1987

Progesterone And Estrogens In Intrauterine Tissues During Pregnancy And Labour

Stephen George Power

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PROGESTERONE AND ESTROGENS IN INTRAUTERINE TISSUES DURING PREGNANCY AND LABOUR

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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April 22, 1987

ISBN 0-315-36082-8

ABSTRACT

A decline in progesterone and a rise in estrogen levels in the maternal and fetal plasmas occur prior to parturition. I examined the hypothesis that similar changes in the ovine intrauterine tissues are associated with labour. I also determined whether the intrauterine tissues could produce progesterone and estrogens and whether these activities change in association with labour.

Samples of maternal and fetal bloods, fetal fluids, myometrium, endometrium, chorion, amnion and allantois were obtained from pregnant sheep of different gestational ages and in labour occurring either at term or induced prematurely by administration of ACTH to the fetus. Steroids were measured using radioimmunoassay techniques. Subcellular and dispersed cell preparations were used to examine conversion of endogenous and exogenous substrates to progesterone and estrogens.

At term, labour was associated with a decline in the levels of progesterone in the maternal and fetal bloods, endometrium and myometrium and a rise in the levels of estrone and estradiol-17β in the myometrium. At ACTH-induced labour, only the rise in the levels of estrogens in the myometrium was reproduced. Concentrations of progesterone and estrogens in fetal fluids and fetal membranes were not affected by labour.

Subcellular preparations of chorion and, to a lesser extent amnion, converted ^{3}H-pregnenolone to ^{3}H-progesterone. At term this conversion in the chorion was quantitatively similar to that in the cotyledons and could be inhibited by several steroids including estrogens. Cells from all tissues converted 20α-dihydroprogesterone to progesterone but only the chorion and endometrium converted preg-

nenolone to progesterone. Cells from all tissues converted estrone sulphate to estrone and estradiol-17β but only the chorion converted androstenedione to estrone. ^{3}H-progesterone was converted to ^{3}H-20α-dihydroprogesterone by all tissues and to ^{3}H-17α-hydroxyprogesterone by chorion. These conversions did not change significantly with labour.

In conclusion 1) labour was associated with alterations in the levels of progesterone and estrogens in the myometrium and endometrium, but not in the fetal membranes; 2) the intrauterine tissues have the steroidogenic potential to alter the local steroid milieu but no change in this potential was identified at parturition, and 3) steroids may inhibit steroid metabolizing enzymes in the intrauterine tissues and alter steroid production.

This thesis is dedicated

to my wife

Lori

Poeragh - aboo

ACKNOWLEDGEMENTS

The studies described herein were performed concurrently with the course work and clinical rotations of the undergraduate medical curriculum at the University of Western Ontario. The M.D./Ph D program was designed under the guidance of Dr. M. Hollenberg, Dean of Medicine, Dr. R. Anderson, Assistant Dean of Medicine, Dr. G. Mogenson, Chairman of the Department of Physiology and Dr. R. Kidd, Assistant Dean of Graduate Studies. Administrative Assistants, Mrs. R. Wright and Mrs. C. Abraham coordinated the medical curriculum. These people allowed the momentum of my prior research training to continue and build while I pursued my medical training. A studentship (1981-1985) from the Medical Research Council of Canada made my efforts financially rewarding.

Mr. Case Van Noord and Mr. Doug Johnson provided sheep of known gestational age. Dr. George Carson and Dr. John Patrick taught the surgical techniques. Mr. Ken McGill and his staff at U.W.O. Animal Centre and Miss Sharon Barwick and Mr. Jim Bousquet and their staff at The St. Joseph's Research Institute housed the animals. Ms. Cher Sprague liasoned with the animal facilities. Dr. Stephen Lye's help at surgery and tissue collection was invaluable. Ms. Dayle Huhtanen and Mrs. Carole Brunette-Cahill helped process samples. Dr. Keith Tanswell permitted use of his Coulter Counter and spectrophotometer. Dr. Peter Mitchell and Dr. Lye gave constructive criticism of the data and its interpretation. Ms. Peggy Balestrini typed this thesis and Mrs. Nan Cumming typed the manuscripts and abstracts. I express my warmest thanks to all mentioned above.

From our first encounter in Montreal in 1977 I have had the honour of knowing my supervisor Dr. J.R.G. Challis He has earned my deepest respect and admiration as a scientist and a friend. From the beginning he has been a source of encouragement, inspiration and wisdom. Thanks, John.

The discipline, determination, and ambition that gives impulse to my work was nurtured by my parents Jean and Hugh Power, my brother, Mike and my sister Joanne. Thank you all.

There is but one person left to acknowledge but the few words on this page could not do justice to my gratitude and love for her. Her technical expertise in the operating room, laboratory, drawing room, and photography room made the long arduous hours bearable. She knew when I needed words of encouragement, she knew when I needed to be alone. I am forever grateful, Lori, thank you for everything.

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LIST OF ABBREVIATIONS

Δ4	androstenedione, androst-4-en-3, 17-dione
ACTH	adrenocorticotrophic hormone
ALF	allantoic fluid
ALL, AL	allantois
AMN, AM	amnion
AMF, AF	amniotic fluid
ATP	adenosine triphosphate
°C	°Celsius
^{14}C	carbon - 14
cm	centimetre(s)
CHOR, C	chorion
CPM	counts per minute
CSSCE	cholesterol side chain cleavage enzyme
D, d	day (of pregnancy)
DHEA(-S)	dehydroepiandrosterone (-sulphate); androst-5-en-3β-ol-17-one
20α(β)-DHP	20α(β)-dihydroprogesterone; pregn-4-en-20α(β)-ol-3-one
DPM	disintegrations per minute
ENDO, E	endometrium
E2 (α, β)	estradiol (17α-, 17β-); estra-1,3,5-trien-3,17α(β)-diol
E3	estriol, estra-1,3,5-trien-3, 16, 17α(β)-triol
E1	estrone; estra-1,3,5-trien-3-ol-17one
Es	estrogens

F	cortisol; pregn-4-en-11β, 17α, 21-triol-3,20dione
g	gravity
gm	gram(s)
h	hours
HPLC	high pressure liquid chromatography
^3H	tritium
3β-HSD	3β-hydroxysteroid dehydrogenase, Δ5-4 isomerase
3α-(17β-, 20α-) HSD	3α-(17β-, 20α-) hydroxysteroid dehydrogenase
IR	immunoreactive
kg	kilogram(s)
Km	affinity constant
Ki	inhibition constant
Krebs GA	Krebs buffer with glucose and albumin
M	molar
min	minute(s)
mg	milligram(s)
ml	milliliter(s)
MPV	maternal peripheral vein
mmHg	millimeters of mercury
MCR	metabolic clearance rate
n	number
ng	nanogram(s)
nm	nanometre(s)
NAD^+	nicotinamide adenosine dinucleotide (oxidized)
P5	pregnenolone, pregn-5-en-3β-ol-20-one
P4	progesterone, pregn-4-en-3,20-dione

pg	picogram(s)
pmole	picomole(s)
PGs	prostaglandins.
PGE(F)	prostaglandin E series (F series)
PGI_2	prostaglandin I2 (prostacyclin)
PGFM	prostaglandin F metabolite
PR	production rate
r	correlation coefficient
RIA	radioimmunoassay
RF	retention factor
SEM	standard error of mean
SL	spontaneous labour at term
TLC	thin layer chromatography
U	urachas
Umb A(V)	umbilical artery (vein)
UV	uterine vein
µg	microgram(s)
µl	microliter(s)
Vol, V, v	volume

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INTRODUCTION

The processes of pregnancy and parturition are unique to viviparous species. Our interest in the physiological mechanisms that control the maintenance of pregnancy and the onset of parturition is fueled by our desire to decrease the incidence of infant morbidity and mortality that is generated primarily by a population of prematurely born infants (Creasy, 1980). To date, our knowledge describes a process of complex interrelationships between the actions of protein and steroid hormones and the fatty acid derivatives, prostaglandins. This knowledge, however, still cannot explain entirely the mechanisms governing the natural and premature onset of labour.

