IN VIVO STEROID BIOGENESIS AND METABOLISM IN THE HUMAN TERM PLACENTA
2. IN SITU PLACENTAL PERFUSION WITH CHOLESTEROL-7α-^{3}H

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ABSTRACT

Two human term placentas have been perfused *in situ* with cholesterol-7α-^{3}H. The perfusions were carried out at the time of elective repeat cesarean section and tubal ligation in term pregnancies. The major tritiated steroid identified was pregnenolone.

It is suggested that the human placenta has the capacity to utilize circulating cholesterol at this stage of steroid biogenesis.

Biosynthetic pathways in most steroid producing organs have been found to involve the conversion of cholesterol to the various steroid hormones characteristic of that organ. In the placenta, the cholesterol so utilized has been found to be derived from a series of precursors including acetate, mevalonate, squalene, and lanosterol (1,2). Placental conversion of cholesterol to pregnenolone[2] and progesterone *in vitro* has been demonstrated (3-5). However, *in vitro* studies demonstrate the capacity for a reaction to occur, without necessarily reflecting the situation extant *in vivo*. Further, the relative contribution

of circulating precursors as opposed to de novo placental synthesis at these stages of steroid biogenesis remains to be ascertained. The present study was devised in an effort to assess the capacity of the human term placenta to utilize circulating cholesterol in steroid biogenesis in vivo, and to ascertain the amount of circulating cholesterol available to the placenta from both the maternal and fetal compartments.

METHODS AND MATERIALS

Perfusion Technique: Studies were carried out on two patients scheduled for elective repeat cesarean section and tubal ligation at term with no other medical or obstetric complications of the present pregnancy. These studies were performed in a manner similar to that previously reported from this laboratory for the placental perfusion of labeled pregnenolone (6). In brief, after catheterization of an umbilical artery and the umbilical vein, whole blood was perfused via the umbilical arterial catheter, and the venous effluent collected in ethanol. A 50 percent (v/v) ethanolic solution of cholesterol-7α-^{3}H (0.8 ml.) was injected into the umbilical arterial catheter at one minute intervals, over a period of seven minutes, followed by an additional two minute perfusion with blood only. At the conclusion of the procedure, the placenta was manually separated from the uterus, its membranes and cord dissected free, and the placenta was then homogenized in 80% ethanol.

