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FORMATION AND METABOLISM OF PROGESTERONE BY THE MOUSE PLACENTA *IN VITRO*

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SUMMARY

The *in vitro* metabolism of radioactive pregnenolone and progesterone by mouse placental tissue has been studied. From the progesterone incubations 5α-pregnane-3,20-dione, 3β-hydroxy-5α-pregnan-20-one, 20α-hydroxy-4-pregnen-3-one, 5α-pregnane-3β,-20α-diol, 3α-hydroxy-5α-androstan-17-one, 4-androstene-3,17-dione, and 21-hydroxy-4-pregnene-3 20-dione were isolated by paper and thin layer chromatography and identified by crystallization to constant specific activity. In addition, the formation of radioactive progesterone, 4-androstene-3,17-dione and 21-hydroxy-4-pregnene-3,20-dione from [4-^{14}C] pregnenolone has been demonstrated. No estrogens were isolated.

PLACENTAL steroidogenesis has been well documented for the human placenta (e.g. [1]) and for many mammalian species [2–5]. Little information on placental steroid biosynthesis, however, has been observed in animals in which ovariectomy terminates pregnancy after a certain stage of gestation.

This report describes *in vitro* studies on the formation and metabolism of progesterone by placental tissue of the mouse, a species in which abortion or resorption of the products of conception occurs irrespective of the time at which the ovariectomy is performed [6].

The present report documents the synthesis of radioactive progesterone from [4-^{14}C] pregnenolone following incubation with mouse placental tissue. In addition, the ability of mouse placental tissue to synthesize androstenedione and deoxycorticosterone from pregnenolone and progesterone is demonstrated. 5α-Metabolites of progesterone are also identified.

MATERIALS AND METHODS

Substrates and reference steroids

The following substrates purchased from the Radiochemical Centre, Amersham, England were used: [4-^{14}C] progesterone (S.A. 69·0 mCi/mg) and [4-^{14}C] pregnenolone (S.A. 76·0 mCi/mg). Steroids were examined for purity in at least 2 chromatographic systems. 20α-Dihydroprogesterone* and 20β-dihydroprogesterone were a gift from Dr. R. Neher, Ciba Ltd. (Basel). 5α-Pregnane-3β, 20α-diol was a gift from Dr. L. Kornel, Chicago. The other reference steroids were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

*Trivial names and abbreviations: 20α-Dihydroprogesterone: 20α-hydroxy-4-pregnen-3-one; 20β-dihydroprogesterone: 20β-hydroxy-4-pregnen-3-one; 16α-hydroxyprogesterone; 16α-hydroxy-4-pregnene-3,20-dione; 17α-hydroxyprogesterone: 17α-hydroxy-4-pregnene-3,20-dione.

PPC: paper partition chromatography; TLC: thin-layer chromatography.

The purity of each steroid was checked by TLC and, in most cases, by melting point determinations.

Paper partition chromatography

All chromatographies were carried out at room temperature, using the following systems:

1. cyclohexane/propanediol
2. isooctane/propanediol for 48 h
3. benzene/propanediol for 24 h
4. benzene/propanediol
5. cyclohexane-benzene (3 : 1, v/v)/propanediol.

Thin-layer chromatography

The specifications of Lisboa[7, 8] were followed, using the systems:

1. *n*-hexane-ethyl acetate (3 : 1, v/v)
2. chloroform–ethanol (19 : 1, v/v)
3. ether saturated with water
4. ethyl acetate–cyclohexane (1 : 1, v/v)
5. chloroform–ether–ethanol (90 : 10 : 1, by vol.).

Chemical reactions

Acetylation was carried out overnight in an acetic anhydride: pyridine mixture [9]. The method of Mattox *et al.*[10] was used for acetylation of hydroxyl groups under mild conditions. Oxidation was performed with chromic acid in acetic acid[11] or in acetone[12]. Oxidations with periodic acid and sodium bismuthate were carried out as described by Bush[11]. Saponification was carried out as described by Meyer[13] or Bush[11].

Counting of radioactivity

Radioactivity was quantitatively determined using a USB-2 (BUTJ-Warsaw) scintillation spectrometer. The scintillation solution was prepared by adding 3·0 g of PPO and 0·3 g of POPOP (Nuclear Enterprises, Scotland) to 1 liter reagent grade toluene containing 1% methanol. Counting efficiency for ^{14}C was 50%. Measurements were carried out to a precision of $\pm 5\%$. The radioactivity in quenched samples was corrected by use of an internal standard.

