Metabolism of [^3H]oestrone sulphate by fetal membranes, placenta and uterine tissues from pregnant rabbits

J. R. G. Challis and Ellen Greenblatt*

*Departments of Obstetrics & Gynaecology and Physiology, University of Western Ontario, University Hospital, London, Ontario N6A 5A5 and *Department of Physiology, McGill University, Montreal, P.Q., Canada*

Summary. The distribution of radioactivity within the fetal membranes and uterine tissues from pregnant rabbits after an intra-amniotic injection of [^3H]oestrone sulphate was determined, and the conversion to oestrone and oestradiol-17 β during in-vitro incubation of these tissues with [^3H]oestrone sulphate was measured. After intra-amniotic injection, radioactive oestrone and oestradiol-17 β were recovered from amniotic fluid, amnion, placenta, decidua and myometrium of the injected fetus, and, in some experiments, from tissues of an adjacent fetus. During incubation, extensive metabolism of oestrone sulphate occurred in placenta and chorion, and oestrone sulphatase and 17-oxidoreductase activities were found in these tissues as well as in amnion, decidua and myometrium. We suggest that oestrone sulphate, present in amniotic fluid, may be accessible for metabolism to unconjugated oestrogen by several intrauterine tissues of the pregnant rabbit.

Introduction

It is generally considered that prostaglandins are involved in the final train of events associated with parturition in various species of animals. One mechanism by which prostaglandin (PG) biosynthesis may be modulated at term is through the influence of different steroid hormones (see Thorburn, Challis & Robinson, 1977). In the rhesus monkey parturition was preceded by an increase in the concentration of PGF in amniotic fluid which was believed to reflect an increase in PG production. The raised PG levels were associated with an increase in the concentrations of oestrone and oestrone sulphate in amniotic fluid (see Challis, Robinson & Thorburn, 1977b). In primates, the fetal membranes are a major site of PG synthesis (MacDonald, Porter, Schwarz & Johnston, 1978), and the possibility has been raised that free or conjugated oestrogen, present in amniotic fluid, might be accessible to the fetal membranes or decidua where, after further metabolism, the steroid could influence PG synthesis (Challis *et al.*, 1977b). Earlier studies had shown that human chorionic tissue was able to hydrolyse dehydroepiandrosterone sulphate and pregnenolone sulphate (Warren & French, 1965; Gant, Milewich, Calvert & MacDonald, 1977) and in a single experiment the conversion of oestrone sulphate to oestrone by human chorion has been demonstrated (Turnbull *et al.*, 1977).

Previous investigators have demonstrated 17 β -oxidoreductase activity in the uterus (Jütting, 1966), and aromatase activity in the placenta (George, Tobleman, Milewich & Wilson, 1978) of the rabbit. In order to examine the possibility that oestrone sulphate from amniotic fluid might be metabolized to unconjugated oestrogen within the pregnant uterus, we have measured the conversion of oestrone sulphate to oestrone and oestradiol-17 β after in-vivo intra-amniotic injection, and during in-vitro incubation of the steroid with fetal membranes and uterine tissues.

Materials and Methods

Animals

Norfolk White rabbits (Canadian Hybrid Farms, Stansted, P.Q.) were mated during a 2 h period of a day that was arbitrarily designated Day 0 of pregnancy. The animals were transported to the laboratory on Day 18 of gestation and housed individually until subsequent experimentation.

Materials

[6,7-³H(N)]Oestrone sulphate, ammonium salt (sp. act. 53 Ci/mmol), [4-¹⁴C]oestradiol-17 β (sp. act. 59.0 mCi/mmol) and [4-¹⁴C]oestrone (sp. act. 59.0 mCi/mmol) were purchased from New England Nuclear, Boston, Massachusetts. The purity of the oestrone sulphate was confirmed by thin-layer chromatography (t.l.c.) in the system benzene:methanol:chloroform:10 M-ammonium hydroxide (48:60:84:8, by vol.), and, after solvolysis, in the t.l.c. system cyclohexane:ethyl acetate (60:40, v/v). Labelled oestrone and oestradiol-17 β were purified by t.l.c. in the system cyclohexane:ethyl acetate (50:50, v/v). Non-radioactive steroids were obtained from Steraloids (New York). Solvents were purchased from American Chemicals, Ltd (Montreal, P.Q.) and were used without further purification. Other chemicals were purchased from Fisher Scientific Ltd.