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

Cholesterol-7α-^{3}H, Lot. No. 184-32-32[3] with a

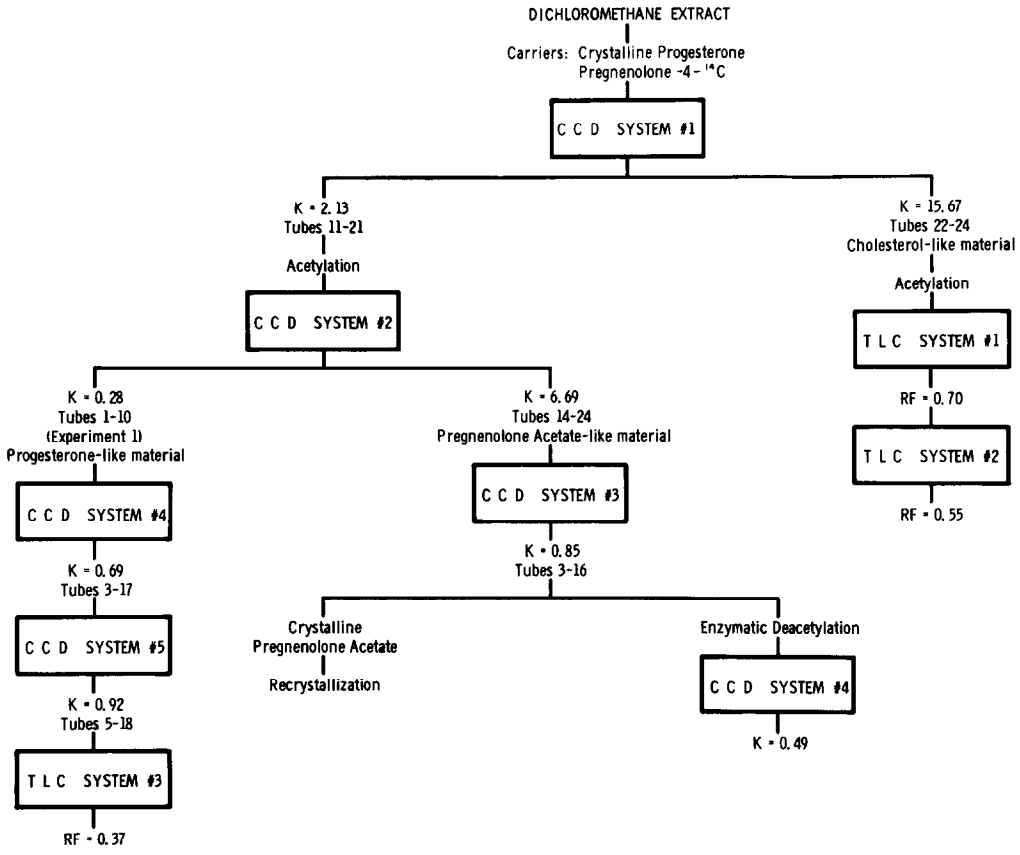

Figure 1: Procedures employed in analysis of the dichloromethane extract of placenta and venous effluent following in situ term placental perfusion with cholesterol - 7α- ^{3}H.

specific activity (S.A.) of 6.2 mc/mg was purified utilizing thin-layer chromatography (TLC) on silica gel G[4] in the following solvent systems: benzene, ethyl acetate, 1:1 (Rf = 0.51) and chloroform, acetone 19:1 (Rf = 0.33). Pregnenolone-4-^{14}C, Lot. No. 79-L39A-8[3] with a S.A. of 1.4 mc/mg was similarly purified in the following solvent systems: cyclohexane, ethyl acetate, ethanol, 9:9:2 (Rf = 0.52) and benzene ethanol 9:1 (Rf = 0.33). The amount of cholesterol-7α-^{3}H perfused was 16.2 and 24.3 μc.

Extraction and Purification: This was carried out as described previously (6). Briefly, placenta and venous effluent were extracted repeatedly with

80% and absolute ethanol. This was followed by acetone extraction and methanol precipitation at -17°C. A dichloromethane-water partition was then employed in a counter-current fashion, using 6 lower phase transfers, and the dichloromethane fraction further analyzed.

An outline of the procedures employed in the analysis of the dichloromethane fraction is illustrated 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 (CCD): Twenty-four 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. Cyclohexane, ethanol, water (10:9:1).

4. n-Hexane, ethanol, water (10:4.8:5.2).

5. Cyclohexane, ethyl acetate, ethanol, water (4:1:3:2)

Derivative formation: Acetylation was carried out with acetic anhydride in anhydrous pyridine (1:2) at room temperature overnight. Enzymatic deacetylation using the enzyme, Acylase[5] was performed in the following manner: the dried steroid extract was dissolved in a drop of ethanol and 1.0 ml. of a 0.1 Molar pH 7.3 phosphate buffer. Three mg of the enzyme, dissolved in 0.5 ml of the buffer were then added, and the solution incubated at 37°C for 4 hours. The reaction was stopped by the addition of water.

Thin Layer Chromatography (TLC): Slurries of silica gel G[4] were applied to glass plates (5 x 20 cm or 20 x 20 cm) and activated at 120°C. Ascending chromatography in the following solvent systems was employed:

1. Chloroform, acetone (19:1).

2. Benzene, chloroform (1:1).

3. Ethyl Acetate, chloroform (50:1).

For detection of radioactivity on the 5 x 20 cm plates, a Packard Model 7201 radiochromatogram scanner was used.

Estimation of C-21 Steroids: Progesterone was measured in ethanol by ultraviolet absorption at 240 mμ using the Allen correction formula (7). For detection of pregnenolone on thin-layer chromatograms either a phosphomolybdic acid or water spray was employed. Progesterone was detected on thin layer chromatograms with ultraviolet light. Measurement of 3β-hydroxy-Δ^5 steroids was carried out as described by Oertel and Eik-Nes (8).

Cholesterol Determinations: Total and esterified cholesterol were measured in serum utilizing the methods described by Connerty et al. (9, 10).

Recrystallization to Constant Specific Activity: Crystalline pregnenolone acetate was added to part of the isolated radioactive material obtained from the third CCD. Successive recrystallizations from 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 made using an internal standard technique. Counting efficiencies were monitored utilizing standards prepared in the same manner as the samples.

RESULTS

The bulk of the converted radioactive material in the "free" (dichloromethane-soluble) fraction in both placenta and venous effluent was identified as pregnenolone. This amounted to 49 and 70% of the free radioactive material recovered from the placenta and 14 and 49% of that in the venous effluent. The bulk of the remaining radioactive material was unchanged cholesterol. The cholesterol-like material from the first CCD was acetylated, and further characterized by TLC in Systems 1 and 2. There was good agreement between the Rf of authentic cholesterol acetate and that of the acetylated radioactive material in both TLC systems.

In Experiment 1, the tritiated progesterone-like material was characterized in CCD Systems 1, 2, 4 and 5 and in TLC System 3. Excellent agreement was noted between partition coefficients and the Rf value of carrier progesterone and the tritiated material. The quantity of radioactive material associated with carrier progesterone was insufficient to permit further identification. In Experiment 2, there was no appreciable radioactivity associated with the carrier progesterone.