Tissue preparation and incubation procedure

Unmated female mice of the R-III, C-57/Bl., CBA and BALB/c strains, about 3 months old, were used. After timed matings each mouse was placed in an individual cage. All mice were sacrificed either on day 10–12 or on day 18–20. At autopsy, the placentae were removed and carefully quartered with fine scissors. The 8 quarters from 2 placentae of each mouse were distributed among 8 flasks containing Krebs–Ringer bicarbonate medium with 0·2% glucose. No cofactors were added to the medium. The amount of tissue and volume of medium varied according to the circumstances of the experiment but usually 200 mg of tissue in 5 ml of medium was placed in each flask. The tissue was preincubated in an atmosphere of 95% O_2–5% CO_2 in a shaking incubator at 37° for 60 min. The

preincubation medium was discarded and replaced with fresh medium for the incubation period of 3h. Radioactive steroids were added to the incubation medium as small volumes (10 μl) of ethanolic solutions. After the incubation, the vessels were cooled 2 min and then chloroform was added.

Extraction and purification of radioactivity following incubation

The placental tissue and media were extracted three times with 2 vol. of chloroform. The chloroform extracts were evaporated in a stream of nitrogen and the residue was partitioned between toluene/N NaOH to yield phenolic and neutral fractions. Purification and separation of the radioactive metabolites present in the neutral fraction was accomplished by PPC and TLC. From paper strips the material was eluted in the apparatus described by Saffran and Sharman [14]. From TL plates steroid spots were scraped off. The silica gel was packed into a small column and extracted with methanol. Reference steroids containing a 4-ene-3-ketone configuration were localized on paper chromatograms by contact photography on photocopy paper using U.V. light. Following TLC, reference standards were localized by exposure of the plates to iodine vapour. The radioactivity on PPC and TLC was localized by means of an automatic chromatogram scanner [15]. The individual labelled compounds were isolated and identified by the procedures presented schematically in Fig. 1. Final identification of the radioactive metabolites obtained after incubation of placentae of R-III strain

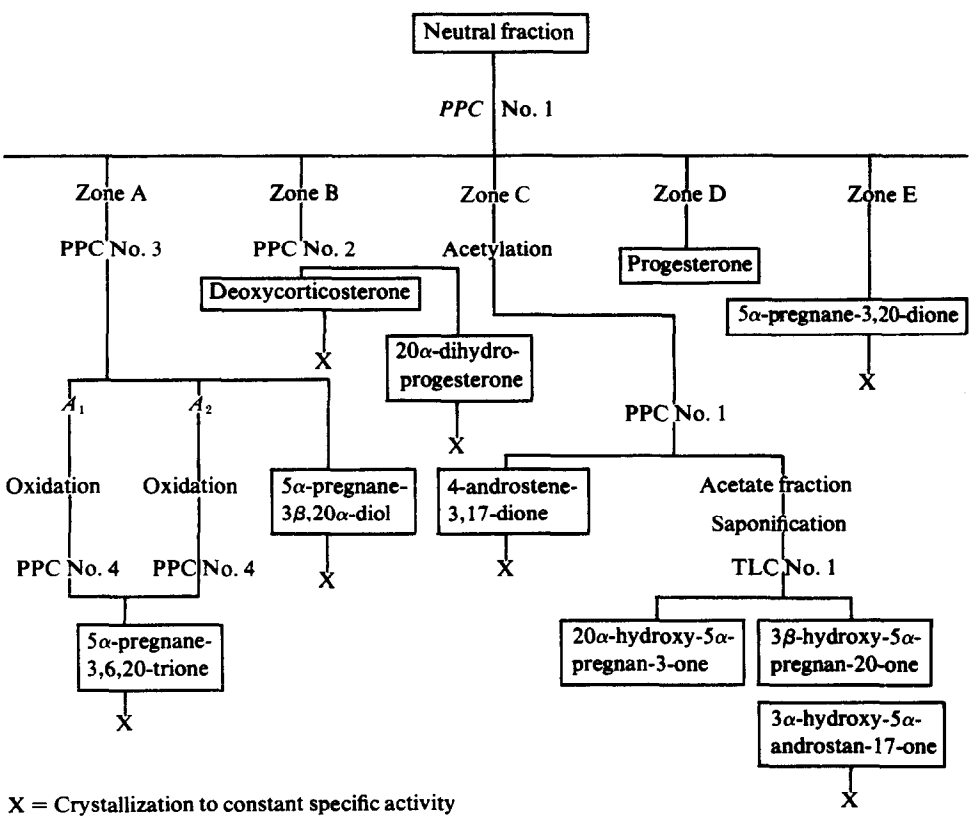

Fig. 1. Scheme of the isolation and identification of the metabolites of [4-^{14}C] progesterone.

mice were crystallized with 8 to 12 mg standard steroid to constant specific activity [16]. Radioactive metabolites obtained after incubations of placentae of other mouse strains were identified chromatographically.

