Conjugated and unconjugated oestrogens in fetal and maternal fluids of the cow throughout pregnancy

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Summary. The changes in concentration of oestrone, oestradiol (-17α and -17β), oestrone sulphate and the oestradiol sulphates have been measured in allantoic and amniotic fluids and in maternal peripheral plasma throughout gestation. Oestrone sulphate was the major oestrone present in all of the fluids. It was measurable in allantoic fluid before Day 52 and reached a peak concentration of 475 ng/ml around Day 133. A lower peak occurred in the amniotic fluid around Day 110. The changes in oestradiol sulphates in allantoic fluid were similar to those of oestrone sulphate but at a much lower level. Considerable fluctuation was observed in the oestradiol sulphate concentrations in amniotic fluid. The ratio of oestradiol- 17α sulphate to oestradiol- 17β sulphate was considerably higher in amniotic fluid than in allantoic fluid. Consistent changes in the levels of oestrone and the oestradiols were found in amniotic fluid but not in allantoic fluid during the second half of pregnancy. In maternal peripheral plasma oestrone sulphate was measurable before Day 72. In the limited number of samples analysed no difference in oestrogen concentration due to the sex of the fetus was evident in any of the fetal or maternal fluids.

Introduction

As part of a comparative study on the role of oestrogens synthesized by the conceptus early in pregnancy in the cow, ewe and sow, Robertson & King (1975) and Robertson, King & Carnegie (1976) have reported that the concentration of oestrone sulphate increased in the chorioallantoic fluid of the pregnant cow during the 2nd month of gestation. Similar observations have been made for the chorioallantoic fluid of the sheep (Carnegie & Robertson, 1978) and the peripheral plasma of the pregnant sow (Robertson & King, 1974; Robertson, King & Dyck, 1978). As it is well established that steroid hormones influence the development and maturation of the fetus during intrauterine life (Jost, 1969, 1970; Gorski, 1971) and, later, the induction of parturition (Liggins, 1969), it was considered expedient to extend our investigations on the concentrations of oestrogens in fetal and maternal fluids of the pregnant cow to cover the complete period of pregnancy. The only work so far reported on oestrogens in fetal fluids of the cow is that of Challis *et al.* (1974) on unconjugated oestrogens in the serum of the fetal calf.

Materials and Methods

Animals

Heparinized samples of maternal blood were collected at the time of commercial slaughter from 22 Holstein–Friesian and cross-bred Friesian dairy cows covering a gestation age of Days 72–270. The samples were immediately chilled in ice-cold water and centrifuged within 4 h of collection. The plasma was transferred in 3 ml aliquots to stoppered glass vials, frozen over solid CO_2 and stored at -20°C until assayed. The uterus was removed within 20 min of death and the

conceptus exposed by carefully dissecting through the uterus along the mesometrial surface, taking care not to rupture the chorioallantois. Samples of allantoic fluid were aspirated with a minimum of contamination using sterile syringes fitted with 18-gauge needles. In some instances the presence of a transparent gelatinous mass in the allantoic sac made it difficult to obtain samples. The chorioallantois was then ruptured to drain off excess allantoic fluid and to expose the amniotic sac. Amniotic fluid samples were collected in a manner similar to that for allantoic fluid. The fluids were frozen over solid CO₂ in 3 ml aliquots in stoppered glass vials and stored at -20°C until assayed.

The fetuses were removed, their sex determined, and the crown-rump length measured. The approximate gestational ages were then determined using the tables compiled by Roberts (1956). For the period between Days 32 and 68, maternal and fetal samples were obtained in a similar manner from 4 Friesian dairy cows of known gestational age (Day 0 = day of insemination).

Oestrogen assay

The rabbit anti-oestrogen serum (courtesy of Dr B. V. Caldwell) used in the assays cross-reacts as follows: oestradiol-17β, 100%; oestradiol-17α, 30%; oestrone, 60%. All the standard curves were prepared by the displacement of [³H]oestradiol-17β. The cross-reactivity with oestradiol-17α was only 30% of that with oestradiol-17β and since the total oestradiol values were determined using an oestradiol-17β standard curve the total oestradiol concentrations would be underestimated if a significant amount of oestradiol-17α were present. The volume of fluid analysed ranged from 0.5 to 2 ml depending upon the concentration present. All assays were carried out in duplicate and have been corrected for individual procedural losses by incorporating a tritiated standard of oestrone, oestradiol-17β, oestradiol-17α or oestrone sulphate.