Investigators have measured the concentrations of hormones in maternal and fetal plasmas throughout pregnancy and labour. They have looked for the origins and targets of these hormones. No one has been able to explain the onset of parturition in all species on the basis of changes in peripheral plasma concentrations of hormones. We are now learning that the tissues of the pregnant uterus (see Appendix 1) produce a vast array of compounds that might influence their concentrations not only in the circulation but also in neighbouring tissues and cells of the same tissue.

In the literature review that follows I will present evidence that implicates several hormones in the endocrine events that characterize viviparity. Particular reference will be given to the domestic sheep as this is a species that has been extensively investigated in this regard and in which I have performed my experiments. The review will contain mostly scientific literature up to 1981, the year I

began my research activities. Reference to more recent studies will be introduced in later chapters.

Progesterone (P4) and estrogens (Es) have been linked to the endocrine changes in late pregnancy and parturition. My investigations have focused on the levels of these steroids in the intrauterine tissues of pregnant ewes. Studies were performed to relate patterns of change for P4 and Es levels throughout gestation in intrauterine tissues to those in the maternal and fetal plasmas and fetal fluids. Other studies have investigated the ability of the intrauterine tissues to modulate the local steroid milieu through their ability to synthesize and metabolize P4 and Es. These studies were performed to support the concept that, superimposed upon the endocrine phenomena influencing the pregnant uterus, there are autocrine and paracrine phenomena as well.

SECTION I

LITERATURE REVIEW AND RATIONALE

CHAPTER 1

LITERATURE REVIEW

1.1 Progesterone and the Maintenance of Pregnancy

It has been known since the early studies of Fraenkel (1905) that ovariectomy of the pregnant rabbit, irrespective of gestational age, is followed by abortion of the conceptus. Corner and Allen (1929, 1930) were able to prevent abortion in ovariectomized rabbits with extracts of corpus luteum. Later it was shown that pregnancy could be maintained in ovariectomized rabbits with authentic progesterone (P4) (Allen and Heckel, 1939) or synthetic P4 (Wu and Allen, 1958). Thus, the importance of P4 from the corpus luteum in the maintenance of pregnancy was established.

Similar experiments have been performed in sheep but with different results. Ovariectomy of the pregnant sheep is followed by abortion if performed before day 50 and a pregnancy of normal gestational length if performed thereafter (Casida and Warrick, 1945; Neher and Zarrow, 1954; Denamur and Martinet, 1955; Foote, Gooch, Pole and Casida, 1957; Moore and Rowson, 1959; Fylling, 1970). Gestational length in the sheep is between 140-150 days. Treatment with exogenous P4 protects the animal from the abortifacient effects of ovariectomy before day 50 (Moore and Rowson, 1959). These results suggest that if P4 is important to the maintenance of ovine pregnancy then a source other than the ovary is producing it in sufficient amounts after day 50.

Studies have been performed on several species describing their pregnancies as either dependent or not dependent on the corpus luteum. In the human female, monkey, mare, guinea pig and cat

removal of the ovaries or corpora lutea during the later stages of pregnancy does not effect the course of gestation, in the rat, mouse, and goat, abortion or resorption occurs irrespective of the times at which the operation is performed (See Amoroso, 1952)

1.2 Progesterone in Ovine Pregnancy

1.2.1 Concentrations

Several investigators have measured the concentration of P4 ([P4]) in the peripheral plasma of pregnant ewes. Whereas some studies showed the [P4] to be constant throughout pregnancy (Short and Moore, 1959; Lindner, Sass and Morris, 1964; Moore, Barrett and Brown, 1972) others have shown the [P4] to rise after day 50 to peak in late pregnancy at values two-fivefold those measured in early pregnancy (Bassett, Oxborrow,Smith, and Thorburn, 1969; Fylling, 1970; Stabenfeldt, Drost and Franti, 1972). Before day 60 the [P4] was similar to that observed during the luteal phase of the estrous cycle (Thorburn, Bassett and Smith, 1969). During the last two weeks of pregnancy the [P4] declined (Bassett et al, 1969; Fylling, 1970; Stabenfeldt et al, 1972; Elsner, Magyar, Fridshal, Eliot, Klein, Glatz, Nathanielsz and Buster, 1980); however, disagreement exists as to the timing of the peak and decline. Those studies reporting constant levels throughout pregnancy used relatively nonspecific estimating techniques or made only few observations at any one time in gestation. Differences observed between profiles of [P4] in late pregnancy may be attributed to high intra- and inter-animal variability (Bassett et al, 1969, Fylling, 1970; Stabenfeldt et al, 1972; Strott, Sundel and Stahlman, 1974). Challis, Patrick, Cross, Workewych, Manchester and Power (1981) showed substantial fluctua-

tions in the [P4] in maternal plasma obtained at frequent intervals over ninety minutes and suggested that a normal biological variation must be considered when evaluating sampling protocols.

The [P4] in the maternal plasma may change in response to a change in the metabolic clearance rate (MCR) or a change in the production rate (PR). Short and Rowell (1962) found no difference between the half-life of P4 measured on day 115 and that measured a few hours before labour. Using tracer kinetic techniques Bedford, Harrison and Heap (1972a) found the MCR to be remarkably constant during most of gestation. The PR of P4 closely reflected changes in blood concentrations. The mean PR was highest during the last two weeks of pregnancy, being nearly five times greater than that in early pregnancy. This increase, however, was only found in sheep bearing fetuses with birth weights greater than 4 kg. Bassett et al (1969) reported that during the last 50 days of pregnancy the mean [P4] in ewes with twins were approximately twice that in ewes with a single fetus. Therefore, the rise in the [P4] after day 50 of pregnancy results from an increase in the PR of P4 which is somehow related to the size of the fetus and degree of multiparity.

The [P4] in fetal plasma is much lower than that in the maternal plasma (Strott et al, 1974; Elsner et al, 1980). Elsner et al (1980) described simultaneous fetal and maternal profiles of the [P4] over the last 18 days of pregnancy. The [P4] fell in both the fetal and maternal plasmas and the mean times of onset of the fall, as determined by time trend analysis, were 2.2 and 3.7 days before delivery, respectively.

1.2.2 Production

The ovaries, adrenals and placenta have been investigated as sources of P4 in the pregnant ewe. The corpus luteum continues to secrete P4 until about two weeks before parturition at which time the concentrations in the ovarian vein blood decrease markedly (Edgar and Ronaldson, 1958). Short and Moore (1959) measured P4 in placentas of intact and ovariectomized ewes and concluded that the placenta may be a source of P4. Homogenates of placental tissue can convert labelled pregnenolone (P5) to labelled P4 (Ainsworth and Ryan, 1967; see figure 1 for steroidogenic pathways). Linzell and Heap (1968) measured P4 in the ovarian, adrenal and uterine vein during pregnancy and estimated placental P4 secretion to be three times ovarian and adrenal secretion. Maternal ovariectomy in late pregnancy had no effect on P4 levels in peripheral plasma. Adrenalectomy and adrenalectomy/ovariectomy lowered P4 levels in peripheral plasma only slightly and had no effect on levels in uterine venous plasma (Thompson and Wagner, 1974). Harrison and Heap (1978) showed that the production of P4 measured in animals with autotransplanted ovaries and adrenals rose between days 66-103 and days 133-145. The production of P4 by the adrenal was low and remained unchanged and that of the ovary fell during this time. The production of P4 by the placenta exceeded that by the ovary after days 66-103 and increased fourfold during the second half of gestation. Ricketts and Flint (1980) measured placental production of P4 in ewes ovariectomized early in gestation and whose pregnancies were maintained by medroxyprogesterone acetate. They reported a small rise in the production of P4 between day 50 and day 70 and a second rise between day 90 and day 120. Therefore, the increase in PR during the second

FIGURE 1. Enzymatic pathways for the production and/or metabolism of progesterone and estrogens.