Experiments

In vivo. Rabbits at Days 24–26 were anaesthetized with pentobarbitone sodium (Nembutal; Abbott) (30 mg/kg). The abdomen was opened under supplemental 1% xylocaine (Astra Pharmaceuticals). One uterine horn was delivered onto a warm, moist gauze, and a small incision was made in the non-placental surface of the myometrium over one fetus. A 21-gauge needle attached to a three-way stopcock was introduced into the amniotic cavity. Amniotic fluid (0.2 ml) was withdrawn into one 1 ml syringe. [³H]Oestrone sulphate (10 μ Ci), dissolved in 0.2 ml saline, was injected through a second syringe, and flushed in with the 0.2 ml amniotic fluid that had been previously aspirated. After 10 min (3 experiments) or 30 min (3 experiments), samples were taken of amniotic fluid, amnion, chorion, placenta and uterine wall (decidua, myometrium) of the injected fetus and of an adjacent fetus. The samples were rapidly frozen in an acetone/solid CO₂ bath and stored at -20°C until their content of [³H]oestrone and [³H]oestradiol-17 β was determined.

In vitro. Tissues were taken from 5 rabbits on Day 24, and from 3 animals on Day 30. The rabbits were anaesthetized with pentobarbitone sodium (30 mg/kg), supplemented with 1% xylocaine hydrochloride along the abdominal midline. The uterus was exposed, and samples were taken of amniotic fluid, amnion, chorion, placenta, decidua and myometrium. The tissues were collected onto ice, rinsed with Krebs–Henseleit–Ringer solution (Dawson, Elliott, Elliott & Jones, 1969), blotted, weighed and homogenized using a Polytron homogenizer (Brinkman Ltd) in Krebs–Henseleit–Ringer solution (500 mg tissue/ml) at 4°C. The tissue homogenates and amniotic fluid were centrifuged at 1000 g for 15 min at 4°C. The supernatant from the equivalent of 200 mg of the original tissue was taken for incubation. With each animal, duplicate incubations were performed in large glass test-tubes containing 0.3 μ Ci [³H]oestrone sulphate dissolved in Krebs–Henseleit–Ringer solution to give a final volume of 2.5 ml. Amniotic fluid (1 ml) was diluted with 1.5 ml Krebs–Henseleit–Ringer solution. Each incubation was performed in a shaking water bath maintained at 37°C, under an atmosphere of 95% oxygen:5% CO₂. After 30 min, the reaction was stopped by rapid freezing in an acetone/solid CO₂ mixture, and the tubes were stored at -20°C until subsequent analysis, usually within 2–3 days of the incubation.

For analysis, the samples were thawed at 4°C. Known amounts (~1000 d.p.m.) of [^{14}C]oestrone and [^{14}C]oestradiol-17 β were added to each sample and the contents of the tubes were mixed. Unconjugated steroids were extracted with 2 $\times$ 2 volumes diethyl ether (Mallinckrodt). The radioactivity in an aliquot of the residual aqueous phase was determined. The combined ether extracts were dried, and the samples were applied to t.l.c. plates (Analabs, NEN-silica gel type GF) with ethyl acetate. Non-radioactive oestrone (25 μg) and oestradiol-17 β (25 μg) were added to each t.l.c. lane. The chromatograms were developed in the solvent system cyclohexane:ethyl acetate (1:1, v/v), and the oestrogens were located either by their absorption at 254 nm, or by a short exposure to iodine vapour. This solvent system separates oestrone, oestradiol-17 α , oestradiol-17 β and 16-oxo-oestradiol-17 β (Challis, Harrison & Heap, 1973b). The zones corresponding to oestrone and oestradiol-17 β were eluted twice with 1 ml ethyl acetate into scintillation vials, and the radioactivity was determined on a Beckman LS 9000 spectrometer using a pre-set double isotope programme to calculate d.p.m. For both steroids, the tritium count was corrected for methodological losses by using the radioactivity present as ^{14}C , and the percentage conversion from [^3H]oestrone sulphate was calculated.