Unconjugated oestrogens. Unconjugated oestrone, oestradiol-17β and oestradiol-17α were determined as described by Abraham, Tulchinsky & Korenman (1970), Robertson, Smeaton & Durnford (1972) and Carnegie & Robertson (1978). The mean recoveries and sensitivities were 80% and 15 pg/tube for oestrone and 68% and 12 pg/tube for oestradiol.

Oestrogen sulphates. Following benzene extraction of the unconjugated oestrogens, conjugated oestrogens in the form of oestrogen sulphates were assayed in both plasma and fetal fluids using the method of Loriaux, Ruder & Lipsett (1971) as modified by Robertson & King (1974) and Carnegie & Robertson (1978). The sensitivity was 40 pg for oestrone sulphate, expressed as the unconjugated oestrone equivalent, and about 45 pg for oestradiol sulphate. It was not established whether the oestradiol sulpho-conjugates were present as the 3 or 17-mono-sulphates and/or 3,17-disulphates. If they arise from the reduction of oestrone sulphate then the sulphate group will occupy the 3 position. In a selected series of samples oestradiol-17β sulphate and oestradiol-17α sulphate were separated after solvolysis by subjecting the mixture to a selective oxidative procedure using the specificity, under certain conditions (Robertson *et al.*, 1972), of a 17β-hydroxysteroid dehydrogenase preparation which converted oestradiol-17β to oestrone leaving the majority (98%) of the oestradiol-17α unchanged. Oestrone and oestradiol-17β were then separated on celite columns, assayed and the results expressed in terms of oestradiol-17β and oestradiol-17α.

Results

For ease of comparison of data, the oestrogen sulphate concentrations have been expressed as the unconjugated oestrogen equivalent. To correct for the actual gravimetric amounts of oestrone sulphate and of oestradiol sulphate (as monosulphate) the values should be multiplied by 1.4.

Table 1. The concentration of oestrone sulphate (expressed as oestrone) in allantoic and amniotic fluids of the cow during early pregnancy

Day of gestation	Oestrone sulphate (pg/ml)	
	Allantoic fluid	Amniotic fluid
32	<40	<40
41	40	<40
52	1200	120
68	7000	19 000

Text-fig. 1. The changes in concentration of oestrone sulphate in the (a) allantoic and (b) amniotic fluids of the cow throughout pregnancy. P = the time of parturition. Values are given as the oestrone equivalent and the curves are fitted by eye.

Allantoic and amniotic fluids

Oestrone sulphate. In allantoic fluid oestrone sulphate was first present in measurable amounts between Days 41 and 52 (Table 1). A peak of 475 ng/ml was reached around Day 132 and this was followed by a sharp drop in concentration to a nadir around Day 170. There appeared to be a second smaller rise and decline (Text-fig. 1a).

In amniotic fluid oestrone sulphate was first detected at Day 52 (Table 1). Concentrations then rose rapidly to reach a peak of about 200 ng/ml around Day 110 (Text-fig. 1b).

Oestradiol sulphates. During the first half of gestation the changes in concentration of the oestradiol sulphates in allantoic fluid were similar to those observed for oestrone sulphate. The earliest sample analysed (Day 72) contained 600 pg/ml (compared with 14.8 ng oestrone sulphate/ml) and the lowest detectable level (45 pg/ml) would probably have occurred around Day 50. The highest concentration (15.8 ng/ml) was found at the same time as the peak in oestrone sulphate (Day 132) but was 30-fold lower (Text-fig. 2a).

The earliest amniotic fluid sample analysed (Day 72) contained 100 pg oestradiol sulphate/ml, considerably less than that in allantoic fluid. Concentrations later in pregnancy were variable (Text-fig. 2b).

Ratio of oestradiol-17 α sulphate to oestradiol-17 β sulphate. Oestradiol-17 α sulphate was present in higher concentration than oestradiol-17 β sulphate in all of the samples analysed (Table 2) and the ratio in amniotic fluid was considerably higher than that in allantoic fluid (means of 50.2 and 7.2 respectively).

Oestrone and oestradiol. The concentrations of the unconjugated oestrogens were very low relative to those of the corresponding conjugated oestrogens (Table 3), with few exceptions being much less than 3% of the conjugated oestrogen concentrations.