(1) 3β-hydroxysteroid dehydrogenase, Δ5-4 isomerase

(2) 20α-hydroxysteroid dehydrogenase

(3) 17β-hydroxysteroid dehydrogenase

(4) Estrone-sulfatase

(5) Estrone-sulfotransferase

(6) 17α-hydroxylase

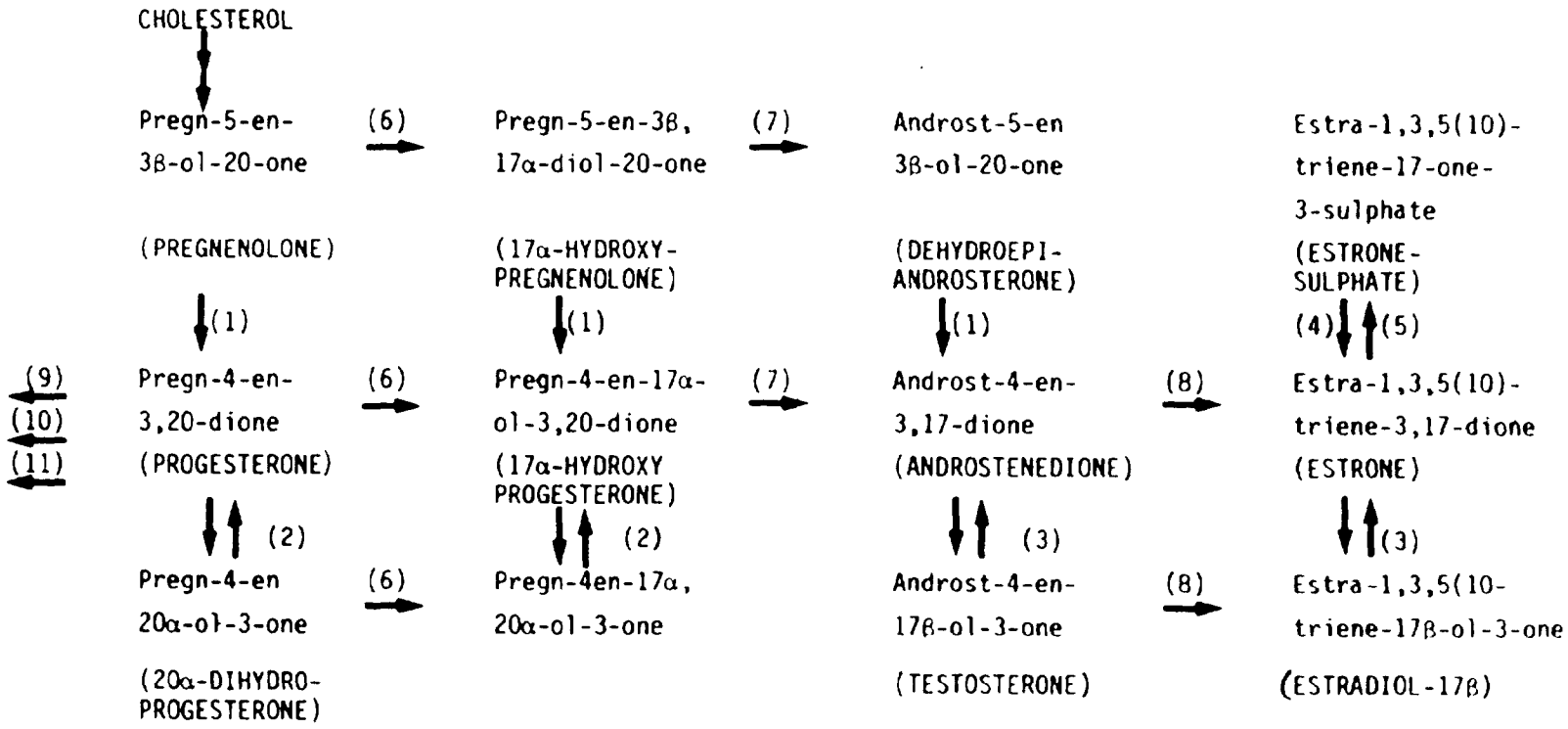
(7) C17-20 Lyase

(8) Aromatase

(9) 5β-reductase

(10) 5α-reductase

(11) 3α-hydroxysteroid dehydrogenase


CHOLESTEROL
Pregn-5-en-3β-ol-20-one
(PREGNENOLONE)
(6)
Pregn-5-en-3β, 17α-diol-20-one
(17α-HYDROXY-PREGNENOLONE)
(7)
Androst-5-en 3β-ol-20-one
(DEHYDROEPI-ANDROSTERONE)
Estra-1,3,5(10)-triene-17-one-3-sulphate
(ESTRONE-SULPHATE)
(1)
(4)
(5)
(9)
(10)
(11)
Pregn-4-en-3,20-dione
(PROGESTERONE)
(6)
Pregn-4-en-17α-ol-3,20-dione
(17α-HYDROXY PROGESTERONE)
(7)
Androst-4-en-3,17-dione
(ANDROSTENEDIONE)
(8)
Estra-1,3,5(10)-triene-3,17-dione
(ESTRONE)
(2)
(3)
Pregn-4-en 20α-ol-3-one
(20α-DIHYDRO-PROGESTERONE)
(6)
Pregn-4en-17α, 20α-ol-3-one
Androst-4-en-17β-ol-3-one
(TESTOSTERONE)
(8)
Estra-1,3,5(10-triene-17β-ol-3-one
(ESTRADIOL-17β)

half of gestation is due to an increase in the secretion of progesterone by the placenta.

1.2.3 Metabolism

Progesterone is rapidly cleared from the maternal plasma (Bedford et al, 1972). The MCR during pregnancy is only slightly greater than that during the luteal phase of the estrous cycle (Bedford et al, 1972). The bulk of radioactivity isolated from maternal plasma following a single injection of [^{14}C]-progesterone (^{14}C-P4) was found in the sulphate fractions from which a major metabolite was identified as 5α-pregnane-3β-ol-20-one (Tsang and Hackett, 1979). Therefore, P4 is cleared from the plasma by 5α reduction of the A ring, and sulfoconjugation.

The placenta contains several enzymatic activities capable of metabolizing P4 including, 5β-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD), and 20α- and 20β-hydroxysteroid dehydrogenase (20α,-20β-HSD). The principal placental metabolite is 5β-pregnan-3α, 20α-diol (Ainsworth and Ryan, 1967) which has been isolated from pregnancy urine (Robertson and Coulson, 1958). Other placental metabolites include pregn-4-en-20α-ol-3-one (20α-DHP), 5β-pregnan-3α, 20α-diol, and pregn-4-en-20β-ol-3-one (20β-DHP). 20α-DHP has been isolated from placenta and peripheral blood of pregnant ewes (Short and Moore, 1959). Constant infusions of [^{3}H]-progesterone (^{3}H-P4) to pregnant ewes resulted in production of ^{3}H-20α-DHP whose concentration closely correlated with that of ^{3}H-P4. Elsner et al (1980) found the fetal:maternal ratio of the [20α-DHP] to be ~3:1, a finding consistent with a high fetal 20α-HSD activity. The ratio of

[P4]:[20α-DHP] was ~1:10 and ~4:1 in the fetal and maternal circulations, respectively.

The major pregnanediols isolated from fetal blood are sulfoconjugates of 5β-pregnan-3β,20β-diol, 5β-pregnan-3β,20α-diol and 5β-pregnan-3α,20α-diol (Dolling and Seamark, 1979). The fetal liver can metabolize P4 to sulfoconjugates of 5β-pregnan-3β,20β-diol and 20α-diol (Nancarrow, 1969; Anderson, Pierrepoint, Griffiths and Turnbull, 1970) 5β-pregnan-3α,20α-diol can be formed by the placenta and several other fetal tissues (Nancarrow, 1969; Ainsworth and Ryan, 1967; Pierrepoint, Anderson, Turnbull and Griffiths, 1973) or be of maternal origin (Robertson and Coulson, 1958, Stupnicki and Williams, 1968; Stupnicki and Stupnicki, 1970).