In two experiments for each tissue, aliquots of the oestrone and oestradiol-17 β eluates from t.l.c. were taken, and the appropriate ^{14}C isotope was added to give $^3\text{H}:^{14}\text{C}$ ratios of between 1 and 10. After the addition of non-radioactive steroid (15 mg), oestrone and oestradiol-17 β were recrystallized 3 times from ethanol/water and the $^3\text{H}:^{14}\text{C}$ ratios in successive sets of crystals and mother liquors were determined.

Radioimmunoassay

Samples of amniotic fluid were collected from the fetuses of 12 rabbits on Day 24. The concentration of oestrone sulphate was measured by radioimmunoassay after extraction and solvolysis (Challis *et al.*, 1977a). The oestrone antibody was prepared against 1,3,5(10)-oestratrien-3-ol-6,17-dione 6-CMO-BSA (Steraloids, N.Y.) in a sheep. It was used at a dilution of 1:8000, and had the following cross-reactivities: oestrone, 100%; epioestrinol, 2.6%; 16-oxo-oestradiol-17 β , 1.9%; oestradiol-17 α , 1.5%; oestradiol-17 β , <0.1%. In a preliminary study, [^3H]oestrone sulphate was added to rabbit amniotic fluid. After extraction and solvolysis, the recovery of radioactivity as unconjugated oestrone was >97%. In subsequent assays, 1000 d.p.m. [^3H]oestrone were added to the samples after solvolysis to monitor the losses during subsequent extraction and microcelite column chromatography. All the samples of rabbit amniotic fluid were measured in one assay. The blank value was not significantly different from zero, and the sensitivity of the assay was about 6 pg/tube (two standard deviations from zero). The intra-assay coefficient of variation for oestrone was 8.8% ($n = 6$).

Results

In-vivo experiments

The average concentration of oestrone sulphate in samples of amniotic fluid collected on Day 24 was 77 ± 10 pg/ml (mean $\pm$ s.e.m., $n = 12$). Insufficient amniotic fluid was obtained on Day 30 for analysis.

At 10 min and 30 min after the intra-amniotic injection of [^3H]oestrone sulphate, radioactivity was isolated as oestrone and oestradiol-17 β in the amniotic fluid, amnion and placenta of the injected fetus, as well as in decidual and myometrial tissue taken from the corresponding uterine segment (Table 1). Radioactive oestrone and oestradiol-17 β were also found in the amnion and placenta of the fetus adjacent to that injected. Considerable variation was encountered between different rabbits, and precluded statistical analysis of these data.

Table 1. Distribution of radioactivity as (A) oestrone and (B) oestradiol-17 β after intra-amniotic injection of 10 μ Ci [3 H]oestrone sulphate into rabbits at Day 24–26 *p.c.*

Exp.	Injection– delivery interval (min)	Injected conceptus					Adjacent conceptus				
		AF	Amnion	Plac.	Dec.	Myom.	AF	Amnion	Plac.	Dec.	Myom.
A. 1	10	3767	1518	10	—	307	nd	56	nd	—	nd
2	10	48252	13882	2198	8311	—	—	12733	1930	5413	—
3	10	550	4187	313	10	28	nd	594	nd	nd	nd
4	30	—	2544	110	—	132	—	207	18	—	29
5	30	1439	1003	132	308	—	614	947	999	264	—
6	30	—	4275	33	132	886	nd	nd	nd	18	100
B. 1	10	789	139	10	—	240	nd	70	nd	—	nd
2	10	35799	9676	1825	6198	—	—	9620	1593	4026	—
3	10	nd	599	150	33	42	nd	200	nd	nd	nd
4	30	—	1547	93	—	150	—	164	23	—	33
5	30	714	351	80	230	—	391	491	82	203	—
6	30	—	1426	43	116	nd	39	12	18	7	66

Values are d.p.m./ml or d.p.m./100 mg. AF, amniotic fluid; Plac., placenta; Dec., decidua; Myom., myometrium; —, no sample collected; nd, no radioactivity detected.

