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CONJUGATED AND UNCONJUGATED ESTROGENS IN THE FETAL AND MATERNAL FLUIDS OF THE PREGNANT EWE: A POSSIBLE ROLE FOR ESTRONE SULPHATE IN PLACENTAL ATTACHMENT

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Abbreviations used in Thesis

CBG	Corticosterone-binding globulin
E_1	Estrone
E_1S	Estrone sulphate
E_2	Estradiol-17
E_2 S	Estradiol-17 sulphate
PGF_2	Prostaglandin F_2
THF:EA	Tetrahydrofuran : ethyl acetate

ABSTRACT

Estrogens (estrone sulphate, estrone, estradiol-17β sulphate and estradiol-17β) were measured in the allantoic and amniotic fluids and the maternal peripheral, uterine venous and ovarian venous plasma of the ewe throughout gestation. Particular emphasis was placed on the interval day 20 to day 60 of gestation since the estrogen profile in the various fluid compartments of the pregnant ewe during the time of the implantation of the fetus into the uterus of the mother was of primary interest in this study.

Estrone sulphate was the most important estrogen with respect to concentration present. It could first be measured in the allantoic and amniotic fluids at day 31 and peaked between days 45 and 50 of pregnancy. It was found that the changes in levels of estrone sulphate, estrone and estradiol-17β sulphate in the allantoic fluid throughout gestation were very similar to what has been reported regarding estrone sulphate in the peripheral plasma of the pregnant pig, in the allantoic fluid of the cow and regarding total estrogens in the urine of the pregnant pig. A comparison of the estrogen levels in the uterine venous, ovarian venous and maternal peripheral plasma of the pregnant ewe implicated the conceptus, rather than the ovaries, as the source of these estrogens produced during gestation.

The possible site or sites of production of these estrogens within the conceptus were discussed along with theories regarding where the conversion of the estrogens to active estradiol-17β might take place. A functional relationship between estrogens and placental vascular development is postulated.

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INTRODUCTION

Research carried out in recent years has sparked much interest in the hormonal regulation of gestation in domestic animals. As various species of animals were studied, controversies arose as to the relative importance of estrogens, especially during early gestation (Nalbandov, 1971; Turner and Bagnara, 1971; Austin and Short, 1972; Aitken, 1974; Finn and Martin, 1974; Robertson and King, 1974; de Hertogh, Thomas, Bretlot, Vanderheyden and Ferin, 1975).

Implantation, that stage in which the conceptus establishes a functional contact with the mother for the purpose of physiological exchange (Shelesnyak, 1960) was found to be a particularly critical period in gestation in domestic animals with regards to embryonic survival (Hafez, 1967; Hafez and Jainudeen, 1974; Robertson, King and Carnegie, 1976). It was determined that 25 - 40% of the embryos of cattle, sheep and swine are lost at this time (Hafez and Jainudeen, 1974). Since studies have shown that the stage of maximum mortality of sheep embryos is the transition from yolk sac to allantoic placentation (Hafez and Jainudeen, 1974), an understanding of the endocrine regulation of this process would certainly prove useful in any attempts to reduce early prenatal mortality.

To date a reasonably complete picture has been obtained of the estrogens present in the peripheral plasma and urine of the

sow throughout gestation (see next section). However in the sheep and the cow, emphasis has been placed on looking at peripheral plasma estrogens within a few days of parturition and very little is known about the levels of estrogens in the maternal or fetal fluids of these animals during the rest of pregnancy. The purpose of this experiment was to study the various fluid compartments of the sheep during the early stages of gestation so that they might be compared with what is known about the pig and perhaps yield some general information regarding patterns of estrogen levels during pregnancy in domestic Artiodactyla.

I. Estrogens in the Urine and Peripheral Plasma of Domestic Animals during Gestation

A) The Sow

Estrogens present during gestation in the sow (term = 115 days) have been investigated quite thoroughly. It was shown, using biological assays and early chemical tests, that estrogenic activity is present in the urine of the sow during two time intervals of gestation. The first occurs between about the 20th and 33rd days, and the second at 11 or 12 weeks (Cowie, 1948). Velle (1958) demonstrated that estrone is the main estrogen excreted by the sow in late gestation and that it is present primarily as a conjugate.

Lunaas (1962) gave support to the early work of Cowie (1948) when he showed that, after acid hydrolysis of the urine, a

peak in estrone occurred between days 20 and 35 of gestation in the sow. Studies have indicated that implantation is in progress in this animal between the 14th and 28th days of pregnancy (Amoroso, 1952). Raeside (1963) confirmed that estrone is the primary urinary estrogen of the sow and gave further evidence that the pattern of excretion of estrone throughout gestation shows two peaks, one between about day 20 and day 35 and another appearing towards the time of parturition.

The peripheral plasma of the pregnant sow was investigated for the presence of estrogens, primarily towards the end of gestation (Shearer, Purvis, Jenkin and Haynes, 1972; Ash, Banks, Bailes, Broad and Heap, 1973; Molokwu and Wagner, 1973). Using radioimmunoassay and related techniques, it was found that unconjugated estrone and estradiol-17β concentrations increased from about 12 days prepartum to shortly before parturition and then declined rapidly as farrowing ensued (Ash et al, 1973; Molokwu and Wagner, 1973). Estrone was found to be the principal estrogen present (Molokwu and Wagner, 1973). Drane and Saba (1967) looked at both conjugated and unconjugated estrogenic activity in the peripheral plasma of the sow throughout gestation. Samples were taken at monthly intervals and it was determined that the levels of conjugated estrogens dropped significantly between day 25 and day 55 of gestation. Estrogenic activity increased again in the last month before term. When Robertson and King (1974) measured

the concentrations of unconjugated estrogens and of estrone sulphate in the peripheral plasma of the gilt throughout gestation, they clearly showed that estrone sulphate levels peaked between days 23 and 30, dropped and then steadily rose as parturition approached. Unconjugated estrogens could not be detected before day 70 of gestation. Conjugated estrone was also detected in the uterine venous plasma and allantoic fluid of the pig during early pregnancy (Lunaas, Refsdal and Schultz, 1973).

B) The Cow and the Ewe

Since urinary and peripheral plasma estrogens in the pregnant cow (term = 282 days) and ewe (term = 148 days) have been found to be present in similar patterns, they will be discussed together. Again, when most of the earlier research was done, the emphasis was placed on estrogens present at or near the time of parturition. In the cow and the ewe estradiol-17α was found to be the main urinary estrogen with estrone the second most abundant estrogen (Nelson and Smith, 1963; Fevre and Rombauts, 1966). Nelson and Smith (1963) measured estrone, estradiol-17α and estradiol-17β concentrations (following acid hydrolysis) in 24-hour urine collections made at days 50, 100, 200 and 275 of gestation in the cow. Levels of all 3 estrogens increased steadily until day 200 after which they rose very sharply until day 275. In the ewe, levels of urinary conjugated estrone and estradiol-17α increased

steadily between days 70 and 140 of gestation and then increased very rapidly until parturition (Fevre and Rombauts, 1966). Studies of estrogens in the peripheral plasma of these two species yielded much the same situation. In the cow, beginning about 3 weeks before term, the levels of unconjugated estrone, estradiol-17α and estradiol-17β started to rise; they peaked just before parturition and by 2 days post partum were scarcely detectable (Henricks, Dickey, Hill and Johnston, 1972; Robertson, 1974). Conjugated estrone concentrations increased from 16 weeks pre-partum until 5 days pre-partum, dropped slightly until 1 day pre-partum and then fell rapidly as calving took place (Robinson, Anastassiadis and Common, 1971). Studies on the ewe were concerned with the last 4 days before lambing. The concentration of plasma unconjugated estrogens (Challis, 1971; Challis, Harrison and Heap, 1971; Robertson and Smeaton, 1973; Tsang, 1974) and estrone sulphate (Tsang, 1974) increased rapidly in the ewe during the last 36 hours before term. Following parturition, the levels again dropped precipitously (Challis, 1971; Challis et al, 1971; Robertson and Smeaton, 1973; Tsang, 1974).

Conjugated estrogens have not been measured in the plasma of the cow or the ewe during early pregnancy. Peripheral plasma taken from cows during the first 39 days of gestation was assayed for unconjugated estrogens but the levels remained at 4 pg/ml or below (Henricks et al, 1972). Sheep plasma has not been tested for

the presence of estrogens during early gestation.

II. Reproduction in the Ewe

A) General Information

The ewe is a seasonally polyestrous animal, being anestrus during spring and summer. The estrous cycle is normally 16 to 18 days and it has been found that estrus lasts for about 29 hours (Swenson, 1970). Gestation is about 148 days.

At the time of ovulation in the ewe, as is the case in the cow and the sow, the first polar body of the egg has been discarded and the egg is at metaphase of the second maturation division which is not completed until fertilization takes place. The shed ovum consists of a nucleus surrounded by vitellus which is in turn surrounded by a vitelline membrane and an outer thick zona pellucida. In the ewe, as in the other domestic mammals studied, the cumulus oophorus is lost after ovulation (Swenson, 1970).

The sheep conceptus enters the uterus from the Fallopian tube at about the third day after fertilization (Davies and Wimsatt, 1966). By the fifth day, the blastocoele has begun to accumulate fluid and by the eighth day it has become significantly enlarged. At about the tenth day the zona pellucida is lost and the blastocyst has become an inner cell mass (future embryonic disc) surrounded by cuboidal trophoblast cells which now are in direct contact with the

uterine epithelium (Amoroso, 1952).

8) Details on Placental Development

The placenta in the sheep consists of many localized sites of interaction between fetal and maternal tissue. The maternal component takes the form of 88 to 96 caruncles (Harvey, 1959), which are round, knob-like projections on the surface of the uterine mucosa extending toward the lumen in both the pregnant and non-pregnant, cycling and non-cycling animal. It is with these caruncles that the trophoblast comes in direct contact when the blastocyst expands. The caruncles are vascularized but contain no uterine glands (Amoroso, 1952).

By about day 12 the blastocyst has begun to elongate rapidly and by day 17 or day 18 it can extend throughout both uterine horns (Davies and Wimsatt, 1966). The nonvascular chorion is established when mesoderm extending out from the primitive streak, (this begins about day 14), fuses with the trophoblast (Amoroso, 1952). The allantois, another mesoderm derivative, grows out from the embryo as a bilobed sac and fuses with the chorion by about day 22. It is in this manner that a vascularized allantochorion is produced.

Studies have shown that the accumulation of fetal fluids occurring between days 16 and 18 presses the allantochorion against the maternal caruncles to which it adheres due to the stickiness of

the outer surface of the chorion (Harvey, 1959). This contact between fetal and maternal tissues is followed by the gradual erosion of the maternal epithelial layer covering the caruncles. In these areas the placentomes will develop and those fetal areas which thicken and develop opposite the maternal caruncles are termed cotyledons. Sheep form syndesmochorial placentae, meaning that of the six possible tissue layers existing in the placenta between fetal and maternal blood supplies (uterine vascular endothelium, uterine stroma, uterine epithelium, fetal allantochorion, fetal stroma, fetal capillary endothelium) only five are retained, the uterine epithelium being lost (Amoroso, 1952; Swenson, 1070). It was demonstrated that the degenerated uterine epithelium is replaced by cells of trophoblastic origin (Harvey, 1959; Davies and Wimsatt, 1966).

Villi begin to develop on the surfaces of the fetal cotyledons by day 31 (Amoroso, 1952; Harvey, 1959). These villi are composed of a central region of vascular mesoderm covered by a layer of trophoblast cells (Amoroso, 1952). They become larger and extensively branched and by interdigitating with corresponding ridges and grooves on the maternal caruncles, bring the two blood supplies into close proximity.

As caruncle and cotyledon enlarge, the caruncle forms a very convex structure into which the cotyledon fits. Davies and Wimsatt (1966) have stated that placentome development is not

complete until about day 90. They have also found that after this time some shrinkage of the placentome often occurs which may be accompanied by some decrease in the total number of placentomes (Davies and Wimsatt).

C. Hormonal Changes During Gestation

After about day 4, progesterone is present in the peripheral plasma of the ewe throughout the rest of gestation (Bassett, Oxborrow, Smith and Thorburn, 1969; Fylling, 1970; Sarda, Robertson and Smeaton, 1973). Sarda et al (1973) ovariectomized sheep early in pregnancy and maintained them on progesterone until day 83 to 94 at which time progesterone therapy was terminated. They found that after removal of the progesterone implants, peripheral plasma progesterone levels in the ovariectomized sheep remained as high as those in the intact animal. This indicates that the placenta plays an important role in the production of the steroid. In fact, it is generally accepted that the ovaries are not needed during the second half of gestation in the ewe (Turner and Bagnara, 1971).

A closer look at the situation has revealed that the peripheral progesterone levels remain fairly constant between day 20 and days 80 to 90 and then show a sharp increase (Sarda et al, 1973) which seems to coincide with the completion of placentome development. There seems to be some controversy over whether there

are two peaks in peripheral progesterone concentration in late gestation (Sarda et al, 1973) or just one (Bassett et al, 1969; Fylling, 1970). Furthermore, while Fylling (1970) found that peripheral progesterone levels began to drop 8 days before parturition, according to Sarda et al (1973), this decline began anytime from 1 to 11 days before term.

It has been found that accompanying the decrease in maternal progesterone levels, there is an increase in the concentration of corticosteroids in the fetal plasma (Bassett and Thorburn 1969; Comline, Silver and Silver, 1970). This is followed by an increase in maternal conjugated (Tsang, 1974) and unconjugated estrogen levels (Challis, 1971; Challis et al, 1971; Robertson and Smeaton, 1973; Tsang, 1974) which is in turn followed by an increase in $PGF_{2\alpha}$ levels in uterine venous plasma (Liggens, Grieves, Kendall and Knox, 1972; Thorburn, Nicol, Bassett, Shutt and Cox, 1972). Although all of these hormonal changes are involved in normal parturition in the ewe, the initial corticosteroid surge appears to be one of the instigating factors. Thus the fetal hypophysis and adrenal glands perform an important role in bringing about the onset of parturition (Flint, Anderson, Steele and Turnbull, 1975).