1.2.4 Other Species

Viviparous species differ with respect to the profile of the [P4] throughout gestation and the source(s) of P4 (see Thorburn, Challis and Robinson, 1977). As in the sheep, the placenta is the major source of P4 in the maternal plasma of several species including humans, monkeys and guinea pigs. In contrast, species including the goat, rabbit and rat depend on the corpus luteum to maintain the [P4] in the plasma. The gestational profile of the [P4] in the maternal plasma of pregnant goats is similar to that of the sheep; however, the [P4] increases during the second half of gestation in response to an increase in luteal output (Thorburn and Schneider, 1972). The [P4] declines abruptly as the corpus luteum regresses during the last few days before parturition (Thorburn and Schneider, 1972; Umo, Fitzpatrick and Ward, 1976). Although plasma concentrations decline prior to parturition in the sheep and goat and

other species including the rabbit, rat and cow, concentrations do not consistently fall in the maternal plasma of guinea pigs and humans (see Heap, Galil, Harrison, Jenkin and Perry, 1977; Bedford, Challis, Harrison and Heap, 1972b). In the pregnant human, the [P4] continues to rise after 6 weeks of pregnancy to peak values at term (see, Thorburn et al, 1977). Although a few authors have reported a decline in the [P4] prior to parturition (Csapo, Knobil, Van der Molin and Wiest, 1971; Csapo, Pohanka, Kaihola, 1973; Turnbull, Patten, Flint, Keirse, Jeremy and Anderson, 1974) most investigators have failed to substantiate these results. P4 in the human plasma is extensively bound, which, perhaps, explains the higher [P4] in pregnant human plasma compared to the [P4] in pregnant sheep plasma. Yanonne, Mueller and Osborn (1969) observed that the portion that was associated with high affinity globulin remained constant at about 91% both antenatally and in labour. Thus the absolute concentration of unbound P4 in human maternal plasma rises with the approach of parturition.

1.3 Estrogens in Ovine Pregnancy

As soon as ovarian hormones were identified and purified, their role in the maintenance of pregnancy and the onset of parturition was sought. Having described the evidence for P4 in the endocrinology of pregnancy and parturition, I will now do so for estrogens (Es).

1.3.1 Concentrations

In 1927, estrogenic activity was demonstrated in extracts of ovine placenta by Parkes and Bellerby. Later, Es were measured in extracts of urine from pregnant ewes and their concentrations shown

to be low for most of pregnancy and to rise slightly in late pregnancy (Whitten 1943; Beck, 1950; Bassett, Sewell and White, 1955). Later studies (Fèvre, Piton and Rombauts, 1965; Fèvre and Rombauts, 1966) did not detect Es in the urine until about day 70 of gestation and then their excretion increased markedly from day 90 to term (Fèvre and Rombauts, 1966). The main Es excreted by the pregnant ewe are conjugates of estrone (E1) and 17α-estradiol (E2α)(Velle, 1963; Fèvre et al, 1965; Fèvre and Rombauts, 1966).

Challis (1971), using radioimmunoassay techniques, measured total unconjugated Es in maternal plasma. The concentrations were less than 5pg/ml until 31 days pre-partum and rose slowly to 20-40pg/ml at 5 days pre-partum. There was a marked increase within 48 hours before lambing. Subsequent investigations focussed on the pattern of changes in the concentrations of specific Es in maternal plasma. The concentrations of E1 ([E1]) and E2 ([E2]) are low in early pregnancy and rise slowly between day 75 and day 140 (Carnegie and Robertson, 1978). The [E1] and [E2β] (Robertson and Smeaton, 1973; Thorburn, Nicol, Bassett, Shutt and Cox, 1972) and [E2α] (Thorburn et al, 1972) increase markedly starting about 48 hours before parturition. The [E2β] rises only within the last 12 hours before lambing (Thorburn et al, 1972). The ratio of E1:E2α:E2β is 2:1:1.

The predominant Es in maternal plasma are sulfoconjugates (Tsang, 1974; Carnegie and Robertson, 1978). The ratio of estrone sulphate (E1-S):E1 (Tsang, 1974; Carnegie and Robertson, 1978) and E2-S:E2 (Carnegie and Robertson, 1978) are 30:1 and 10:1 respectively. The patterns of change for E1-S and E2-S in late

pregnancy are similar to those for E1 and E2 respectively (Carnegie and Robertson, 1978).

In the fetal plasma the major Es are also sulfoconjugates (Findlay and Cox, 1970; Currie, Wong, Cox and Thorburn, 1973). Whereas Findlay and Cox (1970) could not detect unconjugated Es, Thorburn et al (1972) measured concentrations of unconjugated Es of less than 30 pg/ml which changed little before parturition, at a time when maternal concentrations were rising. The [E1-S] and [E2α-S] rose markedly on day 149 and the [E2β-S] rose only marginally. The [E1-S] was slightly higher than the [E2β-S] and both were greater than their respective unconjugates by a factor of 10^2 - 10^3. (Currie et al, 1973).

1.3.2 Production

Challis, Harrison and Heap (1973, a,b,c) explained the sharp rise in the [E1] and [E2] in the maternal plasma within 48 hours of lambing as being caused by an increase in their PR and not a decrease in their MCR. The concentrations of Es in the uterine vein were much higher than the jugular vein during this time (Challis, Harrison and Heap, 1971). Ovariectomy did not effect the uterine venous [E1] (Bedford et al, 1972b; Thompson and Wagner, 1974). Although adrenalectomy lowered the [E1] in the uterine venous plasma, there was still a rise in the [E1] within 24 hours of lambing. These results suggested that a non-ovarian, non-adrenal source of Es, probably the placenta, contributes to the Es measured in the maternal plasma at term.

The placenta has been shown to possess all the enzymes necessary to convert C21 steroids to Es. The presence of these enzymes was

et al, 1979; Green, Bygdeman, Toppozada and Wiqvist, 1976) and urine (Satoh et al, 1979, Hamberg, 1974) during labour compared to levels before labour started. The output of PGs by dispersed cells from amnion and decidual tissue is higher in tissues collected from patients following spontaneous vaginal delivery compared to output by tissues collected at elective caesarian section at term but in the absence of established labour (Skinner and Challis, 1985). Therefore, in the sheep and humans labour is associated with increased levels of PG in intrauterine tissues and maternal and fetal fluids which are probably caused by an increase in the production of PGs from maternal tissues including the myometrium and decidua (endometrium) and fetal tissues including the chorion (chorioallantois) and amnion. At least in the sheep, these events precede the onset of labour.

1.6 Control of Myometrial Activity

The pregnant uterus undergoes a period of relative quiescence which is interrupted at parturition by the onset of enhanced myometrial activity. Csapo (1975, 1977) has championed the "see saw" hypothesis to explain these phenomena. In essence, the activity state of the uterus at any one moment is governed by the balance between suppressor and stimulatory forces acting upon the myometrium. The maintenance of pregnancy would be favoured by an imbalance of suppressor forces and parturition would result from an imbalance of stimulatory forces.

1.6.1 Oxytocin

Oxytocin is a well recognized stimulator of uterine contractions in pregnant and non-pregnant animals. Administration of oxytocin to non-pregnant animals increases uterine activity (Knaus, 1934; Evans

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and 100 of gestation and similar to that in umbilical cord blood, whereas the E1/E2 ratio in the umbilical vein blood was significantly greater than that in the uterine vein in early gestation but fell as pregnancy progressed. These authors presumed these changes reflected changing patterns of estrogen secretion during pregnancy and that in late pregnancy the sheep placenta secretes relatively more E1 than E2 into the maternal circulation.