In-vitro experiments

Table 2 shows the recrystallisation data for oestrone and oestradiol-17 β . Constant $^3\text{H}:^{14}\text{C}$ ratios were achieved for both steroids formed by amnion, placenta, decidua and myometrium and for oestradiol-17 β formed in chorion. There was considerable metabolism of oestrone sulphate, especially by chorion and placenta, to ether-soluble products (Table 3). At both times of pregnancy studied, there was conversion of oestrone sulphate to oestrone and oestradiol-17 β by amnion, placenta, decidua and myometrium. The radioactivity co-chromatographing with oestrone after the incubation of oestrone sulphate with chorion was not identified as oestrone by recrystallization. Under the conditions of these experiments, the conversion of oestrone sulphate to oestrone by myometrium was significantly higher on Day 30 than on Day 24 (*t* test, $P < 0.01$; Table 3).

Table 2. Evidence, as $^3\text{H}:^{14}\text{C}$ ratio, for radiochemical purity of isolated [^3H]oestrone and [^3H]oestradiol-17 β after incubation with [^3H]oestrone sulphate

Tissue	Original	1 X	1 ML	2 X	2 ML	3 X	Mean $\pm$ s.d.*	c.v.
[^3H]Oestrone								
Amnion	7.639	7.725	7.273	7.476	7.338	—	7.490 $\pm$ 0.192	2.56
Placenta	7.561	7.544	6.901	7.407	7.053	7.390	7.309 $\pm$ 0.271	3.71
Decidua	3.483	3.318	3.542	2.513	3.087	2.830	3.120 $\pm$ 0.400	12.82
Myometrium	8.455	7.953	8.194	7.629	8.237	7.773	8.040 $\pm$ 0.310	3.86
[^3H]Oestradiol-17β								
Amnion	1.668	1.628	1.388	1.596	1.812	1.558	1.608 $\pm$ 0.139	8.64
Chorion	1.840	1.777	1.783	1.639	1.767	1.702	1.751 $\pm$ 0.070	3.99
Placenta	1.425	1.326	1.707	1.174	1.337	1.332	1.384 $\pm$ 0.178	12.86
Decidua	1.470	1.484	1.622	1.476	1.348	1.366	1.461 $\pm$ 0.099	6.78
Myometrium	1.652	1.563	1.705	1.598	1.657	1.585	1.625 $\pm$ 0.055	3.38

The original sample was that after extraction and elution from t.l.c. The $^3\text{H}:^{14}\text{C}$ ratio was measured in the crystals (X) and mother liquor (ML) after successive recrystallisations from ethanol:water.

* Mean of all ratios, including original sample.

Small amounts of [^3H]oestradiol-17 β were formed from oestrone sulphate by all tissues examined (Table 3). There was no detectable conversion to oestrone or oestradiol-17 β by amniotic fluid collected on Day 24.

Table 3. Conversion of [^3H]oestrone sulphate to oestrone and oestradiol-17 β and the percentage of radioactivity remaining in the non-ether extractable fraction of tissues from pregnant rabbits

