IN VIVO STEROID BIOGENESIS AND METABOLISM IN THE HUMAN TERM PLACENTA
1. IN SITU PLACENTAL PERFUSION WITH ISOTOPIC PREGNENOLONE

Robert B. Jaffe, M.D. and William J. Ledger, M.D.

Department of Obstetrics and Gynecology
University of Michigan Medical Center
Ann Arbor, Michigan

Received May 24, 1966

ABSTRACT

Three human term placentas have been perfused *in situ* with labeled pregnenolone. Perfusions were performed at the time of elective repeat cesarean section, prior to the onset of labor. Placentas and venous effluents were separately analyzed. As is true in midtrimester placentas, rapid and extensive conversion to progesterone was effected by the placenta. In addition to progesterone, 6β-hydroxyprogesterone was identified.

The rapid and extensive *in situ* conversion of labeled pregnenolone[1)] to progesterone in the midterm human placenta has been demonstrated (1). The capacity for enzyme "maturation" in various human fetal and infant tissues both with advancing gestation and during the neonatal period has also been suggested (2, 3). The following experiment was designed in an effort to assess the qualitative and quantitative aspects of *in vivo* human placental steroidogenesis at term utilizing pregnenolone as substrate, as well as to develop a satisfactory model for the study of *in situ*

steroidogenesis in the human term placenta utilizing a variety of substrates.

METHODS AND MATERIALS

Perfusion Technique: Studies were carried out on three patients scheduled for elective repeat cesarean section at term, with no other medical or obstetric complication of the present pregnancy.

Under tetracaine hydrochloride spinal anesthesia, following delivery of the infant by cesarean section, a sterile infant feeding tube was inserted into one umbilical artery and an adult feeding tube into the umbilical vein. Citrated whole blood, typed and cross-matched against that of the mother, was infused into the umbilical arterial catheter. At one minute intervals, over a period of 7 minutes, 0.1 ml of a 50% ethanol in distilled water (v/v) solution of isotopic pregnenolone was injected into the arterial catheter. This was followed by 2 minutes of additional blood perfusion without further injection of steroid. The venous effluent was collected in a flask of absolute ethanol.

Materials: All organic solvents were redistilled prior to use. Crystalline steroid standards were recrystallized prior to use and corrected melting points determined.

Pregnenolone-7α-^{3}H, Lot No. 66-156A-19[2] with a specific activity (S.A.) of 31.6 mc/mg and pregnenolone-4-^{14}C, Lot No. 79-239A-48[2] with a S.A. of 14.5 mc/mg were purified utilizing thin-layer chromatography on Silica gel G[3] in the following solvent systems: cyclohexane: ethyl acetate: ethanol, 9: 9: 2 (Rf = 0.57) and benzene: ethanol, 9: 1 (Rf = 0.38). An aliquot of this purified material was then mixed with authentic carrier and recrystallized from ethanol and methanol. Initial recrystallization demonstrated agreement within 5% of that of the specific activity of the starting material.

The amount of pregnenolone-7α-^{3}H perfused (Experiments 1 and 2) was 5.0 and 5.8 μc, and that of pregnenolone-4-^{14}C (Experiment 3) was 2.0 μc.

Extraction and Purification: The umbilical venous effluent was collected dropwise in 200 ml of ethanol, which was constantly agitated. After completion of the perfusion, the placenta was manually removed, membranes and cord immediately dissected away and the placenta homogenized in 400 ml of ethanol. Further extraction with ethanol, and methanol precipitation were carried out as described by Mikhail et al. (4).

Separation of "free" from "conjugated" radioactive material was carried out in a countercurrent fashion using a dichloromethane-water partition, with three separatory funnels and six lower-phase transfers. The volume of each phase was 50 ml.

An outline of the extraction and identification procedures employed on the dichloromethane fraction is shown in Figure 1.

Twenty-four hour urine specimens were collected for three days following the procedure, and aliquots of each days urine assessed for their radioactive content.