Parturition in sheep is associated with altered placental enzymatic activities. The levels of placental aromatase (Ash, Challis, Harrison, Heap, Illingworth, Perry and Poysner, 1973; Mann, Curet and Colas, 1975), 17α-OHase (Anderson et al, 1975) and C17-20 lyase (Steele et al, 1976) are elevated in spontaneous labour compared to their levels prior to labour. An increase in the levels of 17α-OHase is supported by the finding of Flint, Goodson and Turnbull, (1975) who observed an increase in the concentrations of pregn-4-en-17α,20α-diol-3-one in the uterine venous plasma. It is conceivable that the prepartum rise in the concentration of Es and the pre-partum decline in the [P4] result from a rerouting of C21 steroids towards C19 estrogen precursors and Es.

1.3.3 Other Species

Other viviparous species differ from sheep and from each other with respect to the gestational profiles of the estrogen levels in the maternal plasma, the site of the production of Es, and the predominant Es secreted. In several species such as the human, goat, cow, guinea pig and rat, the maternal estrogen levels are highest in late pregnancy; however, a surge in the estrogen levels during the last few days of pregnancy, as described previously for the sheep, is

not a consistent finding (see Bedford et al, 1972b; Thorburn et al, 1977).

In the goat, Challis and Linzell (1971) measured concentrations of total unconjugated Es in the maternal plasma that were several times higher than those in the sheep at comparable gestational ages. These authors reported that unconjugated Es appear in the maternal plasma early in pregnancy and then rise steadily through until term before undergoing a more rapid rise during the last 4-5 days. This pre-partum surge is not as marked as that seen in the sheep (Thorburn et al, 1972; Currie et al, 1973; Umo et al, 1976). Currie et al (1973) measured sulfoconjugated and unconjugated E1 and E2 in maternal and fetal plasma but could not detect E2. E2 was the predominant unconjugated estrogen in the maternal plasma. In the fetal plasma the concentrations of estrogen sulfoconjugates were several times higher than the concentrations of unconjugated Es and increased during the last few days of pregnancy. Thus the goat and the sheep are similar in some but not all aspects of estrogen production and metabolism.

In the human, Tulchinsky, Hobel, Yeager, and Marshall 1972 have measured unconjugated E1, E2 and estriol (E3) in the maternal plasma. Throughout pregnancy the major unconjugated estrogen was E2β. Unconjugated Es rose steadily throughout pregnancy. Whereas the [E2β] and [E3] continued to rise through until term, the [E1] plateaued at 30 weeks. No sudden surge of Es during the last few days of pregnancy has been reported (Tulchinsky et al, 1972; Shaaban and Klopper, 1973). In human pregnancy Es are produced by the feto-placental unit (Siiteri and MacDonald, 1966). The fetal and maternal adrenals supply C19 precursors, such as DHEA-S and

16-OH-DHEA-S, to the placenta for subsequent aromatization. The human placenta, unlike the sheep placenta cannot convert C21 steroids through to Es.

1.4 Fetal Adrenal Function

1.4.1 Sheep

Cortisol (F) has been measured in the plasma of chronically catheterized fetal lambs and shown to increase substantially over the last 4-5 days before term (Bassett and Thorburn, 1969; Comline, Nathanielsz, Paisey and Silver, 1970). The rise in the concentration of F ([F]) reflects an increase in PR since the MCR of F is unchanged over the last 10 days of pregnancy (Nathanielsz, Comline, Silver, and Paisey, 1972).

It is now well established that the fetal hypothalamo-pituitary-adrenal axis has an important role in the onset of labour in the sheep. Ablation of the fetal pituitary (Liggins, Kennedy and Holm, 1967) and bilateral adrenalectomy (Drost and Holm, 1968) prolongs pregnancy. The fetal adrenal cortex grows markedly in the last 10 days of intrauterine life (Comline and Silver, 1961) and this growth can be prevented by fetal hypophysectomy (Comline, Silver and Silver, 1970). Fetal hypophysectomy prevents the normal increase in the [F] at term (Robinson, Challis, Pooley and Thorburn, 1977). Parturition can be induced in late pregnancy by the administration of ACTH or glucocorticoids to the fetus (Liggins, 1968, 1969).

In the fetal plasma the concentration of ACTH ([ACTH]) increases in parallel with the [F] during the last week of pregnancy (Rees, Jack, Thomas and Nathanielsz, 1975), at a time when the negative feedback of F on [ACTH] has been demonstrated and when dexamethasone,

a synthetic glucocorticoid, has been shown to suppress the fetal [ACTH] and [F] (Challis, Jones, Robinson and Thorburn, 1977).

The response of the fetal adrenal to ACTH varies with gestational age. Immunoreactive ACTH levels in the fetus were elevated during an episode of hypoxemia at all times during gestation but only after day 139 was the increase associated with elevations in the [F] (Jones, Boddy, Robinson and Ratcliffe, 1977). ACTH infused into the fetus caused little change in the [F] until after day 135-140 (Liggins, 1972). In vitro studies (Wintour, Brown, Denton, Hardy, McDougall, Oddie and Whipp, 1975; Glickman and Challis, 1980) demonstrated the capacity of the adrenal to respond to ACTH with increased production of F before day 90 and after day 130 but not in the intervening period. These results suggested a deactivation/ reactivation process was occuring in the fetal adrenal and that reactivation was followed by a rise in the [F] in the fetal plasma.

The changes in the [Es] and [P4] in late pregnancy are believed to result from the effects of the increased [F] in the fetal plasma. Premature labour induced by ACTH (Thorburn et al, 1972) or dexamethasone (Currie et al, 1973) are preceeded by a fall in the [P4] and a rise in the [Es] similar to that occuring prior to the spontaneous onset of labour. Several in vitro studies have been performed in late pregnancy to investigate the effects of intrafetal glucocorticoids on placental steroid metabolism. The placentas of ewes in dexamethasone induced labour produce similar amounts of E1 from 17α-P4 as those of ewes in spontaneous labour and more than those of non-labouring ewes (Steele et al, 1976). The authors attributed the results to an increase in C17-20 lyase. Intrafetal dexamethasone also increased placental 17α-OHase as evidenced by an

increase in the conversion of P5 and P4 to pregn-4-en-17α, 20α-diol-3-one (Anderson et al, 1975). This increase occured within twelve hours of treatment and prior to the onset of labour. The activities of cholesterol side chain cleavage (CSSC) and 3β-hydroxysteroid dehydrogenase, Δ4-5 isomerase (3β-HSD) were not affected. Infusion of F into the fetus had little effect on aromatase (Anderson, Curet and Colas, 1978a) or C17-20 lyase but increased 17α-OHase (Anderson, Curet and Colas, 1978b). Mann et al (1975) did not find any effect of F on aromatase activity in short term incubation; however, Ricketts, Galil, Ackland, Heap and Flint (1980) did observe an increase in aromatase activity in placental explants after prolonged exposure to F. These findings together suggest that F is responsible for the altered placental enzymatic activity at term that result in the increased production of Es and the decreased production of P4.

1.4.2 Other Species

Evidence for the role of the fetus in the onset of parturition in humans is not as convincing as it is for sheep. Nature has provided us with two experiments, anencephaly and fetal adrenal hypoplasia, to support or refute the importance of the fetal adrenal pituitary axis at parturition. If anencephalics with hydramnios are excluded, the mean gestational length of anencephalic pregnancies is not different from that of normal pregnancies; however, the incidence of premature and post-mature infants is greater (Honnebier and Swaab, 1973). Pregnancies carrying fetuses with adrenal hypoplasia are predisposed to prolongation (Anderson and Turnbull, 1973). The human fetal pituitary and adrenal do not appear to be as essential to the

onset of parturition as they are in the sheep. In humans glucocorticoids have failed to induce parturition (Liggins, Forster, Grieves and Schwartz, 1977). Whereas, in sheep, dexamethasone increased the maternal levels of Es, it decreased them in humans by providing a negative feedback to the release of ACTH, thereby decreasing stimulation of C19 estrogen precursor release by the fetal adrenal (Liggins et al, 1977). Fetal F may not be the trigger to parturition in the human but it has been shown to stimulate organ maturation that occurs around the time of birth (Challis, 1980). Challis (1980) has suggested that at term fetal adrenal production of C19 estrogen precursors may be more important than production of F in the endocrine events that trigger parturition.