RESULTS

I. *Identification of radioactive metabolites present in the neutral fraction after incubation of placental tissue of R-III mouse with [4-^{14}C] progesterone*

After the incubation of [4-^{14}C] progesterone (0·2 μCi) with the mouse placental quarters the neutral and phenolic fractions of the extracts of the incubation medium were separated. Almost all the radioactivity was present in the neutral fraction (95–98%). The small amount of radioactivity in the phenolic fraction did not correspond to estrone, estradiol and estriol in PPC System No. 3. After oxidation with CrO_3 in acetone this material yielded the same radioactive product which exhibited a mobility similar to that of material A_1 and A_2 from the neutral fractions (see below).

Initial PPC of the neutral fractions in System No. 1 resulted in the separation of the material into five major radioactive zones (Fig. 1).

1. *Partial identification of radioactivity present in Zone A.* The material eluted from Zone A of the initial chromatogram was rechromatographed in PPC System No. 3. Three radioactive zones were localized. An aliquot of the radioactivity present in the least polar peak was oxidized and then rechromatographed in TLC System No. 1 (4 developments) along with authentic 5α-pregnane-3,20-dione and its 5β-isomer. The radioactivity was shown to possess a mobility corresponding to the 5α-isomer. TLC of acetylated material from this zone in System No. 1 resulted in a single radioactive peak, which on the basis of its mobility seemed to be a "5α-pregnanediol" metabolite. Upon hydrolysis of the acetate this material was chromatographed in TLC System No. 2 along with 5α-pregnane-3β, 20α-diol and its 3α-isomer. The radioactive product exhibited the polarity of authentic 5α-pregnane-3β,20α-diol. Another aliquot of the radioactive 5α-pregnane-3β,20α-diol was mixed with authentic compound and recrystallized from different solvents (Table 1).

The radioactive compounds (A-1 and A-2) which were more polar in PPC System No. 3 than the material identified as 5α-pregnane-3β,20α-diol have been partially identified. TLC of these materials in Systems 2 and 4 revealed that materials A-1 and A-2 were not identical with either 16α-hydroxyprogesterone or 17α-hydroxyprogesterone. When these metabolites were acetylated, significant loss of polarity occurred in PPC System No. 1. By chromatography after oxidation with CrO_3 in acetone, compounds A-1 and A-2 formed the same radioactive product, with a mobility greater than 5α-pregnane-3,20-dione and progesterone. When chromatographed in TLC No. 2 and PPC No. 5, this material had the same R_F value as authentic 5α-pregnane-3,6,20-trione. 5α-Pregnane-3,6,20-trione was then recrystallized after addition of carrier. Constant specific activity was achieved (Table 1).

2. *Identification of radioactivity present in Zone B.* The material eluted from Zone B of the initial chromatogram was rechromatographed in PPC System No. 2, yielding two radioactive zones. The more polar peak was oxidized with CrO_3. The oxidation product remained on the starting line when chromatographed in PPC System No. 4. A product of the same polarity was obtained when periodic acid or sodium bismuthate was used for oxidation. This is consistent with the

presence of an α-ketolic side chain without 17-hydroxy group and formation of a corresponding etionic acid. On acetylation with either acetic anhydride in pyridine or diluted reagents it gave identical acetates migrating at the same rate as deoxycorticosterone acetate in PPC System No. 1. The acetate, when saponified in methanolic potassium bicarbonate, migrated at the same rate as deoxycorticosterone in PPC System No. 4 and TLC System No. 2. Another aliquot of the radioactive deoxycorticosterone was mixed with authentic compound and recrystallized from different solvents (Table 1).

Table 1. Identification of [4-^{14}C] progesterone metabolites by recrystallization to constant specific activity (cpm/mg)

Metabolite identified	Crystallization No.[a]			
	1	2	3	4
5α-Pregnane-3,6,20-triol[b]	6260 8930[c]	5530 5690[d]	5520 5560[d]	—
5α-Pregnane-3β,20α-diol	12 080 24 850[e]	11 140 12 760[f]	11 000 10 930[f]	11 010 10 860[f]
Deoxycorticosterone	4470 4680[g]	4320 4450[h]	4110 4020[h]	
20α-Dihydroprogesterone	5320 7200[i]	5230 5410[g]	5250 5280[i]	5240 5260[g]
Androstenedione	3330 3750[i]	3210 3170[g]	3170 3230[j]	
3α-Hydroxy-5α-androstan-17-one	6260 8930[i]	5540 5690[d]	5520 5650[d]	
3β-Hydroxy-5α-pregnan-20-one	4070 10 040[h]	4100 4540[h]	4120 4050[h]	
5α-Pregnane-3,20-dione	6600 7220[i]	6540 6580[e]	6510 6530[j]	

[a]Upper figure denotes specific activity of the crystals, lower figure denotes specific activity of the corresponding mother liquor.