Countercurrent Distribution (C.C.D.): In all cases, 24 transfer distributions were carried out in a manual Craig-Post apparatus using the following solvent systems:

No. 1. Cyclohexane, ethyl acetate, ethanol, water (7:3:5:5)
2. Petroleum ether, methanol, water (10:7:3)
3. n-Hexane, ethanol, water (25:12.1:12.9)
4. Methanol, water, carbon tetrachloride (4:1:5)
5. Cyclohexane, ethanol, water (10:9:1)
6. Methanol, water, chloroform, carbon tetrachloride (8:2:1:9)

Derivative Formation: Acetylation was carried out with acetic anhydride in anhydrous pyridine (1:2) at room temperature overnight. Formation of digitonides was performed utilizing the method of Frame (5) and of the bisthiosemicarbazone derivative utilizing the method of Pearlman and Cerceo (6), preparation No. 2.

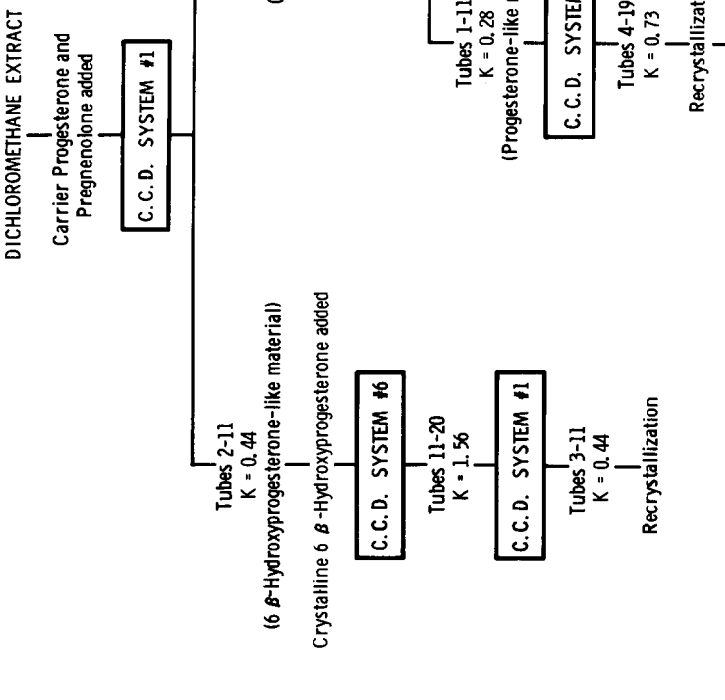

Figure 1: Extraction and identification procedures employed in analysis of dichloromethane extract of placenta and venous effluent following in situ perfusion with labeled pregnenolone.

Estimation of C-21 Steroids: The α, β-unsaturated 3-ketosteroids were detected on thin-layer chromatograms by ultraviolet light and measured by ultraviolet absorption at 240 mμ using the Allen correction formula (7). For detection of 3β-hydroxy-Δ_5-steroids on thin-layer chromatograms, a phosphomolybdic acid spray was employed, and their measurement was carried out by the method of Oertel and Eik-Nes (8).

Recrystallization to Constant Specific Activity: Appropriate carrier steroid was added to the isolated radioactive material derived from the final countercurrent distribution. For each isolated compound, successive recrystallizations from at least three different solvent systems were performed. The carrier was estimated gravimetrically.

Counting Procedure: Counting was carried out in an automated two channel Packard Tri-Carb liquid scintillation counter (series 3000). The tritium window was set from 10 to 100% of the analyzer full scale with a relative gain of 60, and the carbon window was set from 10 to 70% of full scale with a relative gain of 5. Samples were counted in 10 ml toluene containing 3.0 g/l PPO (2,5-diphenyloxazole) and 100 mg/l POPOP (1, 4-bis-2-(4-methyl-5-phenyl-oxazolyl) benzene). Urine samples (0.5 ml) were first dissolved in one ml methanol and then 10 ml of the above toluene scintillant solution added. Appropriate quench corrections were performed using an internal standard technique. Counting efficiencies were monitored utilizing standards prepared in the same manner as the samples.

RESULTS

As seen in Table 1, 47.7 to 66.3 percent of the perfused radioactivity was recovered from the three sources in which it was measured.

Table 2 illustrates the distribution of "free" (dichloromethane-soluble) and "conjugated" (water-soluble) metabolites in the placentas and venous effluents. It can be seen that 0.3 to 5.0 percent

Table 1

Recovery of Injected Radioactivity Following *in situ* Perfusion of Human Term Placentas with Labeled Pregnenolone.