III. Implantation in the Rat and the Mouse

It was first recognized that estrogens and the process of

implantation might be interrelated when the importance of the ovaries for nidation in the rat was studied. Cochrane and Meyer (1957) found that the delay in implantation induced by ovariectomizing or hypophysectomizing pregnant rats and treating them with progesterone could be terminated if estrogen was also administered. Experiments in which embryos from intact rats were transferred by noon of day 6 of gestation to ovariectomized recipients showed that estrogens had to be administered before nidation would take place (Psychoyos, 1967). Further support came from experiments in which rats ovariectomized early in gestation were given various hormone treatments in an effort to induce implantation. Estrogens were always found to be a necessary requisite (Psychoyos, 1962) and the presence of these estrogens has been related to various changes taking place in the size and shape of the blastocyst at this time (Yasukawa and Meyer, 1966). Madhwa Raj, Sairam and Moudgal (1968) inhibited the estrogen surge in rats by giving a "minimum effective dose" of LH antiserum at 10.00 hours on day 4 of pregnancy and in this way were able to inhibit implantation. This could be reversed by giving estradiol-17β 8 hours after the anti-LH treatment (Madhwa Raj et al, 1968).

The same situation exists in mice. After experimentally delaying implantation in pregnant mice (they were ovariectomized on day 3 of gestation and given only a progesterone supplement), Smith and Biggers (1968) were able to induce implantation by giving

estradiol.

IV. Delayed Implantation

Studies of species normally exhibiting delayed implantation have also implicated estrogens in the process of implantation. Daniel (1974) looked at early pregnancy in the Alaskan fur seal, an animal in which blastocyst dormancy occurs between July and the end of October. He noted that a surge of estradiol-17β occurred in the peripheral plasma of the pregnant seal between the middle of October and early November, just prior to embryo reactivation and implantation. This same pattern of an estrogen surge coincident with the termination of blastocyst dormancy occurs in the roe deer, another animal displaying obligatory delayed implantation (Aitken, 1974). Since no obvious changes in the ovaries of the roe deer were apparent at this time (Aitken, Burton, Hawkins, Kerr-Wilson, Short and Steven, 1973), the idea was presented that the estrogen might be fetal rather than ovarian in origin.

V. Source of Estrogens produced during Gestation

A) The Sow

The suggestion that estrogens produced by the roe deer at the time of implantation might be fetal rather than ovarian in origin becomes very interesting when one returns to the subject of early gestation in the pig and what has been discovered regarding the

source of the estrogens present between days 20 and 35. If sows were either ovariectomized or hypophysectomized early in gestation and maintained on progesterone, they proceeded successfully to term and showed the same two-peaked pattern of urinary excretion of estrone as intact animals (Fevre, Léglise and Rombauts, 1968). When estrogens were measured in the various fluid compartments of the sow during early gestation, it was found that an estrone conjugate was present in much higher concentrations in the allantoic fluid and in the uterine venous plasma than in the ovarian venous plasma (Lunaas et al, 1973; Robertson and King, 1974). This estrone conjugate was identified as estrone sulphate and the suggestion was made that it could be involved in implantation (Robertson and King, 1974). These results suggest that the conceptus is the site of the estrogens produced during both early and late gestation in the sow.

This idea became even more acceptable when it was shown that the early pig blastocyst is capable of steroid hormone biosynthesis. Perry, Heap and Amoroso (1973) cultured pig blastocysts taken between days 14 and 16 of gestation and showed that they contained the necessary enzymes to produce estrogens from androgens or progesterone. These observations were supported by the work of Flood (1974). Furthermore, in 1975 Heap and coworkers revealed that, more specifically, 14 to 18 day pig blastocysts produced both estrone (primarily) and estradiol-17β from androstenedione and dehydroepiandrosterone and could convert estrone to estradiol-17β

by 14 days post coitus. It has recently been found that the 12-day pig blastocyst can produce estrone and estradiol-17β from androstenedione and dehydroepiandrosterone (Perry, Heap, Burton and Gadsby, 1976).

B) The Rabbit

Huff and Eik-Nes (1966) demonstrated that six day old rabbit blastocysts could convert acetate to cholesterol and pregnenolone and metabolize other steroid substrates such as pregnenolone and androstenedione.

C) The Rat

Even in the rat, which has been shown to require ovarian estrogens for implantation, Dickmann and Dey (1974) histochemically demonstrated Δ^5-3 -hydroxysteroid dehydrogenase activity in 4- and 5-day blastocysts. Certainly, at least in some species, the potential for steroid hormone biosynthesis does exist in the early conceptus.

D) The Ewe

Indications are that the sheep conceptus can also produce estrogens early in pregnancy. Sheep can be ovariectomized as early as day 4 of gestation and, as long as they are maintained on progesterone, gestation will proceed normally, at least up to

day 60 (Foote, Gooch, Pope and Casida, 1957; Moore and Rowson, 1959; Alexander and Williams, 1966; Bindon, 1971; Sarda et al, 1973; Moore, 1975). Intensive experiments were carried out by Moore (1975) in which embryos were transferred into previously ovariectomized ewes given only exogenous progesterone. Gestation proceeded successfully until day 25 when the animals were slaughtered. Cumming and his associates (1974) ovariectomized and adrenalectomized ewes on day 3 of gestation in order to determine whether estrogens of maternal adrenal origin were necessary for implantation. These animals were maintained only on cortisone,desoxycorticosterone and progesterone and pregnancy was interrupted at either days 21 or 22 or days 35 or 36. The results showed that gestation proceeded normally up to day 36 in these animals. These studies clearly demonstrate that estrogens, either of an ovarian or adrenal source, are not required for the initiation of implantation in the ewe. Furthermore, Challis et al, (1971) measured, between day 102 of gestation and term, the estrogen concentration in the uterine venous plasma of a ewe which had been ovariectomized on day 91 after mating These workers noted that the sharp increase in the level of peripheral estrogens which begins about 36 hours before parturition in the intact animal (Challis, 1971; Challis et al, 1971; Robertson and Smeaton, 1973; Tsang, 1974) occurred without the presence of the maternal ovaries.

VI. Concluding Remarks

In summary, previous research has shown that estrogens, be they of maternal or fetal origin, are present early in gestation in the rat and the pig and indications are that they play a vital role in implantation. Unfortunately there is a paucity of information on estrogens present in the ovine maternal plasma during the first 2/3 of pregnancy and in the fetal fluids throughout gestation. Therefore, in this research project, estrogens, both unconjugated and estrogen sulphates, were measured in the allantoic and amniotic fluids of sheep during the course of pregnancy and special emphasis was placed on the time interval 25 days to 60 days gestation in an effort to elucidate any relationship which may exist between estrogen levels and the process of implantation. Uterine venous and ovarian venous plasma samples were compared in order to indicate the source of the estrogens and the picture was completed by determining what effect pregnancy had on the levels of unconjugated and sulphated estrogens in the maternal peripheral plasma. Estrogen sulphates are of particular interest since they provide a more water-soluble form for the transport of estrogens and since the placenta and the uterus of the human, pig and sheep possess extensive sulphatase activity which would release the active hormone (Diczfalusy, 1964; Pack and Brooks, 1974; Rossier and Pierrepoint, 1974). Furthermore, the human fetus (Levitz, Condon and Dancis, 1961; Diczfalusy, 1964) and the placenta of

the guinea pig (Levitz, Condon, Money and Dancis, 1960), cow (Holcenberg and Rosen, 1965) and ewe (Pierrepoint, Anderson, Harvey, Turnbull and Griffiths, 1971; Rossier and Pierrepoint, 1974; John and Pierrepoint, 1975) have been shown to be capable of sulphurylating estrogens. The results obtained were compared with what is known about estrogen levels during pregnancy in other domestic animals, in particular the pig, to see if a general pattern exists.

MATERIALS AND METHODS

I. Animals, Chemicals and Apparatus

A) Experimental Animals

Forty cross-bred ewes, kept under standard conditions of management, were housed with a vasectomized ram equipped with a marking harness to determine estrus. Once estrous cycling was established, they were exposed to a fertile ram which was similarly equipped for the detection of mating behaviour. The animals were checked twice daily for marking and the first day of marking was taken as day 0 of pregnancy.

B) Radioactive Materials

Progesterone - 1,2,6,7^{3}H (S.A. = 103.7 Ci/mM), corticosterone - 1,2 ^{3}H (S.A. = 48 Ci/mM), estrone - 2,4,6,7 ^{3}H (S.A. = 98.5 Ci/mM), estradiol-17β - 2,4,6,7 ^{3}H (S.A. = 91.3 Ci/mM) and estrone sulphate, ammonium salt - 6,7 ^{3}H (S.A. = 47.9 Ci/mM) were obtained from New England Nuclear. Estrone glucuronide (S.A. = 7.59 X 10^{6} dpm/ M) was kindly provided by Dr. D. G. Williamson, University of Ottawa. All were stored in benzene-ethanol at 4^{o}C and the necessary dilutions were made in methanol and kept at -20^{o}C.

C) Nonradioactive Steroids

Nonradioactive progesterone and estrogens were obtained

in crystalline form from Steraloids, Inc. They were checked for purity by thin layer chromatography. Standard solutions were prepared in methanol and stored at -20°C.

D) Solvents

All solvents, petroleum ether, benzene, tetrahydrofuran, ethyl acetate, iso-octane (2,2,4-trimethylpentane) and ethylene glycol were obtained from Matheson, Coleman and Bell. The petroleum ether was reagent grade and was redistilled to obtain that fraction with b. p. 38-42°C. All of the others were spectroquality.

E) Other Chemicals and Solutions

The florisil (60-100 mesh) was purchased from Fisher Scientific. It was washed 8 times with distilled water followed by 2 washings with methanol to remove the finer particles. It was then dried for at least 24 h in a cool oven (70°C) and stored in a vacuum-tight dessicator.

Celite 545, an analytical filter aid, was obtained from Johns Manville. It was heated in a muffle furnace at 600°C for 18 h to remove any organic contaminants prior to use.

The charcoal (Norit A) was obtained from Matheson, Coleman and Bell and the dextran (T80) from Pharmacia.

The phosphate buffer, (buffer A, ph 7.0) used throughout the estrogen assay was prepared in the following manner; 5.38 g/l

$NaH_2PO_4 \cdot 4H_2O$, 16.35 g/l $Na_2HPO_4 \cdot 7H_2O$, 9g/l NaCl, 1 g/l NaN_3 and 1 g/l gelatin.

F) Corticosterone-binding Globulin (CBG)

The blood plasma of male dogs was the source of the CBG used in the competitive protein assay. The plasma was treated with charcoal to remove any steroids already bound to the globulin and frozen in 1 - 2 ml aliquots. To each 100 ml of distilled water, 0.5 to 1.0 ml of plasma was added to make the CBG solution, depending upon what concentration would give the most sensitive standard curve in the range of 0 to 8 ng, as determined from calibration curves.

G) Estrogen Antibody

Dr. B. V. Caldwell (Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts) kindly provided the estrogen antibody used in this research (sheep antiserum to estradiol-17β-hemisuccinyl bovine serum albumin, no. 029-18). This antibody also cross-reacted with estrone (65 - 70%). It was diluted 1:100 in buffer A and stored at -20°C. For use it was further diluted by a factor of 1:300 with the same buffer, resulting in a final working dilution of 1:30,000.

H) Treatment of Glassware

All glassware was washed thoroughly in detergent (Decon-75 concentrate, BDH) and rinsed many times in regular water, distilled

water and methanol. The columns, culture tubes and pasteur pipets used in the estrogen determinations were siliconized prior to use to prevent any adherance of the estrogens to the walls of the glassware. Since the glassware used for the collection of blood and for the storage of all fluids was not siliconized, there could have been some undetermined loss of estrogens during this stage of the experimental procedure.

I) Liquid Scintillation Counting

The scintillation fluid contained 100 g naphthalene and 8 g omnifluor (98% PPO and 2% Bis-MSB, New England Nuclear) per litre 1,4 dioxane. A Beckman LS-250 scintillation spectrometer, with a counting efficiency of about 40% for ^{3}H was used to measure the radioactivity in each sample. It counted the internal standards to an error of $\pm$ 3% and the assay samples to $\pm$ 1%.

II. Methods

A) Pregnancy Diagnosis

Pregnancy was determined using the competitive protein binding method of Robertson and Sarda (1971) for measuring progesterone in peripheral plasma. Jugular blood samples were collected into heparinized tubes between 08.30 and 09.00 h on days 1, 9 and 17 after marking. The blood was immediately chilled in an ice bucket, spun within one hour of collection in a refrigerated centrifuge (20 min at 2,000 rpm) and stored at -20^{o}C until used.

For each sample, 0.5 ml of plasma was extracted with 10

volumes of petroleum ether. These extracts, along with a 1 ml wash of each extraction tube, were transferred to culture tubes and evaporated under nitrogen. Along with tubes containing 0 to 8 ng of progesterone, for a standard curve, they were then incubated with an aqueous solution (1.0 ml per tube) containing corticosterone-binding globulin (0.5 to 1.0%) and ^{3}H corticosterone (about 25,000 cpm per ml solution) for 5 min at 37°C and 20 min in an ice bath. Removal of the unbound steroid from the CBG-bound was accomplished by adding 80 mg florisil to each tube, mixing for 30 sec and allowing the florisil to settle out of solution for 10 min, while the tubes were still kept at 1°C. Following this, 0.5 ml of the supernatant, containing the bound steroid, was transferred to a scintillation vial and the radioactivity measured.

In the ewe, in a normal estrous cycle, progesterone levels are low (0.1 - 0.2 ng/ml) for about 4 days about the time of estrus, increase to between 1.5 and 2.5 ng/ml by mid-cycle, plateau and then begin to decrease rapidly after day 14 to reach baseline levels by the next estrus (Thorburn, Bassett and Smith, 1969; Robertson and Sarda, 1971). If the animal becomes pregnant, the peripheral progesterone levels remain comparable to those present during the luteal phase of the cycle until day 50 of gestation (Bassett, Oxborrow, Smith and Thorburn, 1969; Robertson and Sarda, 1971). In this pregnancy test, progesterone concentrations were measured at day 1 of the cycle to ensure that the animal was in estrus at the

time of mating, at day 9 to ensure that ovulation had occurred followed by the formation of a functional corpus luteum, and at day 17 to determine whether the corpus luteum had regressed and the animal had returned to estrus or whether a corpus luteum of pregnancy had been formed (Robertson and Sarda, 1971). A correct pregnancy diagnosis was made in 39/40 cases, the only error being made when, in one animal, the levels were borderline and a clear distinction between pregnant and nonpregnant could not be made.