1.5 Prostaglandins and Parturition

The involvement of prostaglandins (PGs) in the control of myometrial activity has been extensively investigated since von Euler (1936) identified these compounds as the active material in seminal fluid that contracted and relaxed uterine strips (Kurzrok and Lieb, 1930). It is now firmly established that PGs have an important role in the biochemical and mechanical events at parturition. In this section I will describe changes in the concentrations of PGs and sites of production at term.

The concentrations of primary PGs and/or their metabolites have been shown to increase in the maternal and fetal plasma and amniotic fluid at spontaneous delivery (Thorburn et al, 1972; Mitchell, Anderson, Brunt, Clover, Ellwood, Robinson and Turnbull, 1979; Mitchell, Robinson and Thorburn, 1977; Mitchell, Ellwood, Anderson and Turnbull, 1978; Challis, Dilley, Robinson and Thorburn, 1976;

Challis, Hart, Louis, Mitchell, Jenkin, Robinson and Thorburn, 1978) and at delivery induced by intrafetal administration of ACTH or glucocorticoids (Currie et al, 1973; Kendall, Challis, Hart, Jones, Mitchell, Ritchie, Robinson and Thorburn, 1977; Mitchell et al, 1979; Olson, Lye, Skinner and Challis, 1984). The PGs measured include prostaglandins of the E series (PGE), prostaglandins of the F series (PGF), a metabolite of PGF (PGFM) and a metabolite of prostacylin (6-keto PGF1α). The rise in the concentration of PGs in the maternal and fetal plasmas and amniotic fluid during the intrafetal administration of ACTH to induce labour occurs before the appearance of labour-like activity (Olson et al, 1984).

The concentrations of PGs in the cotyledons are elevated during dexamethasone (Liggins and Grieves, 1971) and ACTH (Olson et al, 1984) induced labour. Concentrations of PGs in the myometrium are elevated after the establishment of labour (Liggins and Grieves, 1971). Mitchell and Flint (1978a) demonstrated an enhancement of the production of PGs by superperfused cotyledons and myometrium obtained from labouring ewes. High levels of PGs have been measured in the chorioallantois and these levels increased at labour induced by ACTH (Evans, Kennedy, Patrick and Challis, 1981). The output of PGs by dispersed cells from the amnion, chorioallantois and fetal component of the cotyledon were significantly elevated after 72 hours of pulsatile ACTH administration to the fetus (premature labour occured after approximately 100 hours; Olson et al, 1984).

In pregnant women PG levels are also elevated in the amniotic fluid (Keirse, Mitchell and Turnbull, 1977; Satoh, Yasumizu, Fukuota, Kinoshita, Kaneko, Tsuchida and Sakamoto, 1979; Ghodgaonkar, Dubin, Blake and King, 1979) maternal plasma (Satoh et al, 1979; Ghodgaonkar

et al, 1979; Green, Bygdeman, Toppozada and Wiqvist, 1976) and urine (Satoh et al, 1979, Hamberg, 1974) during labour compared to levels before labour started. The output of PGs by dispersed cells from amnion and decidual tissue is higher in tissues collected from patients following spontaneous vaginal delivery compared to output by tissues collected at elective caesarian section at term but in the absence of established labour (Skinner and Challis, 1985). Therefore, in the sheep and humans labour is associated with increased levels of PG in intrauterine tissues and maternal and fetal fluids which are probably caused by an increase in the production of PGs from maternal tissues including the myometrium and decidua (endometrium) and fetal tissues including the chorion (chorioallantois) and amnion. At least in the sheep, these events precede the onset of labour.

1.6 Control of Myometrial Activity

The pregnant uterus undergoes a period of relative quiescence which is interrupted at parturition by the onset of enhanced myometrial activity. Csapo (1975, 1977) has championed the "see saw" hypothesis to explain these phenomena. In essence, the activity state of the uterus at any one moment is governed by the balance between suppressor and stimulatory forces acting upon the myometrium. The maintenance of pregnancy would be favoured by an imbalance of suppressor forces and parturition would result from an imbalance of stimulatory forces.

1.6.1 Oxytocin

Oxytocin is a well recognized stimulator of uterine contractions in pregnant and non-pregnant animals. Administration of oxytocin to non-pregnant animals increases uterine activity (Knaus, 1934; Evans

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and Miller, 1936; Roberts and McCracken, 1976; Soloff, Schroeder, Chakraborty and Pearlmutter, 1977) though the response is much reduced during the luteal phase in the sheep (Roberts and McCracken, 1976) and the cow (Evans and Miller, 1936). During pregnancy the response is low (Fitzpatrick, 1960; Hindson, Schofield and Ward, 1969) but as term approaches the response increases and is maximum at the time of parturition (Fitzpatrick, 1960). Increasing amounts of oxytocin have been measured in the maternal plasma at the time of parturition (Forsling, 1979). Thorburn et al (1977) have suggested that oxytocin is not a prerequisite for parturition but serves a supporting role in labour. Concentrations of oxytocin in the plasma are very low or undetectable in late pregnancy in the pig (Forsling, MacDonald and Ellendorff, 1979), the mare (Allen, Chard and Forsling, 1973) and the goat (Chard, Boyd, Forsling, McNeilly and Landon, 1970) and show a great increase in second stage labour.

1.6.2 Progesterone

Experiments in rabbits established P4 as the classical "suppressor". Uterine activity is high during estrus but declines with the formation of the corpus luteum (Reynolds and Friedham, 1930; Knaus, 1930 quoted by Csapo, 1956) and in particular the increased production of P4 (Reynolds and Allen, 1932). P4 inhibits the response to oxytocin in vitro (Knaus, 1934; Makepiece, Corner and Allen, 1935; Csapo, 1961a) and in vivo (Fuchs and Fuchs, 1958; Csapo, 1961a, 1961b; Csapo and Lloyd-Jacob, 1961). During pregnancy the [P4] in the plasma is high and the responsiveness to oxytocin is diminished whereas at term, the [P4] is lower and the responsiveness to oxytocin increases (Schofield, 1957; Csapo and Lloyd-Jacob, 1961). Effective P4 withdrawal converts a refractory pregnant uterus into an

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active and reactive organ while restoration of P4 restores the suppression of uterine smooth muscle activity (Csapo and Takeda, 1965). Exogenous P4 prolongs pregnancy in the rabbit (Allen and Hechel, 1939).

In the sheep, similar findings support the suppressor function of P4. Uterine activity also decreases during the luteal phase both in vivo (Poloceva, 1942; Mann, 1969; Naaktgeboren, Van Der Weyden, Klopper, Kroon, Schoof and Taverne, 1973; Croker and Shelton, 1973; Lehrer and Schindler, 1974; Ruckebusch and Bueno, 1976) and in vitro (Ambaché and Hammond, 1949; Brinsfield, 1968) when the [P4] is elevated (Stabenfeldt, Holt and Ewing, 1969; Thorburn et al, 1969; Pant, Hopkinson and Fitzpatrick, 1972). The high [P4] in the plasma during pregnancy is accompanied by low levels of uterine activity (Hindson, Schofield, Turner and Wolff, 1965; Hindson, Schofield and Turner, 1968; Hindson, et al 1969; Mitchell, Flint and Turnbull, 1976; Rawlings and Ward, 1976). At term, when the [P4] in the plasma is falling, the uterus no longer behaves as though it were P4-dominated (Bengtsson and Schofield, 1963) and an increase in the response to oxytocin is observed (Hindson et al, 1969).