Experiment	Amount Injected (μc)	Placenta (d.p.m.)	Venous Effluent (d.p.m.)	Maternal Urine (d.p.m.)	Percent Injected dose
1	5.0	2,081,032	679,611	2,528,690	47.7
2	5.8	5,751,597	158,894	2,464,209	66.3
3	2.0	728,423	1,105,879	732,088	59.3

Table 2

Distribution of Dichloromethane-Soluble and Water-Soluble Radioactive Material Following *in situ* Perfusion of Human Term Placentas with Labeled Pregnenolone.

	Placenta			
	Dichloromethane Soluble		Water Soluble	
Experiment	D.P.M.	Percent of Total		Percent of Total
1	2,061,755	99.1	19,277	0.9
2	5,463,440	95.0	288,157	5.0
3	551,952	99.7	1,765	0.3
	Venous Effluent			
1	646,311	95.1	33,300	4.9
2	135,470	85.3	23,424	14.7
3	1,097,568	99.2	8,311	0.8

of the radioactive material in the placenta, and 0.8 to 14.7 percent of that in the venous effluent could not be removed by dichloromethane from the aqueous phase. The material which remained in the aqueous phase was not analyzed further.

The distribution of the major radioactive components in the "free" material in both placenta and venous effluent found after perfusion with pregnenolone-7α ^{3}H and pregnenolone-4-^{14}C is presented in Table 3. Based on calculations derived from the final countercurrent distributions, approximately 60 to 84 percent of the radioactive material present in the "free" fraction of placentas was recovered as progesterone, only 3 to 30 percent as unchanged pregnenolone. In addition, between 17 and 34 percent of the radioactive material recovered from the venous effluent was progesterone and 53 to 73 percent as pregnenolone. 18.6, 26.1 and 10.3 percent of the total injected dose of the pregnenolone perfused was recovered as progesterone in each of the three experiments.

Table 3

Distribution of progesterone and unchanged pregnenolone in placentas and venous effluents expressed as percentage of recovered radioactivity.

Exp.	Placenta		Venous Effluent	
	Progesterone Percent	Pregnenolone Percent	Progesterone Percent	Pregnenolone Percent
1	82.6	3.4	33.8	52.8
2	60.1	29.6	16.8	64.7
3	84.1	6.8	22.9	73.4

The recovery data presented for progesterone and pregnenolone are derived from the final countercurrent distributions since, on subsequent recrystallization there was no appreciable decrease in S.A. from the anticipated values. However, upon recrystallization of 6β-hydroxyprogesterone, a significant drop was noted. Because of a relative paucity of radioactive material in the 6β-hydroxyprogesterone-like fraction, material from this fraction in both placenta and venous effluent was pooled in experiments 1 and 2 prior to recrystallization. Based on the final countercurrent and recrystallization data, and assuming a corresponding decrease in S.A. in both placenta and venous effluent, the 6β-hydroxyprogesterone represented approximately 1.9, 1.9, and 1.5 percent of the radioactive material recovered from the placentas and 2.1, 3.7, and 0.4 percent of that from the venous effluents.

As noted in Fig. 1, identification of progesterone, 6β-hydroxyprogesterone and precursor pregnenolone was based on distribution in multiple countercurrent systems. In each instance, agreement was noted with the authentic carrier substance. Following countercurrent distribution, recrystallization to constant S.A. was effected. The data on recrystallization of

the starting pregnenolone (as the acetate), progesterone and 6β-hydroxyprogesterone is presented in Table 4.

Table 4

Recrystallization Data (c.p.m./mg) derived from in situ human term placental perfusion with labeled pregnenolone.