B) Collection and Treatment of Samples

Prior to sacrifice, laparatomy was carried out under gas anaesthesia (halothane) and heparinized samples of jugular, uterine and ovarian venous blood were obtained. Immediately after the animal was killed, the uterus was removed and within 15 min allantoic and amniotic fluid samples were collected from the exposed conceptus and frozen on solid CO_2. All blood samples were centrifuged within 3 h of collection and all plasma and fetal fluid samples were stored at $-20^{o}C$. Table 1 summarizes the numbers of animals used and the fluids examined at each stage of gestation.

C) Estrogen Radioimmunoassay

1) Unconjugated estrogens

The unconjugated estrogens, estrone (E_1) and estradiol-17β ($E_2\beta$) were measured in plasma and fetal fluids using the method

TABLE 1

Number of animals employed and measurements made at each stage of gestation

Day of Gestation	Number of Animals	Total Number of Fetuses	Fluids Collected	Parameters Determined (see footnote)
17	1		maternal peripheral	a
19	2		maternal peripheral	a
20	1	1	allantoic	a, b, c, d
	1		maternal peripheral	a, b, c, d
21	2		maternal peripheral	a

where a = E_1S, b = E_1, c = $E_2\beta S$, d = $E_2\beta$. Table continues.

22	1		maternal peripheral	a
23	1		maternal peripheral	a
24	1		maternal peripheral	a
25	3	3	allantoic	a, b, c, d
	2		uterine venous	a, b, c, d
	1		maternal peripheral	a
28	1	1	allantoic	a, b, c, d

30	1	1	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
31	1	2	allantoic	a, b, c, d
	1	2	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
33	2	3	allantoic	a, b, c, d
	2	2	amniotic	a, b, c, d
	2		uterine venous	a, b, c, d
	2		maternal peripheral	a, b, c, d

35	3	3	allantoic	a, b, c, d
	3	3	amniotic	a, b, c, d
	2		uterine venous	a, b, c, d
	2		maternal peripheral	a, b, c, d
	1		ovarian venous	a, b, c, d
40	2	2	allantoic	a, b, c, d
	2	3	amniotic	a, b, c, d
	2		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
46	2	2	allantoic	a, b, c, d
	2	3	amniotic	a, b, c, d
	2		uterine venous	a, b, c, d

46	2		maternal peripheral	a, b, c, d
	1		ovarian venous	a, b, c, d
50	2	4	allantoic	a, b, c, d
	2	4	amniotic	a, b, c, d
	2		uterine venous	a, b, c, d
	2		maternal peripheral	a, b, c, d
	2		ovarian venous	a, b, c, d
55	1	2	allantoic	a, b, c, d
	1	2	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d

60	1	1	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
65	1	1	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b
70	1	1	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
	1		ovarian venous	a, b, c, d

80	1	1	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
90	1	1	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
100	1	2	allantoic	a, b, c, d
	1	2	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d

110	7	8	allantoic	a, b, c, d
	4	4	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
	1		ovarian venous	a, b, c, d
120	1	1	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d
130	5	7	allantoic	a, b, c, d
	1	1	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d

130	1		maternal peripheral	a, b, c, d
140	1	2	allantoic	a, b, c, d
	1	2	amniotic	a, b, c, d
	1		uterine venous	a, b, c, d
	1		maternal peripheral	a, b, c, d

described by Robertson, Smeaton and Durnford (1972), as developed from the work of Korenman, Perrin and McCallum (1969) and Abraham, Tulchinsky and Korenman (1970). Some small modifications were made in the procedure where necessary and these are indicated below.

The volume of fluid extracted ranged from 0.5 to 2.0 ml depending upon the concentration present. With the exception of a few ovarian plasma samples, all measurements were carried out in duplicate. With each plasma sample were included approximately 1,000 cpm each of ^{3}H E_1 and ^{3}H $E_2\beta$ (in the form of about 4 or 5 pg of either steroid) which was later used to estimate recovery. Each sample was extracted with 10 volumes of benzene for 2 min and centrifuged at 1400 rpm for 5 min. Since it was found that 90-95% of the E_1 and $E_2\beta$ was recovered in a single extraction of benzene from plasma without adding 0.1 ml of 0.1N NaOH, this step was omitted. The benzene extracts were transferred, along with a 1 ml benzene rinse of the extraction tube, to siliconized culture tubes and the contents were evaporated under nitrogen. The dried extracts were resolubilized in 0.4 ml iso-octane for transfer to celite columns.

Celite was thoroughly mixed in a 2:1 (wt:vol) ratio with ethylene glycol and dry-packed using vacuum suction and a glass rod in 5 ml disposable pipets to a height of 4 cm. A glass bead held the contents in each column. Usually 24 columns were prepared and run each time.

The columns were washed with 2 times 5 ml of iso-octane and a sample applied to each in 1.5 ml of iso-octane. They were then rinsed with 3.5 ml of iso-octane followed by 1.5 ml of 7.5% ethyl acetate in iso-octane to move the estrogens down the columns. E_1 was eluted using 4.0 ml of 15% ethyl acetate in iso-octane and $E_2\beta$ with 4.5 ml of a 30% solution of the same.

The column eluates were evaporated under nitrogen and 0.5 ml methanol was added. The tubes were mixed and half (0.25 ml) was transferred to scintillation vials for the determination of recovery. Methanol, rather than buffer A (Robertson, Smeaton and Durnford, 1972) was used in this step, since the estrogens require several hours to solubilize in buffer A while they dissolve virtually instantly in methanol, allowing a more rapid and accurate determination of recovery to be made. The methanol remaining in the culture tubes was evaporated under nitrogen and 0.5 ml buffer A was added to each tube. This was followed by 0.1 ml of ^3H $E_2\beta$ in buffer A (about 12,000 cpm per 0.1 ml) and 0.1 ml of a 1/30,000 dilution of the antiserum. The standard curves for E_1 and $E_2\beta$ were obtained from tubes containing 0 to 300 pg of either steroid which underwent the same incubation procedure as the samples (Fig. 2).

Incubation was carried out overnight at 4^oC. A solution of 0.5% charcoal and 0.05% dextran in buffer A was used to separate unbound estrogen from bound. A 0.5 ml aliquot of this solution was added to each tube, which was mixed and left for 10 min in an ice

bath and then centrifuged for 2 min to remove the charcoal-steroid complex. The supernatant, containing the bound steroid, was transferred to a scintillation vial and the radioactivity determined.

2) Estrogen sulphates

Following the benzene extraction of unconjugated estrogens, conjugated estrogens, in the form of estrone sulphate (E_1S) and estradiol-17β sulphate ($E_2\beta S$) were measured in both plasma and fetal fluids using the method of Robertson and King (1974). The method originally was used only for the measurement of E_1S; however, it was expanded, in this work, to also obtain estimates of the concentration of $E_2\beta S$.

Hence, in addition to the internal standards for E_1 and $E_2\beta$, about 1500 cpm of E_1S were added to each plasma sample prior to extraction. Since no radioactively labelled $E_2\beta S$ was available, no internal standard for this steroid could be included.

After the unconjugated estrogens had been removed, 10 volumes of a 50:50 solution of tetrahydrofuran and ethyl acetate (THF-EA) were used to extract the estrogen sulphates from each sample. The tubes were mixed for 2 min, 2.0 ml of 0.5M $NaHCO_3$ (pH 8.0) saturated with NaCl was added to each to help disrupt emulsions; they were mixed again and centrifuged for 15 min at 1400 rpm. The THF-EA extract was transferred to 15 ml tubes and evaporated under nitrogen. Hydrolysis was accomplished by adding

1.0 ml of 10% glacial acetic acid (in the 50:50 THF:EA mixture) to each tube and incubating overnight in a shaking water bath maintained at 50°C. The solvolysis mixture was thoroughly dried under nitrogen and the residue was redissolved in iso-octane (0.5 ml per sample).

The columns used for the separation of E_1 and $E_2\beta$ obtained by the hydrolysis of sulpho-conjugates were identical to those used for the benzene-extracted estrogens and transfer of the samples to the columns was carried out in the same manner. The only alteration made in this part of the procedure was the omission of the 7.5% ethyl acetate-iso-octane wash (following the application of the samples to the columns) since it was found that, after hydrolysis, a portion of the E_1 was eluted in this wash. Again, E_1 was eluted using 4.0 ml of 15% ethyl acetate-iso-octane and $E_2\beta$ using 4.5 ml of a 30% solution of the same. The rest of the assay was carried out just as described for the unconjugated estrogens.

D) Calculations

1) Precision

The precision of the assay method was determined using the following formula (Midgley, Niswender and Rebar, 1969).

$\lambda = s/b$ where λ = precision (pg)

s = standard error (pg)

b = slope of regression line

2) Sensitivity

The following formula was used to calculate the sensitivity of the assay method for E_1S, E_1 and $E_2\beta$ (Abraham, 1974).

$$S = \frac{2 \times S.D.}{R \times F}$$

where

S = sensitivity (pg)

S.D. = mean standard deviation of all blank values (pg)

R = recovery (%)

F = fraction of sample used in assay

RESULTS

I. Analysis of the Method

A) Standard Curves

Sample standard curves used in the progesterone (0 to 8 ng) and estrogen assays (E_1 and $E_2\beta$; 0 to 300 pg and 0 to 100 pg) are shown in Fig. 1 and Fig. 2.

B) Recovery of Internal Standards

The mean recoveries of unconjugated estrogens throughout all of the assays were found to be; E_1 - 80% and $E_2\beta$ - 68%. As it was found that 90-95% of either steroid was removed during the initial benzene extraction, the rest of the procedural losses could be attributed to the transfer of samples to and the removal of eluates from the celite columns.

The overall recovery of E_1S from the fluids was found to be 60%. It was found that this could be broken down into a 75-80% recovery from THF:EA extraction and a subsequent 75-80% recovery from acid hydrolysis and separation on celite columns. Although the overall recovery for $E_2\beta S$ is about 50% and therefore slightly lower than that for E_1S, when calculating the levels of both sulphoconjugates in each sample, the recovery for ^3H-E_1S was used for both E_1S and $E_2\beta S$ so that differences in procedural losses between samples could be taken into account for both steroids.

FIGURE 1

Progesterone standard curve - ^{3}H corticosterone (cpm) remaining bound to corticosterone-binding globulin as a function of progesterone added (ng).

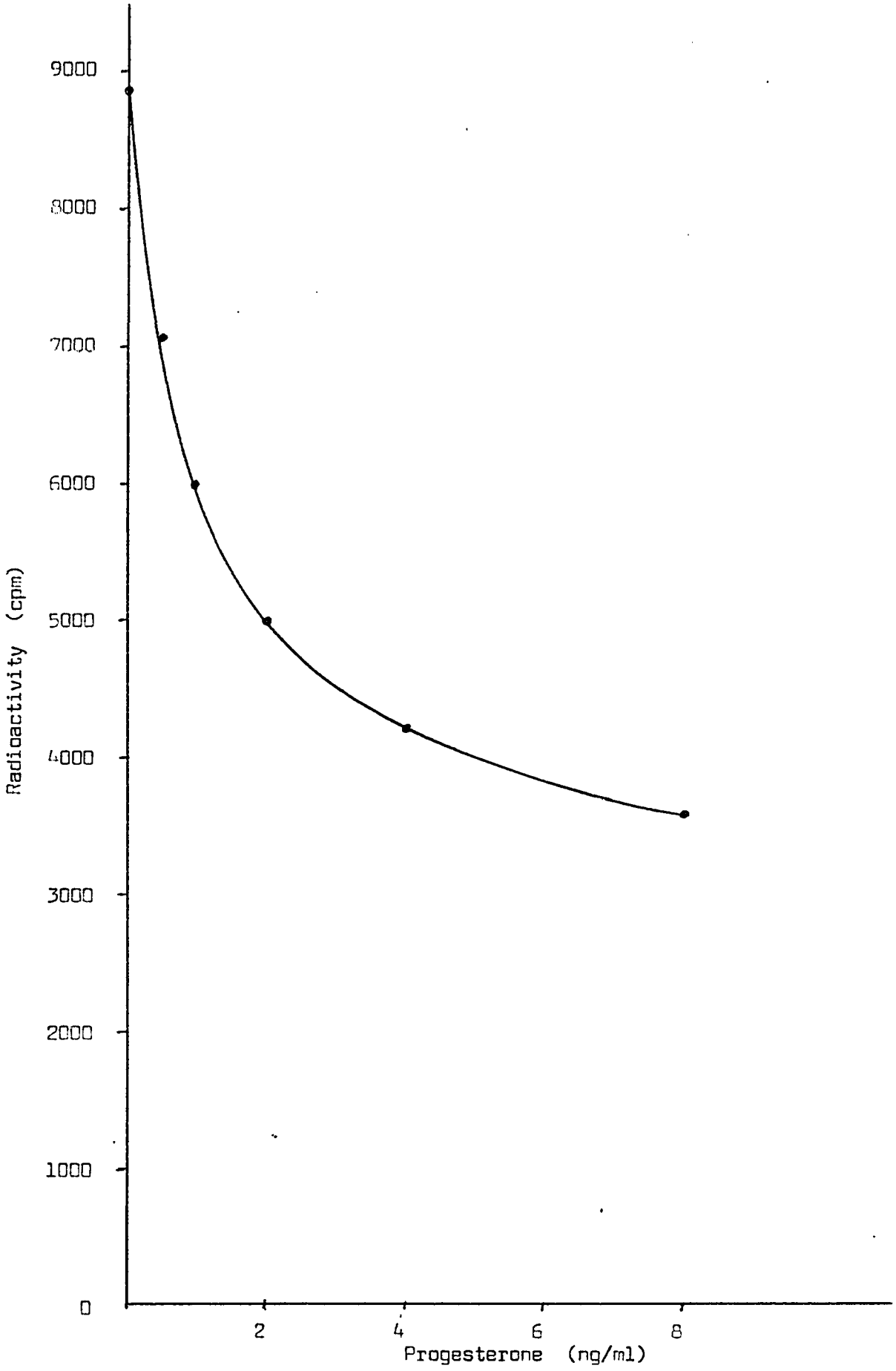

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Radioactivity (cpm)
2
4
6
8
Progesterone (ng/ml)

FIGURE 2a

Estrogen standard curve - ^{3}H $E_2\beta$ (cpm) remaining bound to antibody as a function of E_1 or $E_2\beta$ added (pg). Here, a 1/30,000 dilution of antibody was used.