The effect of exogenous P4 on gestational length is not as clearcut in the sheep as it is in the rabbit. A single injection of P4 (80mg) can delay lambing when given during labour (Hindson et al, 1969), but high doses of P4 (80 or 100mg) or 6-methyl-hydroxy-pregnane-acetate (25-40 mg) administered prior to parturition prevented labour only to a varying extent and was associated with a high degree of fetal mortality. Injections of lower doses were without effect (Bengtsson and Schofield, 1963). Liggins, Grieves, Kendall and Knox, (1972) failed to prolong

pregnancy with 100mg/day progesterone when parturition was induced by intrafetal dexamethasone, but labour failed to start with a dose of 200mg/day even though the [P4] in the plasma in these animals and the labouring animals were not significantly different and were maintained at pretreatment levels. Jenkin, Jorgensen, Thorburn, Buster and Nathanielsz (1985) were able to delay or prevent F induced premature delivery in pregnant sheep by administering twice daily intramuscular injections of 100mg P4 to ewes during the time F was infused into the fetus or with a single intramuscular injection of 250mg medroxyprogesterone acetate given one day prior to the onset of F administration. These experiments were also associated with a high degree of fetal mortality. It was interesting to note that the [P4] in fetal plasma of animals in the P4-treated group and the P4-untreated group fell after cortisol treatment. This occurred while the [P4] in the maternal plasma remained elevated and suggests that P4 in the maternal plasma was not available to the fetal circulation.

1.6.3 Relaxin

Relaxin, a 6000MW polypeptide, has been studied as a suppressor of myometrial activity. Administration of relaxin inhibits spontaneous myometrial contractions in several species (Schwabe, Steinetz, Weiss, Segaloff, McDonald, O'Byrne, Hochman, Carrierre and Goldsmith, 1978; Porter, 1979) including sheep (Porter, Lye, Bradshaw, and Kendall, 1981) and its effects are almost immediate. Relaxin has been measured by radioimmunoassay in the plasma of pigs (Sherwood, Chang, Bevier and Dziuk, 1975) and rats (Sherwood, Crnekovic, Gordon and Rutherford, 1980) throughout pregnancy and during parturition. In both species levels reached a maximum in late pregnancy and fell at parturition. Similar data for sheep are lacking. Downing and

Sherwood (1985 a,b,c) have demonstrated that relaxin inhibits myometrial contractions during the latter half of pregnancy in rats, a species in which progesterone is not inhibitory. In species in which progesterone is inhibitory (cows, sheep) the role of relaxin is less clear; however, Porter (1984) has proposed that relaxin may have a similar effect on the myometrium during waning progesterone domination.

1.6.4 Prostaglandins

Prostaglandins have been shown to tip the "see saw" as either suppressors or stimulators but most evidence supports the stimulator role. They stimulate uterine activity in various species both "in vivo" and "in vitro" (Speroff and Ramwell, 1970).

In the ewe, the uterine response to an infusion of PGs is different depending upon the time of administration during the estrous cycle or pregnancy. PGF2α increases uterine activity when infused during estrus in non-pregnant animals (Rexroad and Barb, 1975; Roberts and McCracken, 1976; Carrick and Cupps, 1976) and during the onset of spontaneous activity in late pregnancy (Mitchell et al, 1976). The uterus is unresponsive to PGF2α during the luteal phase (Roberts and McCracken, 1976) and late pregnancy (Liggins et al, 1972; Oakes, Mofid, Brinkman and Assali, 1973). The importance of PGs as stimulators of myometrial contractions at parturition has been supported by evidence describing prolongation of pregnancy by inhibitors of PG synthesis (Mitchell and Flint, 1978b; Evans, Kennedy, Patrick and Challis, 1982). As mentioned previously concentrations of PGs in the AMF and production of PGs in the intrauterine tissues

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are elevated prior to the onset of uterine activity during induction of labour with ACTH (Olson et al, 1984). Thorburn and Challis (1979) have suggested that PGs produced by the intercotyledonary endometrium and fetal membranes may gain access to the myometrium and increase uterine activity.

Lye and Challis (1982) have demonstrated an inhibitory effect of prostacylin (PGI2) on uterine activity. PGI2 is produced by the myometrium during pregnancy (Jones, Poyser and Wilson, 1977). The concentration of 6-keto PGF1α, the hydrolytic breakdown product of PGI2, in allantoic fluid exceeds that of PGE and PGF, two stimulatory PGs, by several fold for much of pregnancy (Evans, Kennedy and Challis, 1982). The ratio of 6-keto PGF1α:PGE + PGF decreases at the time of labour. These findings led Lye and Challis (1982) to suggest that PG synthesis is altered at term in favour of stimulatory PGs.

1.6.5 Sympathomimetics

Stimulation of sympathetic nerve endings or application of neurotransmitters can exert excitatory or inhibitory effects on the contractile activity of the myometrium (see Marshall, 1970). α-Adrenergic agents produce contraction and β-adrenergic agents produce relaxation. Radioligand binding studies have verified the presence of α- and β-adrenoreceptors in uterine smooth muscle and have further subclassified them as α1 and α2 and β1 and β2 adrenoreceptors (human:Bottari, Vokaer, Kaivez, Lescrainier and Vauquelin, 1983a,b; Bottari, Vauquelin, Lescrainer, Kaivez, and Vokaer, 1983; Falkay, 1985; Bottari, Volkaer, Kaivez, Lescrainier 1985; sheep: Rexroad, 1981; rabbit: Williams and Lefkowitz, 1976, 1977; Williams, Mulliken and LefKowitz, 1977; Roberts, Insel, Goldfien and, Goldfien, 1977; Hoffman, Lesbon, Wood, Schocken, and Lefkowitz, 1979; Hoffman, Lavin, Lefkowitz and Ruffalo, 1981; rat:

Knall, Mori, Tuck, Lisbon, and Koreman, 1978; Maltier and Legrand, 1985). In the human uterus during pregnancy both α1, and α2adrenoreceptors mediate an increase in uterine contractility and are present in a ratio of 60:40 (α1:α2; Berg, Anderson and Ryden, 1986).

During pregnancy in several species there is a loss of adrenergic innervation of the uterus and a subsequent decrease in the concentration of norepinephrine in the myometrium (see Marshall, 1981). The non-pregnant sheep uterus was shown by Sigger, Harding and Summers (1986) to have areas of adrenergic innervation over all regions of the uterus. By day 50 of pregnancy this was unchanged; however, by day 100 areas of innervation were scarce over the whole uterus with the exception of the tubal extremities. The concentration of norepinephrine in the uterus was low and variable in different regions of the uterus in late pregnancy but the greatest concentrations were at the tubal extremities. With pregnancy there is a reduction in the synthesis of norepinephrine by the guinea pig uterus followed by a structural degeneration of nerves in the regions overlying the fetus (Thorbert, 1979; Sporrong, Alm, Owman, Sjoberg and Thorbert, 1981). In humans there is a disappearance of transmitter and transmitter forming enzymes during pregnancy (Thorbert, Alm, Bjorkland, Owman, and Sjoberg, 1977) resulting from a marked reduction in nerve fibres in the myometrium (Wikland, Lundblom, Dahlston, and Hagbid, 1984). Not only is the adrenergic innervation of the pregnant uterus altered during pregnancy but so also is the adrenoreceptor population. Vailliere, Fortier and Bukowiechi (1978) demonstrated a decrease in the binding of dihydroergcryptine (an α-antagonist) to isolated rabbit myometrium in the second week of pregnancy and a return to normal binding shortly thereafter. The

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binding of dihydroallpenalol (a β-antagonist) decreased progressively during pregnancy to 55% of pre-pregnancy levels by the end of gestation.