Progesterone

Solvent	Experiment 1 Placenta	Experiment 1 Venous Effluent	Experiment 2 Placenta	Experiment 3 Placenta + Venous Effluent
Methanol	C_1 2632	C_1 968	C_1 3022	C_1 2422
Ethanol	C_2 2569	C_2 960	C_2 2905	C_2 2485
Acetone/Hexane	C_3 2546	C_3 937	C_3 2849	C_3 2407
	ML 2522	ML 938	ML 2935	ML 2395

6β-Hydroxyprogesterone

Solvent	Experiment 1 Placenta + Venous Effluent	Experiment 2 Placenta + Venous Effluent	Experiment 3 Placenta
Methanol	C_1 517	C_1 450	C_1 224
Ethanol	C_2 277	C_2 268	C_2 204
Acetone/Hexane	C_3 232	C_3 237	C_3 208
Acetone/Hexane	C_4 233	C_4 238	C_4 203
	ML 229	ML 259	ML 198

Pregnenolone Acetate

Solvent	Experiment 1 Placenta + Venous Effluent	Experiment 2 Placenta + Venous Effluent	Experiment 3 Placenta + Venous Effluent
Methanol	C_1 984	C_1 999	C_1 5340
Ethanol	C_2 982	C_2 1000	C_2 5388
Acetone/Hexane	C_3 944	C_3 986	C_3 5309
	ML 942	ML 1007	ML 5112

(C = Crystal, ML = Final Mother Liquor)

Additionally, as seen in Fig. 1, following recrystallization, the bisthiosemicarbazone derivative of progesterone was formed and redistributed in C.C.D. System No. 4. Again, excellent agreement with carrier was noted.

DISCUSSION

Like the midtrimester placenta, the human term placenta has the capacity of converting the bulk of perfused pregnenolone to progesterone *in vivo* and of secreting the progesterone so formed into the fetal compartment. Also similar to the situation obtaining in the midtrimester placenta, this progesterone formation is both rapid and extensive.

The term placenta also has the capacity of converting circulating cholesterol to pregnenolone (9). In addition, there is extensive desulfurylation of pregnenolone sulfate, perfused *in situ* to the human term placenta (10), and conversion of the bulk of the pregnenolone so formed to progesterone. Thus the relative contribution to placental progesterone production by 1) pregnenolone produced in the placenta from circulating cholesterol, 2) circulating pregnenolone sulfate (which has been demonstrated in both pooled peripheral plasma (11) and umbilical

cord blood (12) and 3) "free" circulating pregnenolone (which could not be found in umbilical cord blood although free 17α-hydroxypregnenolone was detected (12)), remains to be ascertained.

The finding by Cassmer (13) of only slight diminution of urinary pregnanediol excretion following interruption of umbilical circulation at midpregnancy, coupled with similar pregnanediol excretion patterns in pregnancies marked by anencephalic fetuses (14) or intrauterine death (15-17), suggests that the progesterone precursor contribution from the fetal compartment is limited. Further, the in vitro demonstration of conversion of acetate and mevalonate to squalene and cholesterol (18, 19) and of cholesterol to pregnenolone and progesterone (20, 21) leaves unresolved the relative contributions of de novo glandular synthesis and circulating precursors to the placental elaboration of progesterone.

Coupled with data pertaining to the fate of dehydroepiandrosterone and its sulfate in pregnancy (22-25), the findings from the human term placental perfusions described above suggest utilization of a variety of circulating Δ^5 3β hydroxylated compounds

in placental steroidogenesis.

Recent studies by Bird et al. (26) have demonstrated that in the seventeenth to twenty-first weeks of pregnancy, some progesterone circulating in the fetal compartment is converted to a variety of free and conjugated steroids, including corticosteroids in the fetal adrenal gland. Whether this utilization of placental progesterone by the fetus occurs *in vivo* at term remains to be ascertained.

Incubations of term placental tissue have demonstrated 6β- (27), 16α- (28, 17α- (29) and 20α- (30) hydroxylation of progesterone when labeled progesterone was used as substrate. Desmolase activity, with conversion of labeled progesterone to adrostenedione has also been demonstrated *in vitro* (28, 31). *In vitro* placental perfusion of progesterone also demonstrated formation of 6-ketoprogesterone (28).

In addition, a variety of progesterone metabolites have been isolated from human placentas. These include 20α- and 20β-hydroxy-pregn-4-ene-3-one, pregnanediol, 3β hydroxy-5-α-pregnane-20-one and 5α-pregnane-3β, 20α-diol (29-31).