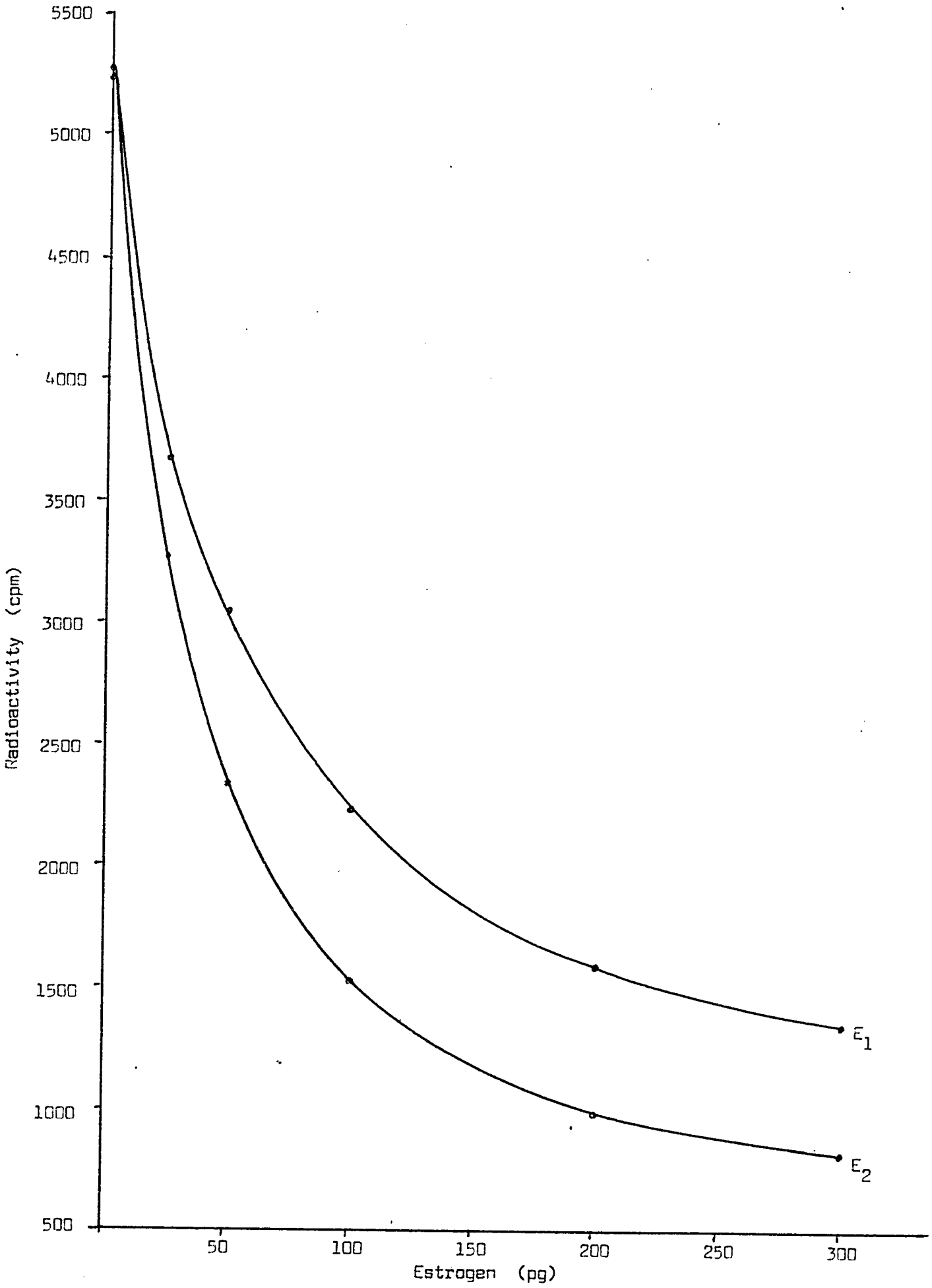
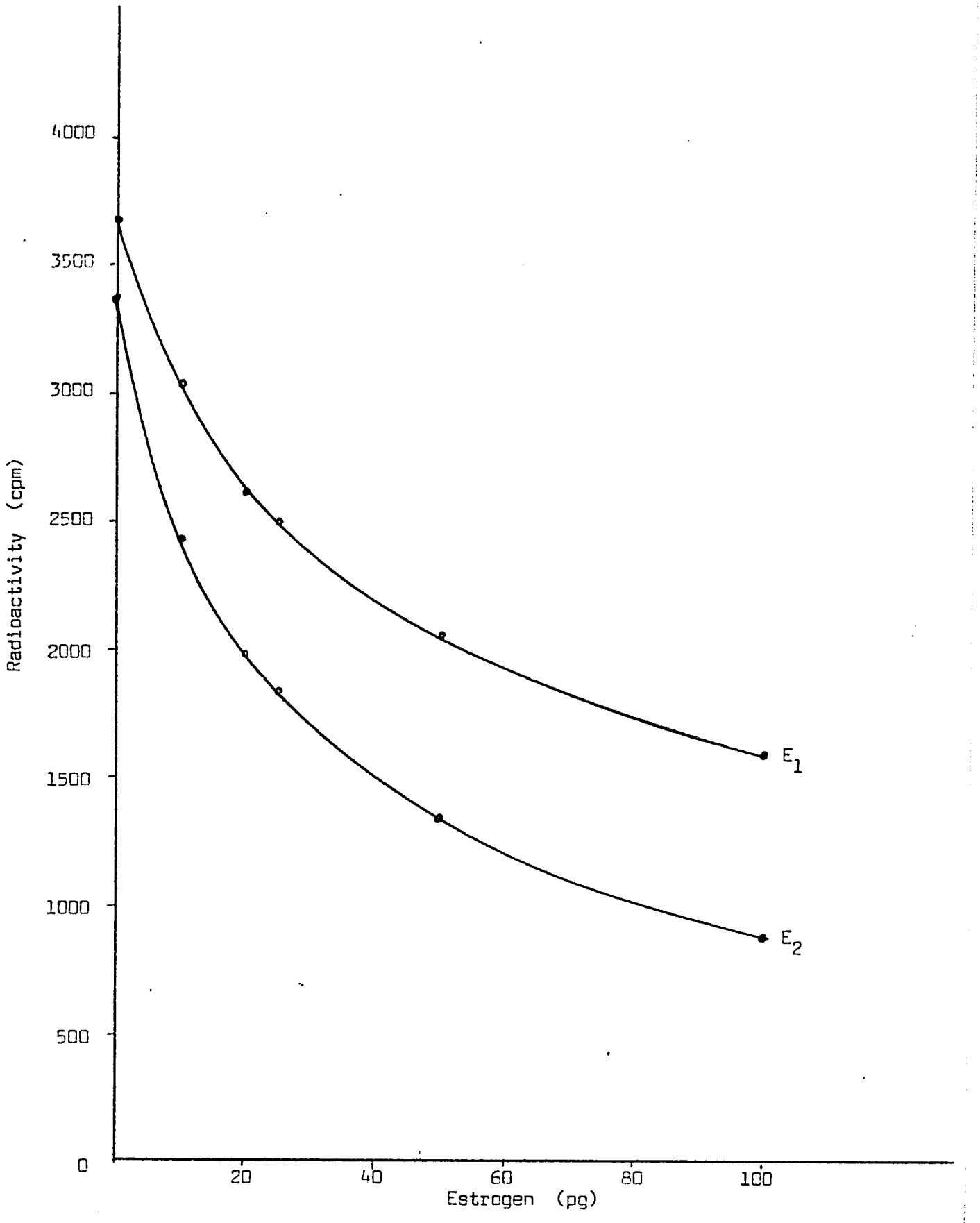

5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
Radioactivity (cpm)
50
100
150
200
250
300
Estrogen (pg)
E1
E2

FIGURE 2b

Estrogen standard curve - ^{3}H $E_2\beta$ (cpm) remaining bound to antibody as a function of E_1 or $E_2\beta$ added (pg). To give a more sensitive standard curve, a 1/60,000 dilution of antibody was used.


4000
3500
3000
2500
2000
1500
1000
500
0
Radioactivity (cpm)
20
40
60
80
100
Estrogen (pg)
E_1
E_2

Using ^{3}H estrone glucuronide, it was determined that only 10% of this compound was recovered using the extraction method for estrogen sulphates and, of this 10%, no significant amount was hydrolysed by the treatment with glacial acetic acid.

C) Blank Values

Blank values were determined by including in each assay two tubes containing only the dried internal standards which were taken through the whole procedure of extraction, separation on columns and incubation with antibody. These values were subtracted from the estimation for each sample as obtained from the standard curve before the corrections for procedural losses were made. The values obtained from the blanks were consistently below the sensitivity of the test but are shown in Table 2 for comparison.

D) Accuracy

The accuracy of the measurements were consistent over the entire experimental range. Known amounts (0, 50, 100, 200 and 400 pg) of each of E_1, $E_2\beta$ and E_1S were added to immature ram plasma and carried through the entire procedure including correction for methodological losses as indicated by the recovery of internal standards. The results of two separate assays for each estrogen are shown in Table 3. Regression equations were determined (Table 4) and estrogen measured was plotted as a function of estrogen added

TABLE 2

Blank values obtained in estrogen radioimmunoassay

	E_1	$E_2\beta$	E_1S	$E_2\beta S$
Mean Blank Value (pg)	8.7	4.3	5.3	7.5
Standard Deviation (pg)	3.0	2.0	3.6	4.3

In each case, n = 15

TABLE 3

Accuracy in determining estrogen levels in plasma

Amount Added (pg)	Mean Amount Measured ± 1 Standard Deviation					
	Estrone		Estradiol-17β		Estrone Sulphate	
	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2
50	60.2 ± 3.3*	44.4 ± 6.3*	55.5 ± 1.9	50.8 ± 3.2		
100	95.2 ± 4.3	106.7 ± 5.8	113.8 ± 4.5	94.5 ± 8.4	108.2 ± 10.6	109.7 ± 23.6
200	199.5 ± 9.6	200.5 ± 12.5	211.7 ± 6.1	239.7 ± 19.8	203.3 ± 24.5	173.5 ± 26.5
400					429.7 ± 34.0	425.8 ± 16.3

* n = 5; in all other cases, n = 6

TABLE 4

Correlation between estrogen added and estrogen measured by radioimmunoassay

	Regression Equation		Correlation Coefficient	
	Assay 1	Assay 2	Assay 1	Assay 2
Estrone	y = 0.95X + 7.1	y = 1.02X - 1.7	0.99	0.99
Estradiol-17β	y = 1.03X + 6.6	y = 1.29X - 21.8	1.00	0.98
Estrone Sulphate	y = 1.08X - 5.0	y = 1.08X - 15.6	0.98	0.97

for each assay (Fig. 3 to Fig. 8). Correlation coefficients did not differ significantly from unity (Table 4).

E) Precision

The precision with which the unconjugated estrogens and estrone sulphate could be measured was calculated using the same cold recovery assays as for the accuracy determinations. Measurements of precision were made at the 50, 100 and 200 pg levels for E_1 and $E_2\beta$ and at the 100, 200 and 400 pg levels for E_1S (Table 5). The values obtained were also expressed as coefficients of variation (%) (Table 5).

F) Sensitivity

The sensitivity of the method was calculated using the 0 to 100 pg standard curve. It was found to be; E_1 - 15 pg, $E_2\beta$ - 12 pg and E_1S - 24 pg. Despite this calculated sensitivity, the minimal detectable level of E_1S in the present study was taken to be 40 pg.

II. Experimental Results

To compare the concentrations of the estrogens on an equivalent basis, the values for the sulphate conjugates are given in terms of the unconjugated estrogen equivalent.

FIGURE 3 and FIGURE 4

Regression curves for E_1.

FIGURE 5 and FIGURE 6

Regression curves for $E_2\beta$.

FIGURE 7 and FIGURE 8

Regression curves for E_1S.

For all of the above, the amount of estrogen measured (pg) (ordinate) was plotted as a function of the amount of estrogen added (pg) (abscissa). See Table 4 for the corresponding regression equations.

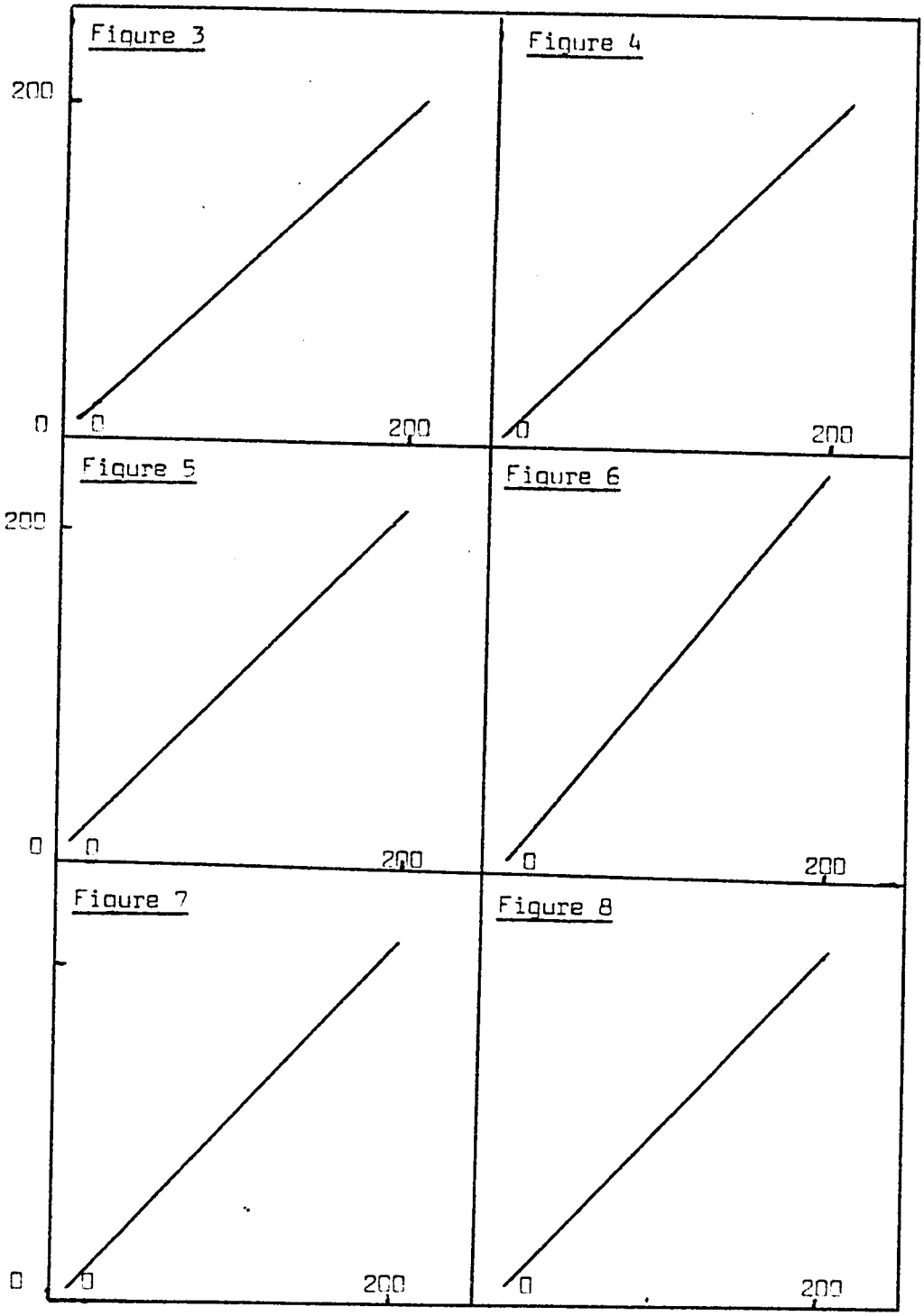


Figure 3
200
0
0
200
Figure 4
0
200
Figure 5
200
0
0
200
Figure 6
0
200
Figure 7
0
0
200
Figure 8
0
200