Maternal jugular venous plasma

Oestrone sulphate. Oestrone sulphate was readily detectable in the earliest sample analysed (Day 72) and the level rose fairly sharply until Day 147, then remained elevated (Table 3).

Oestrone. Oestrone was not measurable until Day 117 and in general the changes paralleled those for oestrone sulphate but at about a 30-fold lower concentration (Table 3).

Oestradiol sulphates. Oestradiol sulphate was first detected at Day 97 (Table 3). A gradual rise occurred over Days 97–177 to reach a concentration of about 1 ng/ml, but considerable fluctuations were then found between Days 177 and 270. Such differences may be due to

Table 2. The relative concentrations of oestradiol-17 β sulphate and oestradiol-17 α sulphate in allantoic and in amniotic fluids of individual cows

Day of gestation	Oestradiol-17 β sulphate (ng/ml)	Oestradiol-17 α sulphate (ng/ml)	Ratio 17 α :17 β sulphate
Allantoic fluid			
132	5.4	30.0	5.6
162	2.6	3.9	1.5
192	0.5	8.9	15.3
222	3.6	24.0	6.7
250	1.8	12.0	6.7
Amniotic fluid			
132	0.75	15.0	20.0
162	0.9	60.0	67.0
192	0.3	30.0	100.0
222	0.45	18.0	40.0
250	0.15	3.6	24.0

Text-fig. 2. The changes in concentration of the oestradiol sulphates ($-17\alpha + -17\beta$) in the (a) allantoic and (b) amniotic fluids of the cow throughout pregnancy. P = the time of parturition. Values are given as the oestradiol equivalent and the curves are fitted by eye.

individual variation during this period or two populations may be represented, one with a high level and one with a low level of oestradiol sulphate.

Oestradiols. Oestradiol was measurable from Day 97 and with one or two individual deviations a fairly steady concentration around 75 pg/ml was maintained until Day 261.

Effect of fetal sex on the unconjugated and sulpho-conjugated oestrogen concentration

No difference in oestrogen concentration in any of the fetal or maternal fluids could be attributed to the sex of the fetus. Oestrogens were produced by the conceptus of both sexes early in pregnancy. Nevertheless, quantitative differences which are not apparent in this study, because of the relatively small numbers of samples analysed at any one stage of pregnancy, may be present.

Table 3. The concentration (pg/ml) of unconjugated oestrogens in bovine fetal fluids, and of unconjugated and sulpho-conjugated oestrogens in maternal plasma, throughout pregnancy

Day of gestation	Allantoic fluid		Amniotic fluid		Maternal plasma			
	Oestrone	Oestradiols	Oestrone	Oestradiols	Oestrone	Oestradiols	Oestrone sulphate*	Oestradiol sulphates†
72	168	<40	<40	<40	<40	<40	82	<40
97	392	<40	786	<40	<40	136	260	68
117	72	<40	—	<40	66	<40	980	88
123	66	<40	160	<40	—	—	—	—
132	144	<40	200	<40	94	176	1520	228
147	144	<40	40	<40	144	74	2610	358
162	<40	<40	48	64	106	126	2500	388
162	<40	<40	92	93	80	108	2290	700
171	2670	450	54	<40	26	<40	1190	400
177	1600	184	—	—	266	76	3130	1140
183	160	<40	214	80	84	74	2820	1260
189	<40	<40	400	293	54	90	1910	900
192	<40	<40	<40	65	174	62	2868	228
198	<40	<40	120	80	214	220	2720	1260
207	<40	<40	692	272	200	94	2220	500
213	<40	52	306	400	120	86	2740	1200
222	84	68	426	200	134	68	3667	368
225	<40	<40	2666	360	62	68	2970	1280
234	1334	144	1600	293	54	70	2330	860
250	<40	<40	94	43	94	81	2875	388
261	<40	<40	1866	200	134	98	3570	1140
270	<40	<40	496	91	186	896	4038	488

* As the unconjugated oestrone equivalent.

† As the unconjugated oestradiol equivalent.

Discussion

The data show that, within the limits of the sensitivity of the assay and of the number of animals sampled, oestrogens were present in the fetal fluids of the cow earlier than Day 52. The principal oestrogen was oestrone sulphate which attained the very high concentration of 475 ng/ml in allantoic fluid around Day 132.