[b]Produced by oxidation with CrO_3 in acetone.

[c]Isooctane/ethyl acetate; [d]n-hexane/ethyl acetate; [e]ethanol/methylene chloride; [f]methanol; [g]80% ethanol; [h]isooctane/methylene chloride; [i]70% methanol; [j]70% acetone.

The less polar peak was subjected to oxidation with CrO_3. The oxidized product migrated as a single zone and with the same R_F value as progesterone in PPC System No. 1. Another portion of the labelled zone was then chromatographed in TLC Systems Nos. 3 and 4 along with 20α- and 20β-dihydroprogesterone. The radioactive material migrated at the same rate as authentic 20α-dihydroprogesterone. The specific activities of the crystals and mother liquors are shown in Table 1.

3. *Identification of radioactivity present in Zone C.* The radioactive material designated as Zone C on the initial chromatogram was eluted, acetylated and rechromatographed in the same system (PPC No. 1). Two radioactive zones were obtained: a more polar zone, which migrated at the same rate as the initial material, and a less polar zone which migrated on the front of the chromatogram.

The nonacetylated material was rechromatographed in TLC Systems Nos. 2 and 5. In both cases the mobility of the labelled compound was the same as that of authentic androstenedione. The data for crystallizations are summarized in Table 1.

The radioactivity associated with the acetylated material was hydrolyzed with methanolic potassium hydroxide and rechromatographed in PPC System No. 5. One peak of radioactivity was observed. Oxidation with CrO_3 in acetic acid followed by chromatography in TLC System No. 1 (3 developments) together with 5α-pregnane-3,20-dione and 5α-androstane-3,17-dione revealed that about 80% of the radioactivity had the same mobility as authentic 5α-pregnane-3,20-dione. The remaining radioactivity corresponded in mobility to authentic 5α-androstane-3,17-dione.

The radioactivity present in the hydrolyzed acetate fraction was chromatographed in TLC System No. 1 (4 developments) with 3β-hydroxy-5α-pregnan-20-one, with its 3α-isomer and with 20α-hydroxy-5α-pregnan-3-one. Two radioactive peaks were observed. The first had the same mobility as 20α-hydroxy-5α-pregnan-3-one whereas the second was asymmetrical and corresponded as well to 3β-hydroxy-5α-pregnan-20-one as to its 3α-isomer. Therefore another portion of this radioactive material was divided into three parts and crystallized with 3β-hydroxy-5α-pregnan-20-one, with its 3α-isomer and with 3α-hydroxy-5α-androstan-17-one. Constant specific activities were obtained for 3β-hydroxy-5α-pregnan-20-one and for 3α-hydroxy-5α-androstan-17-one (Table 1).

Crystallization studies with the material characterized chromatographically as 20α-hydroxy-5α-pregnan-3-one, were not possible due to insufficient amounts of reference 20α-hydroxy-5α-pregnan-3-one.

4. *Identification of radioactivity present in Zone E.* An aliquot of the radioactivity present in this zone was rechromatographed in TLC System No. 1 (3 developments) along with authentic 5α-pregnane-3,20-dione and its 5β-isomer. It was shown to have a mobility corresponding to the 5α-isomer. The remaining radioactivity was mixed with reference 5α-pregnane-3,20-dione and recrystallized to constant specific activity (Table 1).

II. *Identification of metabolites (progesterone, androstenedione and deoxycorticosterone) after incubation of placental tissue of R-III strain mouse with [4-^{14}C] pregnenolone*

Quarters of placenta obtained from 20 days pregnant R-III mice were incubated with [4-^{14}C] pregnenolone (0·2 μCi). After incubation, extraction and evaporation of solvent, the residue was subjected to PPC in System No. 5. The zones of radioactivity with the mobilities of reference progesterone, androstenedione and deoxycorticosterone were eluted and submitted to further characterization. Dehydroepiandrosterone was not detected by PPC of extracts from any of the tissue preparations.