As noted in Figure 1, the identification of pregnenolone was based on an initial CCD as the free compound. The pregnenolone-like material was then acetylated and two CCD's of the acetate of pregnenolone carried out. Figure 2 illustrates a representative countercurrent distribution curve obtained in CCD System 3, immediately prior to recrystallization. The lower curve is that of authentic "carrier" pregnenolone acetate-4-^{14}C, the free form having been added at the initiation of the processing of the dichloromethane extract. The upper curve, with an identical partition coefficient (K = 0.82), is that of the tritiated material derived from the perfusion.

Following the third CCD, an aliquot of the radioactive material was mixed with crystalline pregnenolone-acetate carrier, and recrystallized to constant S.A. The recrystallization data are presented in Table 1. Insufficient radioactive material precluded recrystallization in the venous effluent of Experiment 2.

An additional aliquot of the radioactive pregnenolone acetate-like material was deacetylated, and a final countercurrent distribution in System 4 performed. Again, there was excellent agreement

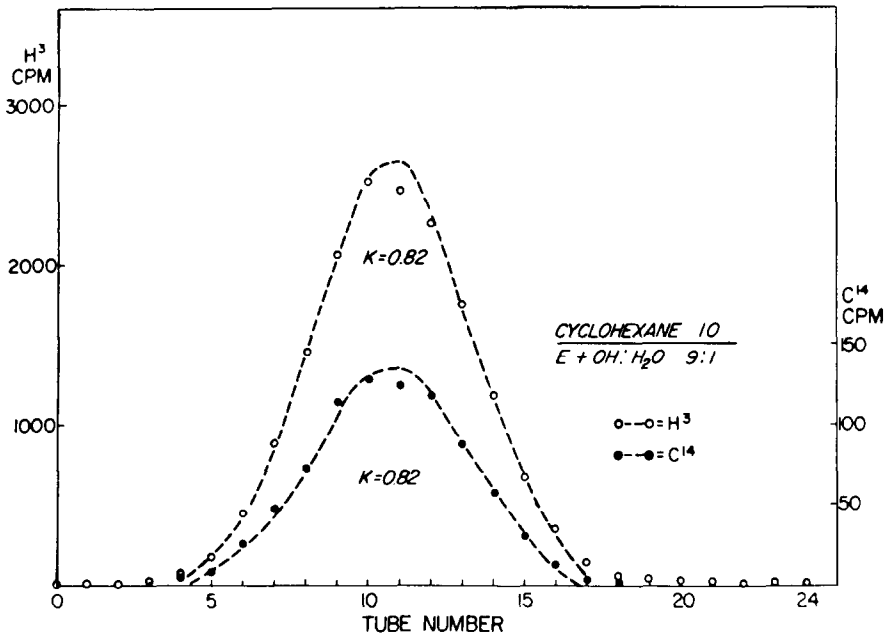

Figure 2

Countercurrent distribution of acetylated radioactive material derived from in situ perfusion of human term placenta with cholesterol-7α-^{3}H. Carrier steroid is pregnenolone acetate-4-^{14}C.

Table 1

Recrystallization Data (c.p.m./mg.) of pregnenolone (as acetate) derived from in situ human term placental perfusion with cholesterol 7α-^{3}H

Solvent	Placenta Exp. 1 + 2	Venous Effluent Exp. 1[a]
Methanol	C_1[b] 2333	C_1 195
Ethanol	C_2 2107	C_2 178
Acetone	C_3 2259 ML_3 2322	C_3 185
Methanol		C_4 180
Acetone		C_5 171 ML_5 188

a) Insufficient radioactive material precluded recrystallization of pregnenolone acetate in the venous effluent of Exp. 2

b) C = Crystal ML = Mother Liquor

between carrier and tritiated material. Recovery of radioactive material in these experiments was very low; in the 2 experiments, 0.2 and 0.9 percent of the injected radioactivity were recovered as pregnenolone in the placenta and venous effluent.

As expected, since very little cholesterol is excreted in the urine, there was no significant radioactivity measured in the small urinary aliquots (0.5 ml) taken for counting. Analysis of large pools of maternal urine for steroid content was not undertaken.