Day of gestation	Tissue	Percentage of initial radioactivity present		
		Oestrone	Oestradiol-17 β	Non-ether extractable
Day 24 (N = 5)	Amniotic fluid	N.D.	N.D.	96.78 $\pm$ 3.06
	Amnion	12.09 $\pm$ 3.34	0.16 $\pm$ 0.09	72.22 $\pm$ 9.26
	Chorion	No X	0.24 $\pm$ 0.18	67.38 $\pm$ 4.30
	Placenta	10.76 $\pm$ 2.85	0.12 $\pm$ 0.05	60.08 $\pm$ 6.82
	Decidua	11.28 $\pm$ 1.48	0.27 $\pm$ 0.13	85.58 $\pm$ 2.23
	Myometrium	6.68 $\pm$ 1.56	0.30 $\pm$ 0.11	74.45 $\pm$ 1.96
Day 30 (N = 3)	Amnion	1.88 $\pm$ 0.30	0.29 $\pm$ 0.27	80.63 $\pm$ 3.07
	Chorion	No X	0.30 $\pm$ 0.17	50.67 $\pm$ 6.78
	Placenta	4.21 $\pm$ 1.25	0.22 $\pm$ 0.19	66.03 $\pm$ 3.30
	Decidua	10.79 $\pm$ 0.55	0.05 $\pm$ 0.02	82.10 $\pm$ 1.67
	Myometrium	16.59 $\pm$ 0.20*	0.08 $\pm$ 0.01	68.43 $\pm$ 1.20

Values are mean $\pm$ s.e.m. Each incubation was performed in duplicate on the number of rabbits indicated (N). The [^3H]oestrone and [^3H]oestradiol-17 β formed when [^3H]oestrone sulphate was incubated with Krebs–Ringer solution alone and subsequently processed (blank value) was subtracted in each individual experiment.

No X = radiochemical purity not achieved during successive recrystallisations. N.D., no detectable conversion.

* Conversion significantly different ($P < 0.01$) between Days 24 and 30.

Discussion

After an intra-amniotic injection of oestrone sulphate, unconjugated oestrone and oestradiol were found in several intrauterine tissues, including the fetal membranes. Because of the variation between experiments, probably partly associated with differences in the amount of mixing with amniotic fluid that was accomplished on injection of the isotope, and the difficulties of sampling from exactly the same sites in successive studies, the results could not be interpreted quantitatively. However, it is of note that Diczfalussy *et al.* (1963) found unconjugated radioactivity in the placenta and fetal membranes, 45–50 min after the intra-amniotic injection of [4^{14}C]oestrone sulphate to women requesting termination of pregnancy at 12–20 weeks. This pattern contrasted with that in other fetal tissues (e.g. skin, lungs, liver) in which most of the radioactivity was not ether-extractable. Further identification of the unconjugated radioactive compounds was not reported in that study.

Other investigators have shown that human fetal membranes, especially chorion, have the capacity to hydrolyse dehydroepiandrosterone sulphate and pregnenolone sulphate (Warren & French, 1965; Gant *et al.*, 1977). These structures are known to possess other steroid metabolizing activities (Gant *et al.*, 1977), and may be of importance in regulating the intra-uterine hormonal milieu of late pregnancy. The present studies suggest that the rabbit might be a suitable animal in which to study aspects of the in-vivo regulation of steroid metabolism in the fetal membranes and other intrauterine tissues. Jütting (1966) and Russell & Thomas (1973) have previously shown the ability of the rabbit uterus to interconvert oestrone and oestradiol-17 β . We did not add NADPH, and the incubations were not performed at optimum substrate

concentrations, thus probably accounting for the lower formation of oestradiol *in vitro* than in our in-vivo experiments and in earlier reports. Indeed, Jütting (1966) showed that the equilibrium between oestrone and oestradiol progressively favoured oestradiol formation by the uterus with advancing gestation, and measurements of the endogenous oestrone and oestradiol concentrations in myometrial tissue taken from pregnant rabbits were consistent with that finding (Challis, Davies & Ryan, 1973a).

It is evident (Table 3) that unconjugated steroids other than oestrone and oestradiol-17 β were formed during the course of the incubations with oestrone sulphate. This was particularly true of placenta and chorion, the most active sites of sulphotase activity. Oestradiol-17 α is of major quantitative importance in the rabbit (Paquet & Layne, 1975), although the recrystallization data show that this oestrogen was not a contaminant in our measurements of oestrone and oestradiol-17 β . The nature of these other steroids, and their possible biological functions, will be of interest in future studies.