The peak in the concentration of oestrone sulphate in amniotic fluid at Day 110 precedes that in the allantoic fluid by some 23 days although the reverse has been found for the ewe (Carnegie & Robertson, 1978). The initial rise in oestrone sulphate concentrations can be attributed to the increase in the mass of the placental tissue, membranes and/or fetus. At present the source of the oestrogens is not known. Gadsby, Burton, Heap & Perry (1976) failed to obtain evidence for oestrogen synthesis *in vitro* by either tissues or membranes of the early bovine or ovine conceptus although Attal (1969) had detected oestrogen precursors, androstenedione and testosterone, in the testes of fetal lambs by Day 30 of intrauterine life.

No explanation can be offered for the precipitous drop in concentration of oestrone sulphate observed in bovine fetal fluids between Days 132 and 170 when the mass of the conceptus is still increasing. There appears to be a marked depression of oestrogen synthesis since there is no evidence for a diversion of oestrogens into other fluids at this time. It is possible that the second peak of oestrone sulphate which is observed in the allantoic fluid arises from a new source. This pattern of oestrone sulphate concentrations in the allantoic fluid of the cow, i.e. a sharp peak relatively early in pregnancy followed by a second rise commencing around mid-pregnancy, and a subsequent decline towards late pregnancy, is similar to that found in the allantoic fluid of the ewe (Carnegie & Robertson, 1978) and in the peripheral plasma of the pregnant sow (Robertson

& King, 1974; Robertson *et al.*, 1978) although the initial peaks occur at different times of gestation (Day 135 for the cow, 45 for the ewe and 30 for the sow). In contrast to the pattern observed in the maternal peripheral plasma, there is no evidence for an increase in oestrone sulphate levels in samples of allantoic and amniotic fluid obtained during late gestation. A similar observation has been reported for the ewe (Carnegie & Robertson, 1978).

This drop in the concentration of the oestrogen sulpho-conjugates late in pregnancy is in keeping with an observed fall in the concentration of the unconjugated oestrogens in the blood of fetal calves during the 8th month (Challis *et al.*, 1974) and of the sulpho-conjugates in fetal ovine blood (Wong, Cox, Durrie & Thorburn, 1972; Findlay & Seamark, 1973). Findlay & Seamark (1973) also found a rise immediately before parturition.

When representative samples of amniotic and allantoic fluids were analysed for oestradiol-17 α sulphate and oestradiol-17 β sulphate it was found that their ratio was relatively constant for each fluid but was higher in amniotic fluid. Since the samples tested covered the period from Day 132 to 250 the predominance of oestradiol-17 α sulphate in the amniotic fluid at this time is indicative of fetal metabolism. The unconjugated oestrogens present in the fetal fluids at relatively low concentrations generally did not show any recognizable pattern and may be artefacts arising from a low degree of solvolysis of the sulpho-conjugates.

In the maternal blood the concentration of unconjugated oestrogens was low throughout pregnancy. The sulpho-conjugated oestrogens were measurable before Day 72 and the high concentration of oestrone sulphate relative to that for oestrone, oestradiol and the oestradiol sulphates present from Day 132 onwards is in keeping with the finding of Tsang, Hackett & Turner (1975) that at parturition oestrone sulphate was the major oestrogen in the plasma of the pregnant cow. Differences in the concentrations of unconjugated oestrogens in fetal blood have been reported for female and male fetuses, with females having the higher concentration (Challis *et al.*, 1974). It was not possible to confirm this finding from the limited data available.

Comparison of the present data with studies in the sow, in which oestrogen synthesis by the conceptus has been demonstrated *in vitro* as early as Day 12 (Perry, Heap & Amoroso, 1973) and oestrogens of embryonic origin have been detected in the maternal peripheral plasma by Day 17 (Robertson & King, 1974; Robertson *et al.*, 1978), suggests that the first appearance of oestrogens in the conceptus of the cow occurs much later in relation to the start of attachment to the maternal uterus. The cow may therefore be more like the ewe in which evidence for oestrogen synthesis by the conceptus was first found around Day 31 (Carnegie & Robertson, 1978). The failure to obtain evidence for oestrogen synthesis *in vitro* by tissues and membranes of the early bovine conceptus (Gadsby *et al.*, 1976), and the present data, tend to argue against oestrogens of embryonic origin being involved as an antiluteolytic or luteotrophic factor in the cow, but the role of these oestrogens, particularly the sulpho-conjugates, during early pregnancy is still obscure.

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