For identification of androstenedione and deoxycorticosterone the same procedures were used as described in Section I (Table 2). The zone of radio-

Table 2. Identification of three [4-^{14}C] pregnenolone metabolites by recrystallization to constant specific activity (cpm/mg)

Metabolite identified	Crystallization No.[a]		
	1	2	3
Progesterone	4770	4570	4370
	5400[h]	4570[h]	4420[h]
	3620	3240	3200
Androstenedione	13 720[i]	3460[g]	3450[g]
Deoxycorticosterone	2100	2120	2090
	3450[g]	2330[h]	2110[g]

[a,g,h,i] See foot-notes to Table 1.

active progesterone was acetylated and chromatographed in PPC System No. 1 and TLC System No. 3. The zones containing progesterone were eluted and the material crystallized to constant specific activity after addition of reference progesterone (Table 2).

III. *Chromatographic identification of metabolites of* [4-^{14}C] *progesterone after incubation of placental tissue of C-57/B1., CBA and BALB/c mice*

The purpose of these experiments was to find out if placentae of 20 days pregnant C-57/B1., CBA and BALB/c mice convert [4-^{14}C] progesterone to the same metabolites as identified in placental tissue of 20 days pregnant R-III mice. For the same purpose similar experiments were also conducted with 10 days pregnant R-III mice. Each metabolite of [4-^{14}C] progesterone was characterized chromatographically in several PPC and TLC systems as described in section I.

It was found that placentae of the mice under study convert [4-^{14}C] progesterone to the same metabolites as were observed in placentae of R-III mice 20 days after mating.

DISCUSSION

The results of this study demonstrate biochemically that mouse placental tissue *in vitro* can form progesterone from pregnenolone. This indicates the presence of the 3β-hydroxysteroid dehydrogenase/$\Delta_5 \rightarrow \Delta_4$ isomerase enzyme system in the placenta. The progesterone is metabolized under influence of several other enzymes present in the placenta, *viz.*, 5α-reductase, 3β-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase, 21-hydroxylase, 6-oxygenase and enzymes which convert C_{21} precursors to androstenedione. The identification of the metabolites was based on chromatographic purification procedures and recrystallization of the isolated compounds to constant specific activity. These methods of identification, however, do not rule out the possibility that radioactive precursors are converted in smaller amounts to other metabolites.

In the mouse placenta progesterone is metabolized primarily to saturated 5α-pregnane derivatives. This observation demonstrates once more the species differences in progesterone metabolism in the placenta. In similar studies, for

Table 3. Percentage conversion of [4-^{14}C] progesterone to the various metabolites in placentae of R-III mice

Metabolites	10 days after mating	20 days after mating
A_1	20·9[a]	21·2
A_2	10·8	5·9
5α-Pregnane-3β,20α-diol	16·0	10·0
Deoxycorticosterone	1·2	3·1
20α-Dihydroprogesterone	5·5	3·4
4-Androstene-3,17-dione	7·8	9·8
3β-Hydroxy-5α-pregnan-20-one		
3α-Hydroxy-5α-androstan-17-one	26·2	33·7
20α-Hydroxy-5α-pregnan-3-one		
Progesterone[b]	6·3	3·3
5α-Pregnane-3,20-dione	6·0	9·7

[a]The percentage conversion of [4-^{14}C] progesterone was calculated by eluting radioactive peaks present on chromatograms (Average of 3 incubations). The flasks contained 200 mg of placental tissue. For other details of incubation see text.

[b]The presence of iso-polar metabolites of progesterone zone was excluded by using TLC and PPC, by acetylation of this material and by measurement of retention of radioactivity on the chromatograms.

instance, it has been shown that sheep and bovine placental tissues metabolize progesterone mainly to saturated 5β-pregnane derivatives [3].

The fact that the mouse placenta is capable of forming androstenedione from pregnenolone and progesterone is of special interest.

The presence of 17α-hydroxyprogesterone in mouse placentae has not been detected in this study and no suppositions about the biosynthetic pathway leading to androstenedione can be made. The androstenedione formed by mouse placentae is probably not converted to estrogens since no labelled phenolic compounds were isolated. No such conversion was observed, either, when mouse placental tissue was incubated with [4-^{14}C] testosterone (Rembiesa *et al.*, unpublished data). Our results agree with the observation of Vinson and Jones [17] that mouse placentae do not readily produce estrogens. These authors, however, reported that phenolic compounds appeared when fetal tissue was incubated with progesterone. These observations provide suggestive evidence that the fetus may convert placental androstenedione to estrogens.

Another interesting metabolite of pregnenolone and progesterone is deoxycorticosterone. It was the first corticosteroid to be identified in mammalian placenta. The mouse placenta seems to be unusual among the placentae of mammals in that it apparently synthesizes steroids belonging to both groups: androgens and corticosteroids. Furthermore it differs from the placentae of other rodents in being richer in steroid metabolizing enzymes [18, 19].

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