Following digitonin precipitation in the present

experiments, no precipitable radioactive material was found other than the precursor pregnenolone. This suggests that, under the conditions of these experiments, no 3β-hydroxy-Δ^5 metabolites were present. Further, neither 16α- nor 17α- hydroxyprogesterone, androstenedione, nor 20α- or 20β-hydroxy-pregn-4-ene-3-one could be detected. In addition, because of the finding of placental 6α-hydroxylation of estrogens (36, 37) 6α-hydroxyprogesterone was also sought, but was not detected.

Non-digitonin precipitable radioactive material which had the mobility of 6β-hydroxyprogesterone in two countercurrent systems was detected. It was possible to further identify this material as 6β-hydroxyprogesterone by recrystallization to constant specific activity. However, as mentioned earlier, a significant decrease in specific activity from starting material to final crystals was observed. In addition, the countercurrent distribution curves of the 6β-hydroxyprogesterone-like material were "broader" than those of the carrier material. These observations suggest the presence of another metabolite with similar chromatographic mobilities to that of 6β-hydroxyprogesterone in the counter-

current systems utilized. Insufficient material precluded further analysis of this fraction. Six β-hydroxylation of progesterone has been reported after incubation with stromal and corpus luteal tissue from human ovaries (38) and hypertrophic and carcinomatous human prostatic tissue, (39) as well as in placental incubation (27). Increasing urinary excretion of 6-oxygenated metabolites of progesterone with advancing gestational age has been reported by Fotherby et al. (40). Zander (41), and Bird et al. (26) have demonstrated the human fetal capacity to form 6β-hydroxyprogesterone. Thus, both fetal and placental 6β-hydroxyprogesterone may contribute to this 6-oxygenated metabolite pool.

ACKNOWLEDGMENTS

This study was supported in part by U.S.P.H.S. Grant No. 01776 from The National Institute of Child Health and Human Development, and by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan.

The expert technical assistance of Mrs. Ulla Lindholm is gratefully acknowledged.

We are indebted for generous gifts by Dr. Seymour Bernstein, Lederle Laboratories of 16α- and 6α-hydroxyprogesterone and to Dr. John Babcock, Upjohn Laboratories, for 20α- and 20β-hydroxy-pregn-4-en-3 one.

REFERENCES

1) The following trivial names have been used in this paper: pregnenolone (3β-hydroxy pregn-5-ene-20-one), 17α-hydroxypregnenolone (3β-17α-dihydroxy pregn-5-ene-20-one), progesterone (pregn-4-ene-3, 20-dione), 17α-hydroxyprogesterone (17α-hydroxy pregn-4-ene-3, 20 dione), 6β-hydroxyprogesterone (6β-hydroxy pregn-4-ene-3, 20-dione), 6α-hydroxyprogesterone (6α-hydroxy pregn-4-ene-3, 20-dione), 16α-hydroxyprogesterone (16α-hydroxy pregn-4-ene-3, 20-dione), androstenedione (androst-4-ene-3, 17-dione), pregnanediol (5β-pregnane-3α, 20α-diol) and dehydroepiandrosterone (3β-hydroxy-androst-5-ene-17-one).

2) New England Nuclear Corp., Boston, Mass.

