C
	Pregnenolone					*Progesterone*			
	SM	1564	3770	0.41		SM	286	284	1.01
Methanol	C_1	867	3927	0.22	Methanol, Ligroine	C_1	190	243	0.78
Ethanol	C_2	846	4094	0.21					
Methanol	C_3	881	4060	0.22	Ethanol, Ligroine	C_2	147	234	0.63
Ethanol	C_4	839	3888	0.22					
Methanol	C_5	811	4218	0.20	Methanol, Ligroine	C_3	129	239	0.54
	ML_5	822	4112	0.20					
					Ethanol, Ligroine	C_4	109	218	0.50
	17α-Hydroxypregnenolone					ML_4	275**	257	1.07
	SM	1682	2254	0.75					
Methanol	C_1	1705	2223	0.77		*17α-Hydroxyprogesterone*			
Ethanol	C_2	1662	2256	0.74		SM	752	1420	0.50
Methanol	C_3	1643	2258	0.73	Methanol	C_1	752	1452	0.50
	ML_3	1633	2189	0.75	Ethanol	C_2	718	1425	0.50
						ML_2	708	1492	0.47
	Dehydroepiandrosterone					*Cortisol*			
	SM	781	2919	0.27		SM	338	2106	0.16
Methanol	C_1	774	2962	0.26	Methanol	C_1	164	2048	0.08
Ethanol	C_2	738	2855	0.26	Ethanol	C_2	127	1977	0.06
	ML_2	764	2947	0.26	Methanol	C_3	159	1885	0.08
					Ethanol	C_4	127	1927	0.07
						ML_4	148	1976	0.07
	Androstenedione					*11β-Hydroxyandrostenedione****			
	SM	714	2461	0.30		SM	559	1016	0.52
Methanol	C_1	620	2064	0.30	Methanol, Ligroine	C_1	360	736	0.49
Ethanol	C_2	620	2032	0.31					
Methanol	C_3	586	2064	0.28	Methanol, Ligroine	C_2	291	654	0.44
Ethanol	C_4	588	2036	0.29					
	ML_4	616	2059	0.30	Methanol, Ligroine	C_3	219	516	0.42
					Methanol, Ligroine	C_4	260	564	0.46
						ML_4	219	555	0.04

[a] SM = starting material, C = crystal; ML = mother liquor; * as the acetate; ** see text for details; *** derived from combination of radioactive material found in both experiments.

rhesus monkey (Hopper and Tullner, 1967) as compared to the human.

Comparison of the findings reported herein with our studies in the human fetus suggests that the monkey adrenal has a greater capacity to hydrolyze PS, greater 3β-hydroxysteroid-dehydrogenase activity, and minimal, if any, evidence of 16 hydroxylation. Both the monkey and human fetal adrenal have sulfokinase, 17α-hydroxylase, and 17-21-desmolase capacity.

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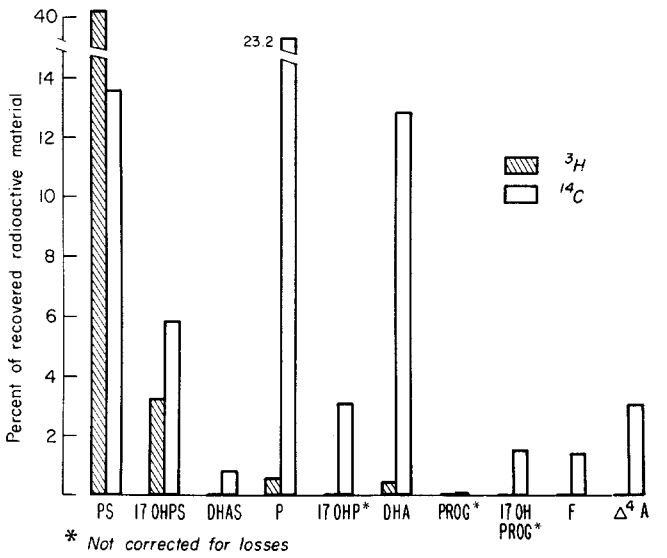

FIG. 3. Relative incorporation of pregnenolone-4-^{14}C and pregnenolone-7α-^{3}H-sulfate into identified metabolites in fetal monkey adrenal incubation.

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