TABLE 5

Precision of radioimmunoassay for estrogens

	Level of Estrogen (pg)	Precision (↗) within - pg Assay 1	Assay 2	Coefficient of Variation (%) Assay 1	Assay 2
Estrone	50	1.6*	2.8*	3.1	5.5
	100	1.9	2.3	1.9	2.3
	200	4.1	5.0	2.0	2.5
Estradiol-17β	50	0.8	1.0	1.6	2.0
	100	1.8	2.7	1.8	2.7
	200	2.4	6.3	1.2	3.2
Estrone Sulphate	100	4.0	8.9	4.0	8.9
	200	9.3	10.3	4.7	5.4
	400	12.9	6.2	3.2	1.6

* n = 5; in all other cases, n = 6

A) Allantoic and Amniotic Fluids

1) Estrone sulphate

E_1S levels in the fetal fluids of the ewe sampled at intervals throughout gestation fluctuated with time (Fig. 9). It was first detectable at day 31 (123 pg/ml), peaked at day 46 (14.2 ng/ml), dropped to around 850 pg/ml at day 55 and gradually rose to form a second, much broader peak in the interval day 100 to day 110 (approx. 11.3 ng/ml). Since the allantoic fluid of the first animal sampled at day 110 of gestation contained very high levels of E_1S (36.7 ng/ml), the allantoic fluid of several other animals at this stage of gestation was investigated in an effort to determine if such high amounts were usually present at this time. The findings are presented in Table 6. It can be seen that the levels of E_1S fluctuate widely at this time (0.2 to 36.7 ng/ml) and do not appear to be dependent on the sex of the fetus. The mean level (11.3 ng/ml) was comparable to that measured at days 98 and 100 of gestation.

In the amniotic fluid, estrone sulphate again was detected first at day 31 (85 pg/ml) and also demonstrated an early peak (10.8 ng/ml at day 50), but no second peak appeared in late gestation. The amniotic fluid also was assayed from several animals at day 110 to see if fluctuations in E_1S levels comparable to those found in the allantoic fluid were present (Table 7). Instead, it was found that the concentration of E_1S showed very

FIGURE 9

Estrone sulphate in the allantoic (-o-) and amniotic (-●-) fluid of the ewe from day 20 to day 140 of gestation. The ↓ indicates the usual time of parturition.

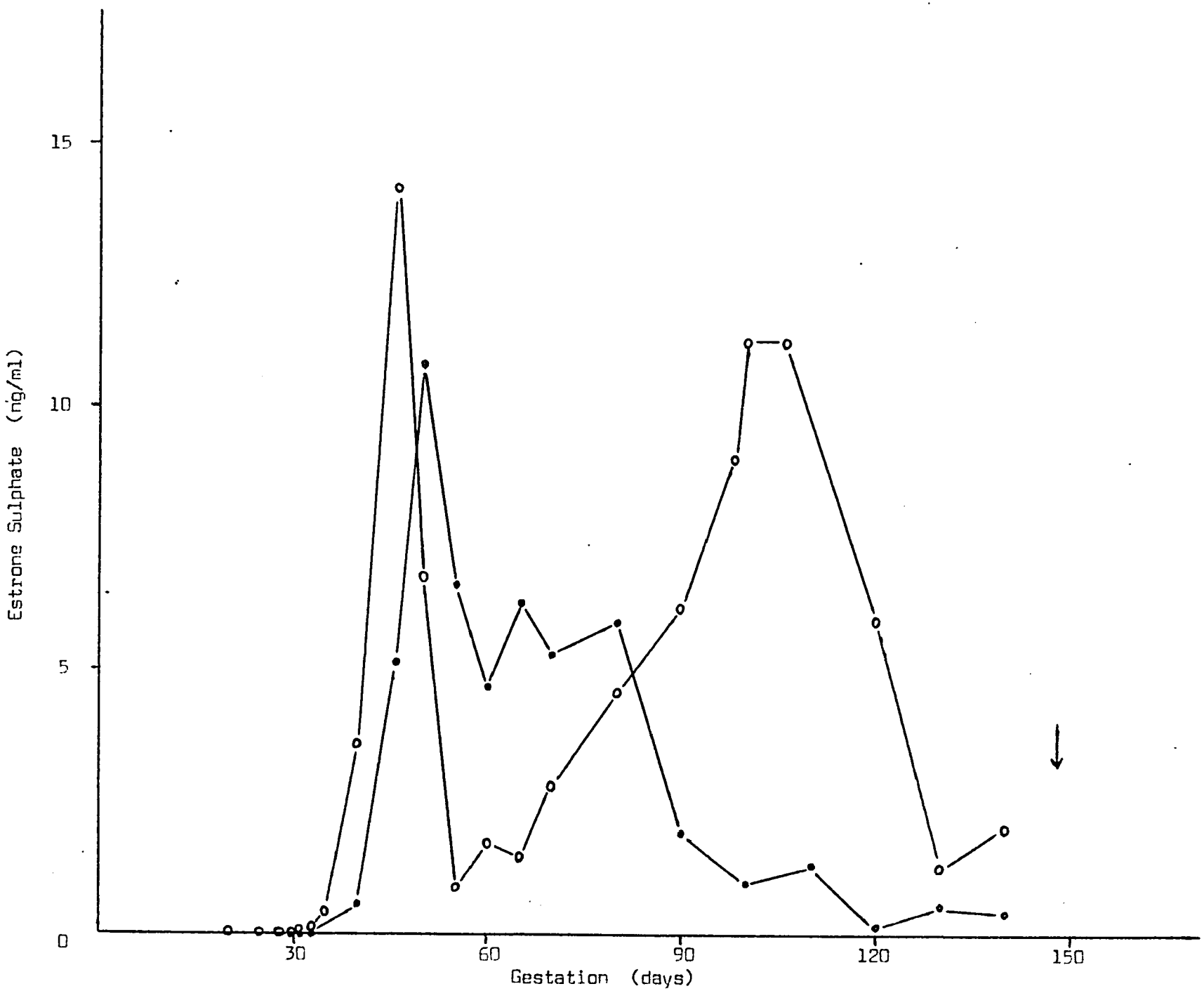

Estrone Sulphate (ng/ml)
15
10
5
0
30
60
90
120
150
Gestation (days)

TABLE 6

Estrone sulphate in the allantoic fluid of the sheep at 110 days gestation

Ewe Number	Sex of Fetus	Estrone Sulphate (ng/ml)
71-1704	female	0.2
71-2043	female	8.0
71-2043	female	17.1
74-1750	female	15.8
72-8084	female	36.7
74-1437	male	1.0
74-1545	male	4.8
73-1394	male	6.0

TABLE 7

Estrone sulphate in the amniotic fluid of the sheep at 110 days gestation

Ewe Number	Sex of Fetus	Estrone Sulphate (ng/ml)
71-1704	female	1.43
71-2043	female	1.04
72-8084	female	1.50
74-1437	male	1.08

Mean = 1.26 $\pm$ 0.20 ng

little change from one animal to the next and, again, the levels did not appear to be sex-dependent.

2) Estrone

Allantoic fluid E_1 (Fig. 10) remained at quite low levels throughout most of gestation. It was first measurable at day 33 (25 pg/ml) and the highest levels were recorded at day 46 (448 pg/ml). In the amniotic fluid, E_1 could first be measured at day 35 (23 pg/ml), formed a small peak at day 46 (64 pg/ml) and a second peak at day 110 (61 pg/ml).

3) Estradiol-17β sulphate

$E_2\beta S$ followed much the same pattern in the allantoic fluid as E_1S with an initial peak at day 46 and a second larger peak at day 110 (Fig. 11). It was first detectable at day 35 at a concentration of 52 pg/ml. In the amniotic fluid, $E_2\beta S$, first measured at day 40 (133 pg/ml), formed a well-defined peak at day 50, following which the levels fluctuated without any consistent pattern until day 140 (Fig. 11).

4) Estradiol-17β

$E_2\beta$ levels remained extremely low throughout all of gestation in both embryonic fluids. The highest concentration recorded was 52 pg/ml with the average being 15 pg/ml.

FIGURE 10

Estrone in the allantoic (-o-) and amniotic (-●-) fluids of the ewe from day 20 to day 140 of gestation.

The ↓ indicates the usual time of parturition.

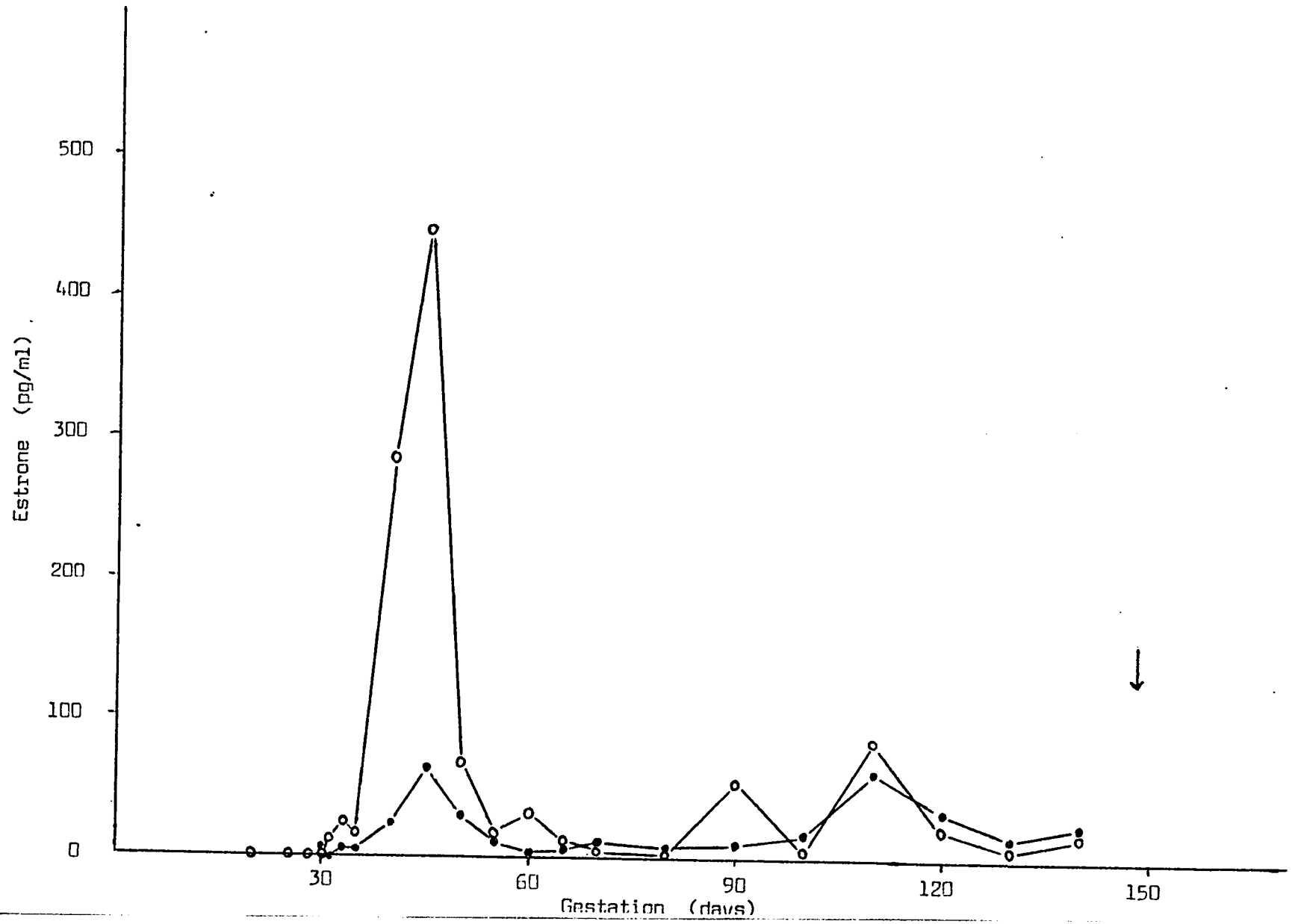
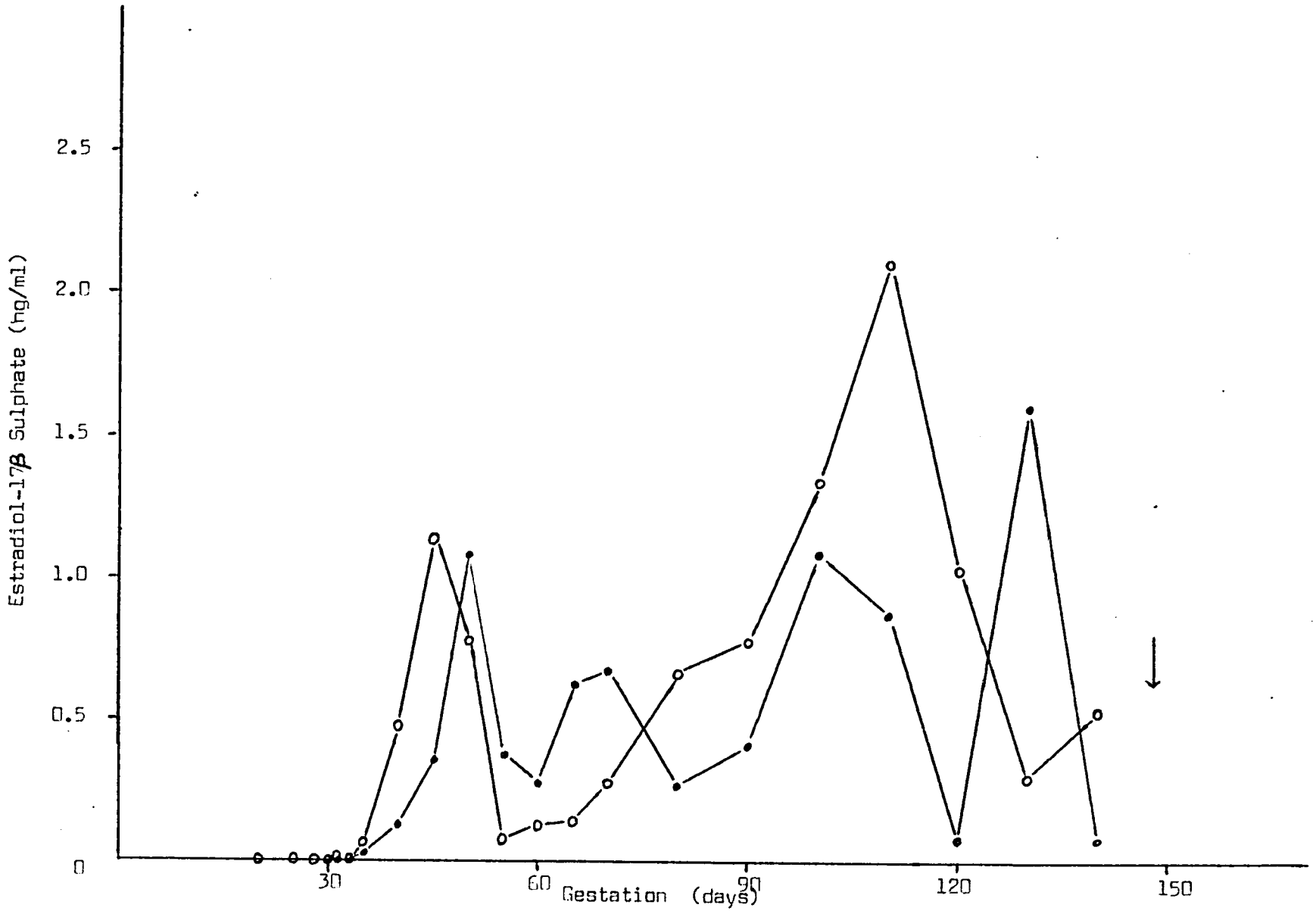