We thank Dr T. G. Kennedy for his helpful discussions. This work was supported by Grant No. MA-6070 from the Medical Research Council (Canada) and by the Jean and Richard Ivey Fund. J.R.G.C. is a Scholar of the Canadian Medical Research Council.

References

- Challis, J.R.G., Davies, I.J. & Ryan, K.J. (1973a) The concentrations of progesterone, estrone and estradiol-17 β in the myometrium of the pregnant rabbit and their relationship to the peripheral plasma steroid concentrations. *Endocrinology* **95**, 160–164.
- Challis, J.R.G., Harrison, F.A. & Heap, R.B. (1973b) The kinetics of oestradiol-17 β metabolism in the sheep. *J. Endocr.* **57**, 97–110.
- Challis, J.R.G., Hartley, P., Johnson, P., Patrick, J.E., Robinson, J.S. & Thorburn, G.D. (1977a) Steroids in the amniotic fluid of the rhesus monkey (*Macaca mulatta*). *J. Endocr.* **73**, 355–363.
- Challis, J.R.G., Robinson, J.S., & Thorburn, G.D. (1977b) Fetal and maternal endocrine changes during pregnancy and parturition in the rhesus monkey. In *The Fetus and Birth*, pp. 211–255. Eds J. Knight & M. O'Connor. Elsevier, Amsterdam.
- Dawson, R.M.C., Elliott, D.C., Elliott, W.H. & Jones, K.M. (1969) *Data for Biochemical Research*, 2nd edn. Oxford University Press, London.
- Diczfalussy, E., Tillinger, K.G., Wiquist, N., Levitz, M., Condon, G.P., & Dancis, J. (1963) Disposition of intra-amniotically administered estriol-16-C¹⁴ and estrone-16-C¹⁴ sulfate by women. *J. clin. Endocr. Metab.* **23**, 503–509.
- Gant, N.F., Milewich, L., Calvert, M.E. & MacDonald, P.C. (1977) Steroid sulfatase activity in human fetal membranes. *J. clin. Endocr. Metab.* **45**, 965–972.
- George, F. W., Tobleman, W.T., Milewich, L. & Wilson, J.D. (1978) Aromatase activity in the developing rabbit brain. *Endocrinology* **102**, 86–91.
- Jütting, G. (1966) Die Wirkung von Oestrogenen auf die 17 β -Hydroxysteroid: NAD-Oxydoreduktase des Myometriums von Kaninchen. *Geburtsh. Frauenheilk.* **26**, 636–639.
- MacDonald, P.C., Porter, J.C., Schwarz, B.E. & Johnston, J.M. (1978) Initiation of parturition in the human female. *Seminars in Perinatology* **2**, 273–286.
- Paquet, A. & Layne, D.S. (1975) The diglycoside of 17 α -estradiol from rabbit urine: comparison with material prepared by synthesis. *Can. J. Biochem.* **53**, 293–297.
- Russell, S.L. & Thomas, G.H. (1973) The inter-conversion of oestrone and oestradiol-17 β and the concentration of oestradiol-17 β receptors in cultured rabbit uterus. *J. Endocr.* **56**, 203–211.
- Thorburn, G.D., Challis, J.R.G. & Robinson, J.S. (1977) Endocrine control of parturition. In *Biology of the Uterus*, pp. 653–732. Ed. R. M. Wynn. Plenum Press, New York.
- Turnbull, A.C., Anderson, A.B.M., Flint, A.P.F., Jeremy, J.Y., Keirse, M.J.N.C. & Mitchell, M.D. (1977) Human parturition. In *The Fetus and Birth*, pp. 427–452. Eds J. Knight & M. O'Connor. Elsevier, Amsterdam.
- Warren, J.C., & French, A.P. (1965) Distribution of steroid sulfatase in human tissues. *J. clin. Endocr. Metab.* **25**, 278–282.

Received 29 January 1979