3) Merck AG, Darmstadt, Germany

1. Pion, R., Jaffe, R., Eriksson, G., Wiqvist, N. and Diczfalusy, E., ACTA ENDOCR. (KBH.) 48, 234 (1965).

2. Villee, D. B., Engel, L. L., Loring, J. M. and Villee, C. A., ENDOCRINOLOGY 69, 354 (1961).

3. Reynolds, J. W., J. CLIN. ENDOCRINOL. & METAB. 25, 416 (1965).

4. Mikhail, G., Wiqvist, N. and Diczfalusy, E., ACTA ENDOCR. 42, 519 (1963).

5. Frame, E. G., ENDOCRINOLOGY 34, 175 (1944).

6. Pearlman, W. H. and Cerceo, E., J. BIOL. CHEM. 203, 127 (1953).

7. Allen, W. M., J. CLIN. ENDOCRINOL. & METAB. 10, 71 (1950).

8. Oertel, G. W. and Eik-Nes, K. B., ANALYT. CHEM. 31, 98 (1959).

9. Jaffe, R. B. and Peterson, E. P., Unpublished data.

10. Jaffe, R. B. and Ledger, W. J., To be published.

11. Begue, J. A., C. R. ACAD. SCI. (PARIS) 260, 3777 (1965).

12. Eberlein, W. R., J. CLIN. ENDOCRINOL. & METAB. 25, 1101 (1965).

13. Cassmer, O., ACTA ENDOCR. (KBH.) Supp. 45 (1959).

14. Frandsen, V. A. and Stakemann, G., ACTA ENDOCR. (KBH.) 38, 383 (1961).

15. Appleby, J. I. and Norymberski, J. K., J. ENDOCR. 15, 310 (1957).

16. Klopper, A., Macnaughton, M. C. and Michie, E. A., J. ENDOCR. 22, 14 (1961).

17. Huis in't Veld, L. G. and Kloosterman, G. J, BULLETIN OF THE RIJKS INSTITUUT VOOR DE VOLKSGEZONDHEID, UTRECHT (1964).

18. Levitz, M., Emerman, S. and Dancis, J., MEETING ON BIOLOGICAL AND CLINICAL ASPECTS OF PLACENTAL STEROIDOGENESIS, The Williams and Wilkins Company, 1964, pp. 4-12.

19. Van Leusden, H. and Villee, C. A., STEROIDS 6, 31 (1965).

20. Solomon, S., THE PLACENTA AND FETAL MEMBRANES, Villee, C. A. (Ed.), The Williams and Wilkins Company, 1960 pp. 201-202.

21. Morrison, G., Meigs, R. A. and Ryan, K. J., STEROIDS Supp. II, 177 (1965).

22. Bolte, E., Mancuso, S., Eriksson, G., Wiqvist, N. and Diczfalusy, E., ACTA ENDOCR. (KBH.) 45, 560 (1964).

23. Bolte, E., Mancuso, S., Eriksson, G., Wiqvist, N. and Diczfalusy, E., ACTA ENDOCR. (KBH.) 45, 576 (1964).

24. Siiteri, P. K. and MacDonald, P. C., STEROIDS 2, 713 (1963).

25. Baulieu, E. E. and Dray, F., J. CLIN. ENDOCRINOL. & METAB. 23, 1298 (1963).

26. Bird, C. E., Solomon, S., Wilson, R., Wiqvist, N. and Diczfalusy, E., ENDOCRINE SOC. ABSTRACTS No. 96, 68 (1965).

27. Berliner, D. L. and Salhanick, H. A., J. CLIN. ENDOCRINOL. & METAB. 16, 903 (1956).

28. Little, B., Shaw, A. and Purdy, R., ACTA EDOCR. (KBH.) 43, 510 (1963).

29. Little, B. and Shaw, A., ACTA ENDOCR. (KBH.) Supp. 51, 713 (1960).

30. Little, B., Dimartinis, J. and Nyholm, B., ACTA ENDOCR. (KBH.) 30, 530 (1959).

31. Warren, J. C. and Cheatum, S. G., CANAD. J. BIOCHEM. 42, 143 (1964).

32. Hagopian, M., Pincus, G., Carlo, J. and Romanoff, E. B. ENDOCRINOLOGY, 58, 387 (1956).

33. Salhanick, H. A., Jones, J. E. and Berliner, D. L., FED. PROC. 15, 160 (1956).

34. Pearlman, W. H. and Cerceo, E., J. BIOL CHEM. 194, 807 (1952).

35. Zander, J., Forbes, T. R., von Munstermann, A. M. and Neher, R., J. CLIN. ENDOCRINOL. & METAB. 18, 337 (1958).

36. Cedard, L. and Knuppen, R., STEROIDS 6, 307 (1965).

37. Alonso, C. and Troen, P., BIOCHEM. 5, 337 (1966).

38. Sweat, M. L., Berliner, D. L., Bryson, M. J., Nabors, C., Jr., Haskell, J. and Holmstrom, E. G., BIOCHIM. BIOPHYS. ACTA. 40, 289 (1960).

39. Acevedo, H. F. and Goldziehev, J. W., BIOCHIM. BIOPHYS. ACTA. 111, 294 (1965).

40. Fotherby, K., James, F., Kamyab, S., Klopper, A. I. and Wilson, G. R., J. ENDOCR. 33, 133 (1965).

41. Zander, J., CIBA FOUND. STUDY GROUP 9, 32 (1961).