500
400
300
200
100
0
Estrone (pg/ml)
30
60
90
120
150
Gestation (days)

FIGURE 11

Estradiol-17β sulphate in the allantoic (-o-) and amniotic (-•-) fluid of the ewe from day 20 to day 140 of gestation. The ↓ indicates the usual time of parturition.


Estradiol-17β Sulphate (hg/ml)
2.5
2.0
1.5
1.0
0.5
0
30
60
90
120
150
Gestation (days)

B) Uterine Venous Plasma

1) Estrone sulphate

E_1S was first detectable in the uterine venous plasma of the ewe (Fig. 12) at day 30 of gestation (70 pg/ml). A small peak was found at day 46 (0.25 ng/ml). The levels remained low during mid-gestation and then gradually rose to a second peak towards the end of pregnancy (2.0 ng/ml at day 120).

2) Estrone

E_1 levels remained lwo throughout most of gestation, being measurable only by day 110 (25 pg/ml) and not rising above 75 pg/ml form then until day 140 (Fig. 12).

3) Estradiol-17β sulphate

The levels of $E_2\beta S$ parallelled those of E_1S but at about one-half the concentration (Fig. 13).

4) Estradiol-17β

The change in level of $E_2\beta$ followed a pattern similar to E_1 but at about one-half the concentration (Fig. 13).

C) Jugular Venous Plasma

1) Estrone sulphate

E_1S appeared to be present early in gestation, but the

FIGURE 12

Estrone sulphate (-□-) and estrone (-■-) in the uterine venous plasma of the ewe from day 25 to day 140 of gestation. The ↓ indicates the usual time of parturition.

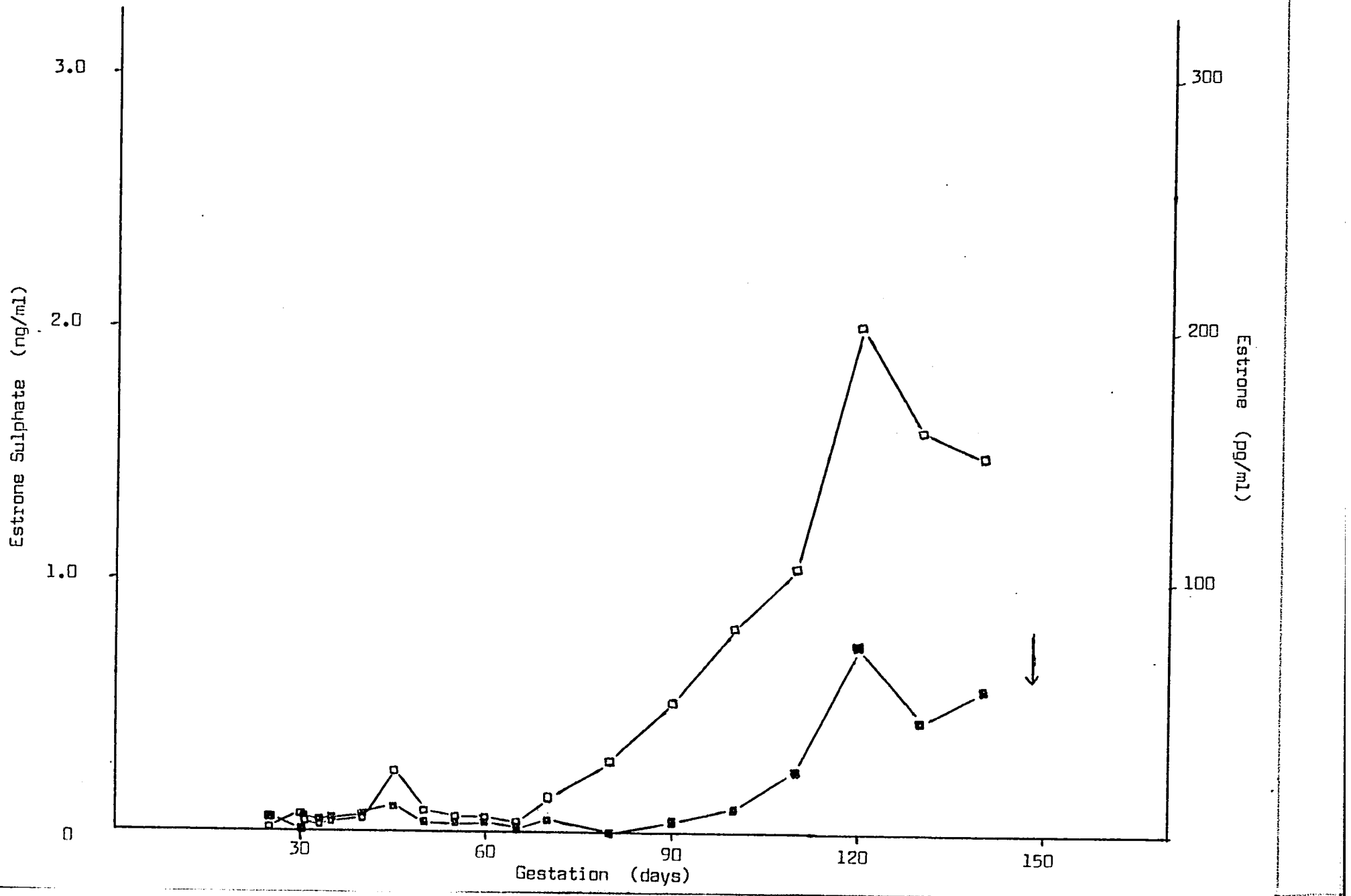
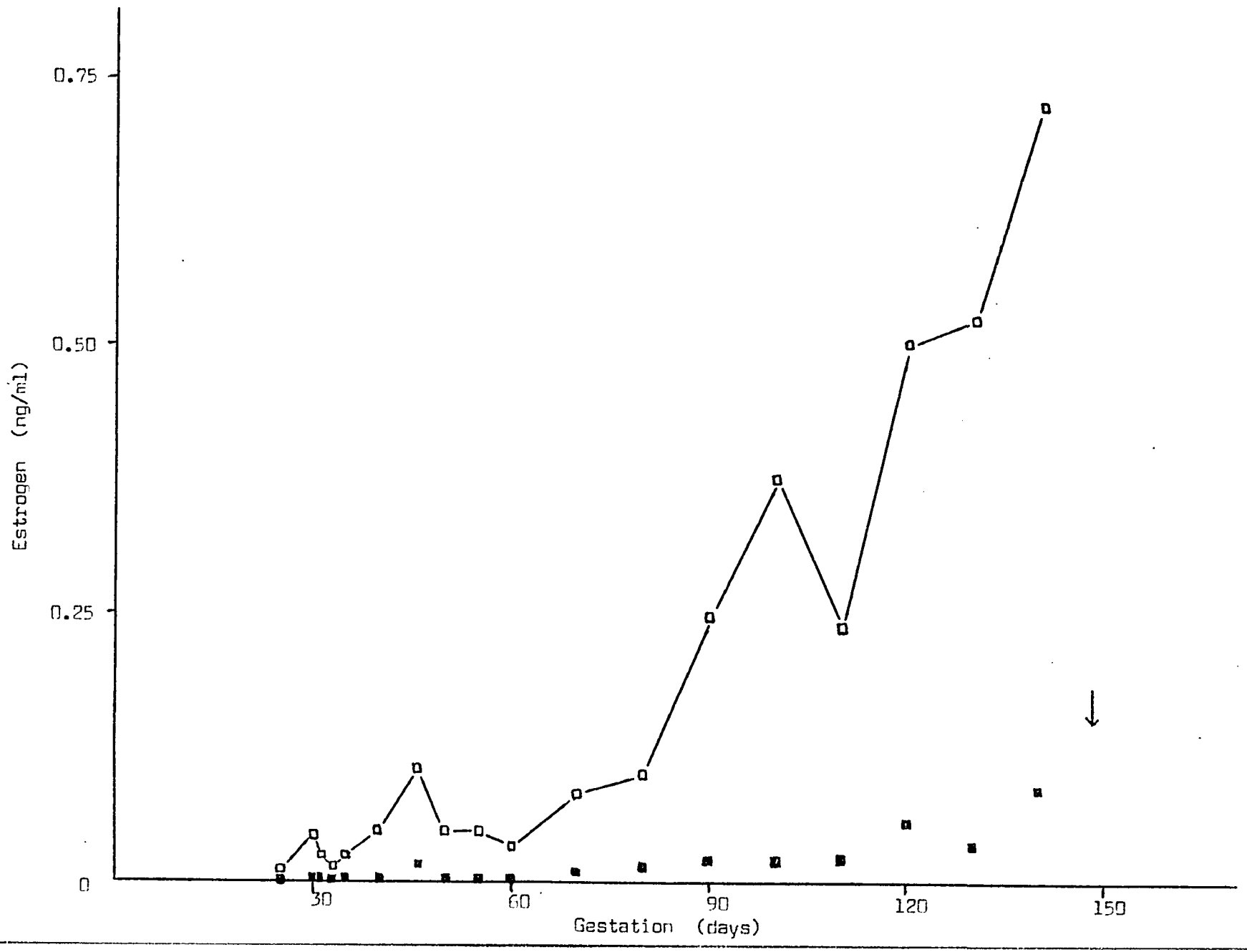

3.0
2.0
1.0
0
Estrone Sulphate (ng/ml)
300
200
100
Estrone (pg/ml)
30
60
90
120
150
Gestation (days)

FIGURE 13

Estradiol-17β sulphate (-□-) and estradiol-17β (-■-) in the uterine venous plasma of the ewe from day 25 to day 140 of gestation. The ↓ indicates the usual time of parturition.


0.75
0.50
0.25
0
Estrogen (ng/ml)
30
60
90
120
150
Gestation (days)

concentration was so low that a definite pattern could not be discerned (Table 8). A gradual increase in the concentration of E_1S began at day 80 (Fig. 14) and by day 140 was 1.9 ng/ml.

2) Estrone

E_1 remained at a concentration below the sensitivity of the method until day 110 when it was measured at 20 pg/ml (Fig. 14). The level gradually increased until, at day 140, the concentration was 59 pg/ml.

3) Estradiol-17β sulphate

$E_2\beta S$ was first detectable (Fig. 15) at day 31 (56 pg/ml). Levels began to increase by day 80, peaked at day 120 (544 pg/ml) and were still high at day 140 (472 pg/ml).

4) Estradiol-17β

$E_2\beta$ was first measurable at day 90 (15 pg/ml) and reached a concentration of 54 pg/ml by day 140 (Fig. 15).

D) Ovarian Venous Plasma

Ovarian venous plasma was sampled at various time intervals throughout gestation (Table 9) and it was found, except for the four instances cited in the table, that the levels were comparable to those found in corresponding jugular venous plasma samples.

TABLE 8

Estrone sulphate in jugular venous plasma of the ewe during gestation

Ewe No.	Day of Gestation	E_1S (pg/ml)	Ewe No.	Day of Gestation	E_1S (pg/ml)
66-9611	17	49	66-9611	25	>40
66-9610	19	>40	81	25	53
81	19	>40	66-9610	31	44
66-9611	20	42	65	33	67
66-9610	21	54	43	33	41
81	21	>40	72-8012	35	78
66-9611	22	>40	73-8011	35	63
81	23	>40	73-8119	40	56
66-9610	24	>40	09-8135	46	43

Ewe No.	Day of Gestation	E_1S (pg/ml)
09-8357	46	88
72-8071	50	57
73-8093	50	83
67-9701	60	77
09-8213	70	59
73-8327	80	137

FIGURE 14

Estrone sulphate (-Δ-) and estrone (-▲-) in the jugular venous plasma of the ewe from day 20 to day 140 of gestation. The ↓ indicates the usual time of parturition.