To assess the extent to which cholesterol is available for placental utilization in both the maternal and fetal compartments, in 40 term pregnancies terminated by vaginal delivery, the concentration of total and esterified cholesterol was determined in umbilical venous blood. In twelve of these, umbilical arterial samples were also obtained. In addition, cholesterol levels were measured in maternal antecubital vein blood in 19 cases. In the umbilical artery, the mean concentration of total cholesterol was 88 mg/100 ml serum (range 52-142) and in the vein it was 74 mg/100 ml (range 44-137). The mean esterified cholesterol fraction in the umbilical artery was 79

percent, and that in the vein was 77 percent. When paired samples were considered, arterio-venous differences were not statistically significant. In the maternal venous blood, the mean total cholesterol was 196 mg/100 ml (range 110-290) with 63 percent esterified.

DISCUSSION

It is becoming increasingly apparent that blood-borne precursors, reaching the placenta from the maternal and/or fetal compartments may be utilized in the placental elaboration of certain estrogens (11-15). That circulating precursors, at least from the fetal compartment, are necessary for the placental production of progesterone seems less certain for reasons detailed in earlier communications (6, 16).

In addition, as noted previously, in vitro placental conversion of acetate and mevalonate to lanosterol and cholesterol has been demonstrated (1, 2). Further, incubation of mitochondrial preparations of human term placentas with labeled cholesterol and a TPNH generating system by Morrison et al. (5) gave rise to both pregnenolone and progesterone, the latter in high yield. An in vitro perfusion study by Solomon (3) also demonstrated this conversion, although in low yield.

However, as has been demonstrated recently *in vivo* by Jaffe and Ledger (6) the human term placenta is capable of utilizing circulating pregnenolone in the production of progesterone. And it effects this conversion in a rapid and efficient manner. Circulating pregnenolone sulfate is similarly metabolized (17, 18). With the relatively large quantities of cholesterol in both the maternal and fetal circulation, it was of interest to ascertain whether the placenta was also capable of utilizing circulating cholesterol in steroidogenesis; the presently described experiments demonstrate this capacity to be present and suggest that circulating cholesterol may contribute at least some of the starting material for placental steroid synthesis. To assess the capacity of placental transfer of maternal cholesterol, experiments are now in progress in which tritiated cholesterol preincubated with maternal serum (19), has been injected into the maternal circulation and a search made for pregnenolone and progesterone metabolites.

The utilization of circulating cholesterol in adrenocortical steroid synthesis in the non-pregnant individual has been demonstrated (20). Bloch's classic work (21) also demonstrated the conversion,

in pregnancy, of orally administered deuterium labeled cholesterol to deuterium labeled pregnanediol.

Under the conditions employed in the presently described experiments, the bulk of the converted radioactive material was found to be pregnenolone, with progesterone being detected in small quantities in one experiment. In view of the relative efficiency of placental conversion of pregnenolone to progesterone (6), the reason for the failure to demonstrate larger amounts of progesterone is not immediately apparent. A similar finding was noted upon perfusion of a midtrimester placenta with cholesterol (22).

That circulating cholesterol is available to the placenta both from the maternal and fetal side is apparent from the concentration of plasma cholesterol in these two compartments.

The mean umbilical venous cholesterol values agree closely with those of Mortimer (23), Rafstedt (24) and Lloyd (25), and are slightly higher than those of Brody and Carlson (26). The authors are unaware of other studies of paired umbilical arterial and venous samples. The concentration of cholesterol in the maternal compartment has been found to increase from the second trimester to term (27). This increase is

present in both the free and esterified fractions. The values obtained in this study are somewhat lower than those found by de Alvarez et al. (27). Sample size in the present study was small, and it is known that there is wide variation in maternal cholesterol levels.

Circulating cholesteryl sulfate has been found in human plasma (28) and umbilical cord blood (29). Whether it is utilized in placental steroidogenesis remains to be ascertained.

It should be noted that while extraction procedures for both placenta and venous effluent were essentially the same as those employed for evaluation of other steroid precursors, both phenolic (30) and neutral (6, 16), under similar experimental conditions, recoveries were far less complete when cholesterol was perfused. This may be a reflection of either solubilization factors, membrane permeability, precipitation of sterols with methanol, rapid passage across the placenta or the ubiquitous pathways of cholesterol metabolism in the placenta, only the steroid fraction being extracted by our techniques. Experiments are being undertaken involving placental perfusion of tritiated cholesterol

preincubated with serum (19) in an attempt to increase recovery and more closely approximate the physiologic situation. It is felt that this reaction may be of importance, in spite of these low recoveries, since even a low rate of conversion might explain a significant amount of the placental steroid elaboration extant in pregnancy.

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2) The trivial names used throughout this paper are: pregnenolone (3β-hydroxy pregn-5-en-20-one) and progesterone (pregn-4-ene-3, 20-dione).

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

4) Merck and Co., Darmstadt, Germany.

5) Sigma Chemical Co., St. Louis, Mo. We are grateful to George Mikhail, M.D. for furnishing the details of this method.

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