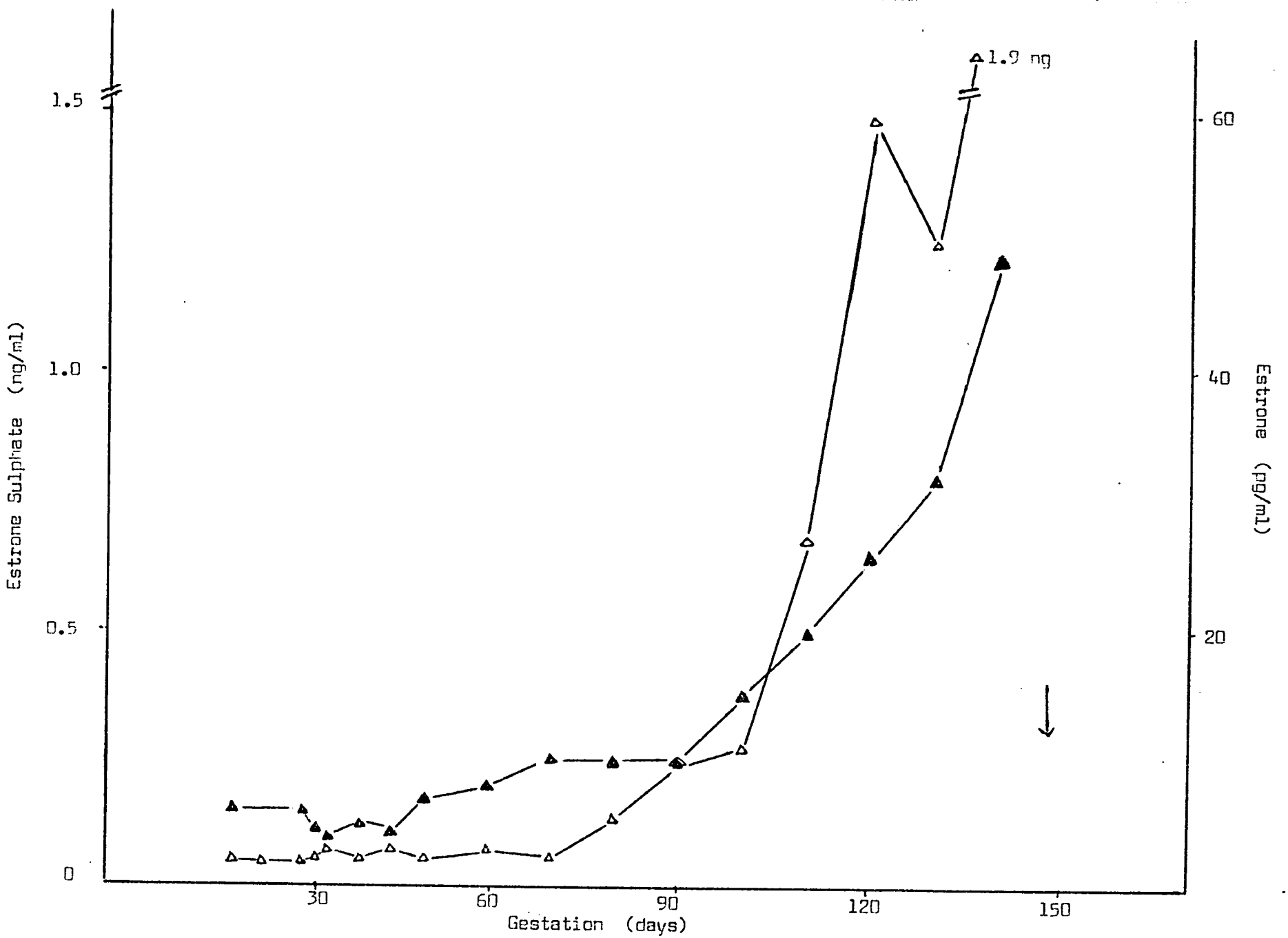

1.9 ng
1.5
1.0
0.5
0
60
40
20
Estrone Sulphate (ng/ml)
Estrone (pg/ml)
30
60
90
120
150
Gestation (days)

FIGURE 15

Estradiol-17β sulphate (-▽-) and estradiol-17β (-▼-) in the jugular venous plasma of the ewe from day 20 to day 140 of gestation. The ↓ indicates the usual time of parturition.

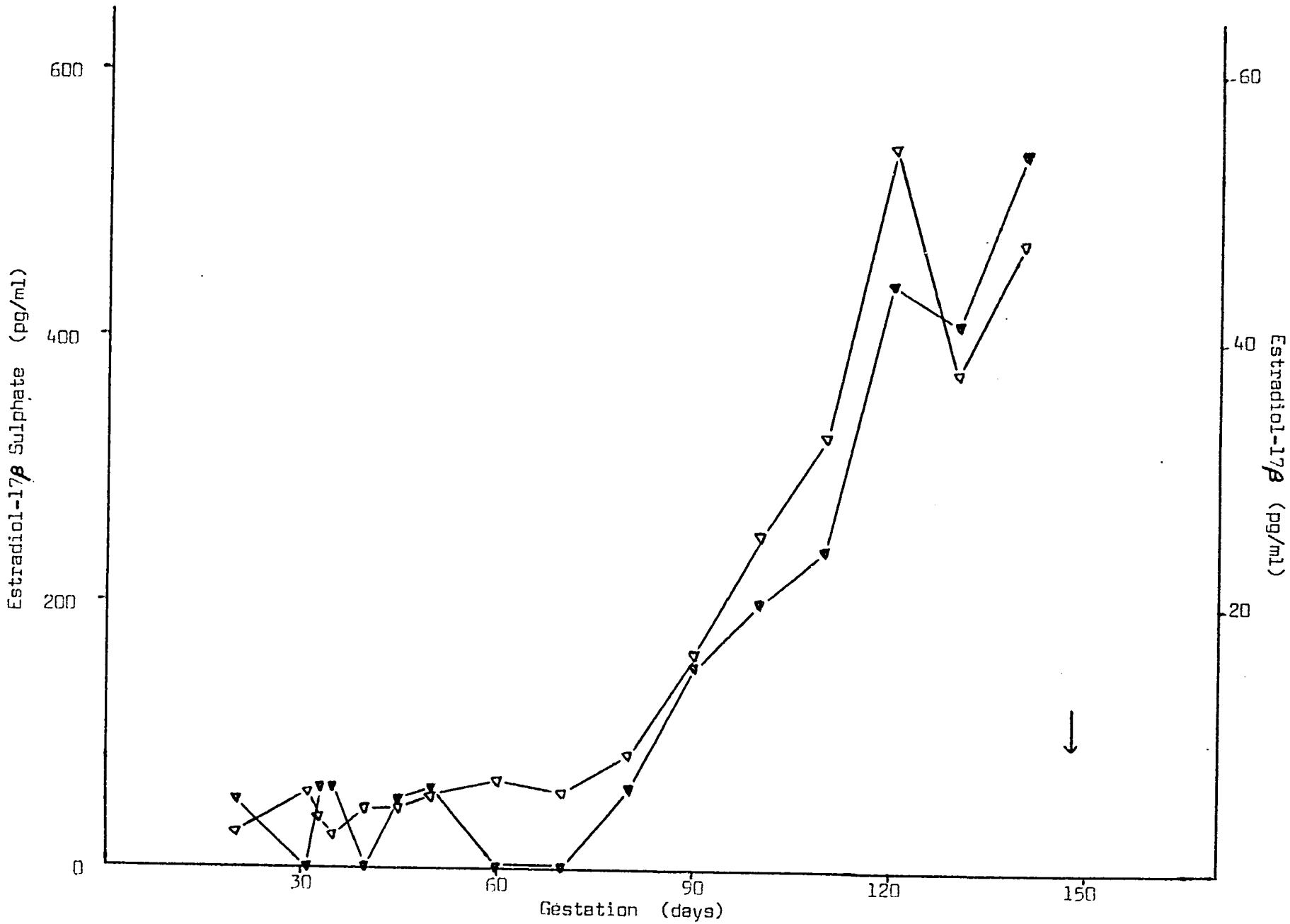

600
400
200
0
Estradiol-17β Sulphate (pg/ml)
60
40
20
Estradiol-17β (pg/ml)
30
60
90
120
150
Gestation (days)

TABLE 9

Estrogens in the ovarian venous plasma of the ewe during gestation

Ewe No.	Day of Gestation	E_1S (pg/ml)	E_1 (pg/ml)	$E_2\beta S$ (pg/ml)	$E_2\beta$ (pg/ml)	No. of C.L.	No. and Size of Follicles
73-8011	35-lt. ovary	>40	15	>40	200	1	1-6mm; 1-4mm
09-8135	46-lt. ovary	78	15	54	13	2	no follicles
09-8135	46-rt. ovary	80	15	>40	12	1	no follicles
72-8071	50-lt. ovary	72	15	53	137	0	several - 3 mm
72-8071	50-rt. ovary	91	22	60	25	2	several - 2-3 mm
73-8093	50-lt. ovary	60	0	>40	16	0	several - 2 mm
73-8093	50-rt. ovary	65	0	>40	12	4	several - 2mm
09-8213	70-lt. ovary	83	15	49	103	0	several - 2mm
09-8213	70-rt. ovary	100	19	53	261	2	several - 2-3 mm
72-8084	110-lt. ovary	439	15	208	42	0	3 or 4 - 2mm
72-8084	110-rt. ovary	600	34	218	16	3	6 - 2mm

C. L. = corpus luteum

DISCUSSION

Estrogens are present in the fetal fluids of the sheep early in gestation (Fig. 9, 10, 11). Using this radioimmunoassay procedure, E_1S could first be detected at day 31 (123 pg/ml) and when the values for E_1S during early pregnancy were plotted on a semi-logarithmic scale (Fig. 16), it was found that they did not rise above the limit of sensitivity (40 pg/ml) until day 30.

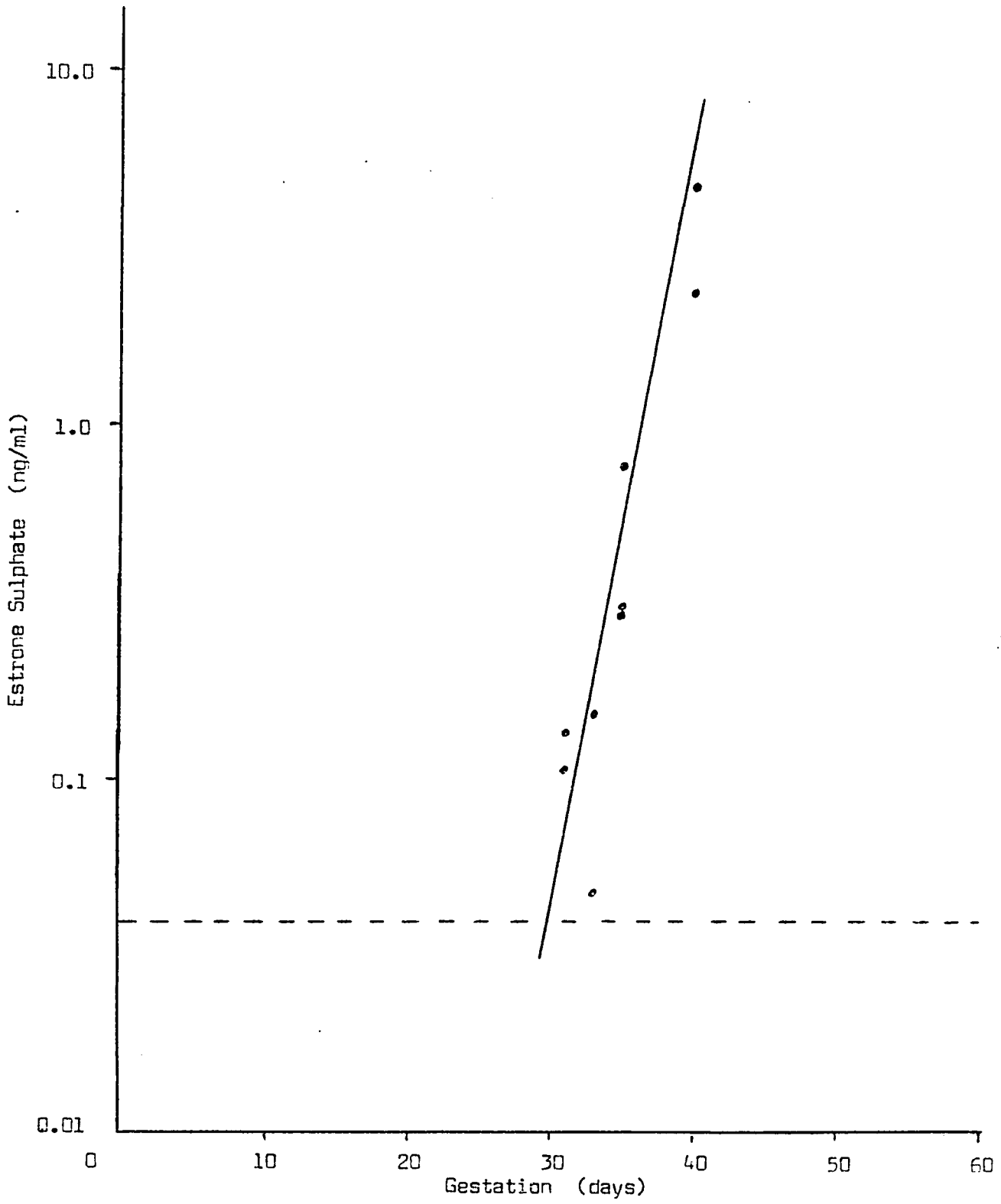
A comparison of uterine venous (Fig. 12, 13) and ovarian venous (Table 9) plasma samples throughout gestation implicates the conceptus, rather than the maternal ovaries, as the site of release of these estrogens. It has been shown by Huff and Eik-Nes (1966) that 6 day old rabbit blastocysts are capable of sume steroidogenic activity, and studies on the early pig blastocyst (days 12 to 16 of gestation) give ample evidence of its ability to produce, in vitro, estrogens from androgens and progesterone (Perry et al, 1973; Flood, 1974; Heap et al, 1975; Perry et al, 1976). Furthermore, in the specific case of the ewe, experiments involving ovariectomy within a few days of conception (Foote et al, 1957; Moore and Rowson, 1959; Alexander and Williams, 1966; Bindon, 1971; Sarda et al, 1973; Moore, 1975) have shown that pregnancy proceeds normally, at least up to day 60, in the absence of a maternal ovarian estrogen source. Challis et al (1971) also showed that the preparturition rise in estrogens

FIGURE 16

Semi-logarithmic plot of estrone sulphate in the allantoic fluid of the ewe from day 31 to day 40 of gestation.

The equation of the line is $\log y = 0.19X - 4.12$.

The correlation coefficient is 0.88.


10.0
1.0
0.1
0.01
Estrone Sulphate (ng/ml)
0
10
20
30
40
50
60
Gestation (days)

in the uterine venous plasma still occurred in an animal which had been bilaterally ovariectomized at day 91.

Concerning Table 9, some comment should be made regarding the four instances in which substantial amounts of $E_2\beta$ were measured in the ovarian venous plasma of the pregnant ewe. During the estrous cycle in the ewe, the estrogen concentration in the ovarian venous vessels can reach 100 to 400 pg/ml (Scaramuzzi, Caldwell and Moor, 1970). Several cycles of follicular development take place during the estrous cycle of the ewe (Smeaton and Robertson, 1971) with these follicles reaching 4 to 5 mm in diameter (Hutchinson and Robertson, 1966). If ewes are mated during the breeding season (the animals used in this work were bred between October, 1974 and early March, 1975), this cyclical follicular development is carried over into pregnancy; however the diameter of the follicles decreases as gestation proceeds (Williams, Garrigus, Norton and Nalbandov, 1956). Follicles which are 2 mm or less in diameter generally do not appear to be active in estrogen production (Table 9); however, those with a diameter of 3 mm or greater display varying degrees of synthesis of $E_2\beta$. Comparing maternal ovarian venous (Table 9) and jugular venous E_1S (Table 8) and E_1 (Fig. 14) concentrations, one can see that the former is simply a reflection of the latter, indicating that there is no real contribution by the maternal ovaries to circulating levels of E_1S throughout most, if not all,

of gestation.

With respect to amounts present, E_1S appears to be the predominant estrogen in both fetal and maternal fluids and will be emphasized in this discussion. With the exception of the fetal fluids in late gestation, the pattern for the presence of unconjugated E_1 in both fetal (Fig. 10) and maternal fluids (Fig. 12, 14) is very similar to that for E_1S (Fig. 9, 12, 14), but at a fraction of the concentration. Caution should be exercised when interpreting the results for $E_2\beta S$ since recovery values for individual samples could not be obtained in this assay. However, with the exception of the amniotic fluid in mid to late gestation, a similar pattern is again observed, but at 10 to 25% the concentration of E_1S. Unconjugated $E_2\beta$, the active form of the steroid, was rarely found at a measurable concentration in any of the fluids.

Two peaks in the concentration of E_1S were found in the allantoic fluid, but only one peak was found in the amniotic fluid (Fig. 9). Although a definite peak in maternal plasma E_1S corresponding to the high concentration of this steroid found in the fetal fluids at day 46 is not evident (Fig. 14), it is likely that this is a function of the sensitivity of the assay method. Such a peak has been shown to occur in the maternal plasma of the sow in early pregnancy (Robertson and King, 1974). In fact, a point of considerable interest is that the changes in pattern for the

concentrations of E_1S, E_1 and $E_2\beta S$ in the allantoic fluid of the sheep during the first two-thirds of pregnancy are similar with those described for E_1S in the peripheral plasma of the pregnant pig (Robertson and King, 1974), in the allantoic fluid of the cow (Robertson and King, 1976) and for total estrogens in the urine of the pregnant pig (Raeside, 1963). This pattern is a sharp peak early in pregnancy followed by a second later rise in concentration.

In the case of the pig, a close similarity of profiles between the concentration of E_1S in maternal plasma, urine and allantoic fluid indicates that changes in estrogen levels in the allantoic fluid would appear to reflect changes in the rate of estrogen synthesis by the conceptus rather than changes in the rate of storage, at least up to the preparturition rise in plasma estrogen. This reasoning can also be extended to the ewe, certainly in late gestation and also for the early peak in allantoic fluid estrogen, since the release of estrogens from a very small volume of allantoic fluid to a much larger volume of maternal plasma would result in a considerable dilution with the effect that the radioimmunoassay method may not be sufficiently sensitive to show whether or not these estrogen sulphates are reaching the maternal circulation.

Towards the end of pregnancy, differences are evident. Whereas plasma (sheep, pig and cow) and urinary (pig) levels of

estrogens continue to rise until parturition, the concentration of estrogens in the allantoic fluid of the sheep (Fig. 9, 10, 11) falls towards the end of pregnancy. But it was not possible in the present work to establish whether an immediate preparturition rise occurred in the allantoic fluid analogous to that reported in the peripheral plasma (Challis, 1971; Challis et al, 1971; Robertson and Smeaton, 1973; Tsang, 1974) at this time. Although the concentration of E_1S and of E_1 in the uterine venous plasma appears to plateau around day 120 (Fig. 12), the secretion rate may be remaining constant or even continuing to increase since the blood flow through the uterus is increasing at this time.

A time discrepancy appears to exist in that E_1S levels peak for the second time in the allantoic fluid at 110 days and in the maternal peripheral and uterine venous plasma at 120 days. However, in the fetal peripheral plasma (not recorded here since only 10 animals were sampled between days 80 and 140) the E_1S levels remained at a concentration of about 4 ng/ml between days 100 and 130 and it is possible that this is a source of the maternal peripheral E_1S.

It was indicated previously in this discussion that the estrogens found in both embryonic and maternal fluids were fetal rather than maternal in origin. The next question which presents itself is what fetal endocrine organ or organs might be involved in this estrogen biosynthesis? In the case of the human, a fetoplacental unit has been proposed which involves the placental production of pregnenolone from cholesterol followed by its conversion to dehydroepiandrosterone and subsequent sulphurylation in the fetal adrenal glands and final return to the placenta for

the aromitization step, resulting in the production of estrone and estradiol-17 (Diczfalusy, 1969). Some evidence has been presented, however, that such a fetoplacental unit does not exist in the sheep. It has been well extablished that sheep placentae in vitro can convert C-19 steroids such as androstenedione (Ainsworth and Ryan, 1966; Pierrepoint, Anderson, Griffiths and Turnbull, 1970; Pierrepoint et al, 1971), dehydroepiandrosterone (Ainsworth and Ryan, 1966) and epitestosterone (Pierrepoint et al, 1970; Pierrepoint et al, 1971) to estrogens. With regards to other domestic animals it has been shown that placental preparations from the cow, horse and sow can convert dehydroepiandrosterone and androstenedione to estrogens (Ainsworth and Ryan, 1966; Perry et al, 1976), and that placentomes from the goat when incubated with androstenedione produce estrone, estradiol-17 and estradiol-17 (Ainsworth and Ryan, 1970). This capacity is similar to what has been found in human placentae.

However, when John and Pierrepoint (1975) incubated late pregnancy sheep placentae with 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, both substrates were extensively metabolized yielding a variety of C-18 and C-19 products. Of these, estrone sulphate was the primary metabolite, followed by estrone and epitestosterone. In all cases 17α-hydroxypregnenolone appeared to be utilized to a greater extent over 17α-hydroxyprogesterone in the formation of these products. Although these results would tend to indicate an ability of the ovine placenta to produce C-18 steroids from C-21 precursors, the situation does not seem to be that straightforward. When Davies, Ryan and Petro (1970) incubated the placenta or the fetal adrenal glands from a sheep in late

gestation with ^{3}H-pregnenolone, there was no conversion of this substrate to estrone or estradiol. But when the placenta plus fetal adrenal glands were incubated together with the same substrate estrone was produced. Comparing the results of Davies et al (1970) with those of John and Pierrepoint (1975) suggests that one reason why Davies et al got no estrogen production from isolated placentae incubated with pregnenolone was that these placentae lack the necessary 17αhydroxylase enzyme. Hence, although some evidence indicates that there does not exist in the sheep a fetoplacental unit identical to that which has been described for the human (John and Pierrepoint, 1975), it is still quite possible that some fetal component (i.e. fetal adrenal glands) may also be involved in the production of these C-18 steroids from progesterone, specifically in the initial conversion of pregnenolone or progesterone to its 17α hydroxylated form.

The appearance of two separate peaks in estrone sulphate concentration in the allantoic fluid (Fig. 9) presents the possibility of two separate sources of estrone sulphate; one functional during the first trimester and the second during mid to late gestation. However, until the question of the extent of the steroidogenic capabilities of the ovine placenta during both early and late pregnancy is settled it is difficult to elaborate on this idea. It could be very illiminating to incubate early ovine placentomes (day 40 or 50 of gestation) with pregnenolone

and see what products are formed.

Another observation which can be made from Fig. 9 is that in the second half of gestation, allantoic and amniotic fluid E_1S concentrations are very different. From about day 18 to day 90 of gestation fetal urine in the sheep passes into the allantoic cavity; after this time the urachus closes and urine passes via the urethra to the amniotic sac (Davies, 1952; Wales and Murdoch, 1973). However, the fact that the concentration of E_1S in the amniotic fluid is low from day 90 onwards does not rule out the possibility that estrogens could be present as some other conjugate, such as glucuronides. A greater understanding of the relationships between amniotic and allantoic fluids and between them and the fetal and maternal circulatory systems has to be reached before an awareness of the significance of the differences in estrogen levels between these fluid compartments can be obtained.

Classical experiments on the rat have demonstrated that in this species implantation will not occur in the absence of ovarian estrogen (Cochrane and Meyer, 1957; Psychoyos, 1962; Shelesnyak, Kraicer and Zeilmaker, 1963; Nutting and Meyer, 1964; Yasukawa and Meyer, 1966; Madhwa Raj et al, 1968). Conversely, neither ovarian (Foote et al, 1957; Moore and Rowson, 1959; Alexander and Williams, 1966; Bindon, 1971; Robertson and Smeaton, 1973; Moore, 1975) or adrenal estrogens (Cumming, Baxter and Lawson,

1974) are required by the ewe during the time of placental attachment. However, the fact that estrogens arising from the conceptuses in the pig can be detected in the urine (Kust, 1931; Struck, 1931; Lunaas, 1962; Raeside, 1963) and in the maternal plasma (Robertson and King, 1974) of this species at the time of the attachment of the embryo raises the question of a possible role for estrogens synthesized by the conceptus in the process of placental attachment in some species, in this case the sheep. It has been suggested in this discussion that E_1S is being produced by the sheep conceptus at about day 30 (Fig. 16), but since by this time the attachment of the conceptus to the maternal uterus is already in progress, it is not possible to state from the present finding whether estrogens, primarily E_1S, are secreted by the conceptus prior to the initiation of this attachment or arise as a consequence of it. However, by analogy with the rat the circumstantial evidence is weighted in favour of the hypothesis that estrogens may be involved in initiating placental attachment in the ewe or are involved in some aspect of its early development.

An interpretation of what role estrone sulphate plays in placental attachment is made difficult by the prolonged period of time during which initial attachments occur and subsequently during which new attachment sites are progressively formed. Since E_1S is readily water soluble, one could speculate that it diffuses to the maternal uterus where some or all of it is deconjugated by a

uterine sulphatase and reduced to the biologically active $E_2\beta$. Steroid sulphates in general are being treated with increasing importance as transport forms of hormones and control of sulphatase activity is being regarded as a mechanism of regulation of steroid hormone action (Dominguez, Valencia and Loza, 1975; Notation, 1975). Rossier and Pierrepoint (1974) showed that sheep uterine myometrium obtained at days 119 and 127 of gestation, when incubated with E_1S, produced high levels of both E_1 and $E_2\beta$. It would be interesting to see if early pregnancy uterine myometrium or uterine caruncles were also active in this regard.

What might be the function of the active $E_2\beta$ once it is produced? It has been shown that, in the hamster, ovarian estrogens are required for proper vascular development of the placenta during early gestation (Pijenborg, Robertson and Brosens, 1975). It has also been found that estrogens induce uterine hyperemia in both the pregnant and nonpregnant ewe (Greiss and Marston, 1965; Anderson and Hackshaw, 1974; Resnic, Kellam, Battaglia, Makowski and Meschia, 1974). Certainly vascular development plays an integral role in the process of implantation.

In the presence of such high sulphatase activity, how does one explain the high levels of E_1S found in the peripheral plasma of the sow during the time of implantation and in that of both the ewe and the sow during late gestation? It has been demonstrated that the guinea pig placenta is impermeable to

estrogen sulphates (Levitz et al, 1960). Whether this is also the case for domestic mammals remains to be seen. Sulphurylation activity has been observed in the placenta of the guinea pig (Levitz et al, 1960), the cow (Holcenberg and Rosen, 1965) and the ewe (Pierrepoint et al, 1971; Rossier and Pierrepoint, 1974; John and Pierrepoint, 1975) and in the uterine myometrial and endometrial tissue of the sow (Perry et al, 1976). In the case of the guinea pig, it has been shown that estrogen sulphates present in the maternal circulation could not traverse the placental barrier, but unconjugated estrogens appeared in the fetal circulation as their corresponding sulpho-conjugates. Could such a system be functioning in domestic mammals in that unconjugated estrogens produced in the placenta are then sulphated and by this process of sulphurylation, aided in traversing the placental barrier so that they can travel in the maternal circulatory system to their target organs?

In conclusion, estrogens are being produced by the sheep conceptus at least twice during gestation. The early peak coincides with the time of implantation and it is possible, especially when considering the similarity between estrogen profiles in the sheep and the pig, that these estrogens are involved in placental attachment, perhaps by influencing the vascular development of the placentomes. More information must be obtained regarding sulphatase and sulphurylation enzymes, the relationships

between fetal and maternal fluids and the steroidogenic capabilities of the various endocrine organs of pregnancy before a clear picture can be drawn of where the estrogens are produced, by what means they reach their target organs and what their effects are on these target organs during both early and late gestation.

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