The functional significance of these changes and their relation to the maintenance of pregnancy and onset of parturition are unclear; however, the use of β-agonists as tocolytics has been investigated (Beniarz, Motew and Scommegna, 1972; Liggins and Vaughan, 1973; Wallace, Caldwell, Ansbacher and Otterson, 1978).

1.6.6 Mechanisms of Action

The effects of oxytocin to increase myometrial activity may in part be mediated by release of PGs. Concentrations of PGF are elevated in the plasma of estrogen-treated anestrous ewes (Sharin and Fitzpatrick, 1974) and late pregnant sheep (Currie, 1974; Mitchell, Flint and Turnbull, 1975) following intravenous oxytocin. A marked increase in the release of PGs occurs immediately before delivery during the second stage of labour (Currie et al, 1973). The release of oxytocin in response to vaginal distension is followed by an increase in uterovarian venous concentrations of PGF (Flint, Forsling, Mitchell and Turnbull, 1975). However, the increased uterine activity following an injection of oxytocin was not inhibited in the ewe when the production of PGs was suppressed by indomethacin (Roberts and McCracken, 1976). It appears that an increase in the synthesis of PGs is not essential for the stimulatory effects of oxytocin; however, oxytocin and PGs may act synergistically to increase myometrial activity.

Uterine contractions are generated by the Ca^{2+}-activated interaction between the contractile proteins of the uterus, actin and myosin, and energy rich phosphate, ATP (Csapo, 1961b, 1969a, 1971).

Therefore, the only direct stimulator of uterine contractions is Ca^{2+}. Csapo (1977) has suggested that P4 enhances the binding of intracellular Ca^{2+}, thus decreasing its availability to the contractile machinery and PGs enhance the influx of Ca^{2+} through and from cell membranes. Thus, only when the uterus is free from the influence of P4 is the effect of PG sufficient to elevate free intracellular Ca^{2+} to a level required for contraction. PGs are effective stimulators of uterine contractions during the estrous cycle and during pregnancy when the [P4] is low or is declining (see above).

1.7 Actions of Progesterone and Estrogens

1.7.1 Prostaglandin Production

Es have been implicated in the control of the production of PGs and hypothesized to cause the increased release of PGs at term. The concentrations of Es and PGF in the uterovarian vein rise in parallel during the last 24 hours of pregnancy (Challis, Harrison, Heap, Horton and Poyser, 1972; Currie et al, 1973). Diethylstilbesterol, a synthetic estrogen, administered on day 130 elevated the concentrations of PGF in maternal cotyledons, myometrium and uterovarian venous plasma (Liggins et al, 1973). In the non-pregnant ewe, an injection of E2β leads to an increase in the concentrations of PGF2α in the caruncles and uterine venous plasma provided that the animals are primed with P4 (Caldwell, Tillson, Brock and Speroff, 1972; Ford, Weems, Pitts, Pexton, Butcher and Inspeek, 1975; Louis, Parry, Robinson, Thorburn and Challis, 1977; Scaramuzzi, Baird, Boyle, Land and Wheeler, 1977; Rexroad, 1978).

Several lines of evidence argue against Es as being the cause of the increased production of PGs at term. Rawlings, Pant and Ward (1978) observed that passive immunization against unconjugated Es did not affect gestational length or the normal rise in maternal venous plasma concentrations of PGF during parturition. Kendall et al (1972) showed that the levels of PGFM in maternal plasma rose during labour induced prematurely by ACTH administered to hypophysectomized fetuses despite the absence of any change in the concentration of Es. These results suggested that the production of PGs at term is not dependent on any change in the concentration of Es and that other mediators of PG release are present.

The role of P4 in the control of the production of PGs is unclear. In addition to its "priming" role in estrogen-stimulated release of PGs, P4 also increases the production and release of PGs from caruncles (Louis et al, 1977). However, a decline in the [P4] in the plasma in pregnant and non-pregnant ewes leads to further release of PGs (Challis, Forster, Furr, Robinson and Thorburn, 1976). Mitchell and Flint (1977) showed that a temporary reduction of P4 in maternal plasma following administration of cyanoketone, an inhibitor of the conversion of P5 to P4, led to an increase in the concentration of PGs in the maternal plasma; however, peak concentrations of PGs were achieved at a time when the [P4] had returned to pretreatment values. There was no change in the levels of unconjugated Es in uterovarian venous plasma in this study. In high doses, P4 blocks the release of PGs following intramaternal diethylstilbesterol and intrafetal dexamethasone administration (Liggins et al, 1973).

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P4 and Es can influence the output of PGs from endometrium. E2β stimulates output of PGF2α from secretory endometrium obtained from non-pregnant women maintained in tissue culture (Abel and Baird, 1980). In the same study P4 inhibited output of PGF2α and blocked the stimulatory effect of E2β. Schatz and Gurpide (1985) localized the effects of E2β to the glandular cell epithelium. Using decidual cell preparations from tissue obtained from pregnant women at elective cesarean section, Olson, Skinner and Challis (1983), demonstrated a modest increase in the output of PGE2 and a reduction in the output of 6-keto-PGF1α, a breakdown product of PGI2, from cells exposed to E2β. These authors reasoned that such an effect could alter the ratio of stimulatory PG's (PGE2) to inhibitory PG's (PGI2). To date no studies have been performed assessing the direct effects of P4 and Es on PG output by fetal membranes or any of the intrauterine tissues of pregnant sheep.

Regulators of the release of PG may influence the stability of lysosomes which contain the enzyme, phospholipase A2, that releases arachidonic acid, a precursor of PG synthesis, from phospholipid stores. Under the influence of P4, lipid droplets accumulate in endometrial epithelial cells of ovariectomized sheep, whereas, Es cause their depletion in P4-pretreated ewes. The possibility that these droplets contain fatty acid precursors for the synthesis of PGs has been suggested (Louis et al, 1977). In humans, decidual and amnion lysosomes are particularly susceptible to fracture at the time of labour (Gustavii, and Brunk, 1974; Schwarz, MacDonald and Johnston, 1980). Studies are still required to demonstrate direct effects of P4 and estrogens on lysosomal stability. Recently, Saitoh, Hirato, Tahara, Ogawa, Noguchi, Yanaihara, and Nakayama

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(1984) demonstrated that phospholipase A2 activity in human amnion could be stimulated by DHEA-S, P5-S and E1-S.

1.7.2 Oxytocin Receptors

The responsiveness of the uterus to oxytocin is influenced by P4 and Es (see above). The high concentrations of Es which occur at estrus and at parturition are associated with high uterine responsiveness to oxytocin, but the high [P4] during the luteal phase and throughout pregnancy is associated with little or no response to oxytocin (see above). Administration of estrogen to ovariectomized ewes increases uterine responsiveness to oxytocin (Hays and Van Demark, 1953a,b; Fitzpatrick, 1960; Follett and Bently, 1964; Jones and Knifton, 1975; Carrick and Cupps, 1976; Parkington and Lipton, 1976) while administration of P4 decreases responsiveness (Fuchs and Fuchs, 1958; Jones and Knifton, 1975; Carrick and Cupps, 1976). In other species such as the rat and guinea-pig the uterus remains responsive to oxytocin during some or all of pregnancy and, in the guinea-pig, administration of P4 does not decrease oxytocin responsiveness (Newton, 1933; Bell and Robson, 1936; Grieg, 1939; Fuchs, 1969; Porter, 1970, 1971).

The effects of steroids on responsiveness to oxytocin may be mediated by altering the concentration of the receptors for oxytocin. During pregnancy the concentrations of oxytocin receptors in the uterus are low but, at term, rise abruptly to reach a peak at labour before declining significantly by 24 hours post-partum (Soloff, 1979). Administration of estrogen increases concentrations of oxytocin receptors and administration of P4 reduces them (Soloff, 1975, 1979; Nissenson, Flouret and Hechter, 1978).

1.7.3 Adrenergics

The adrenergic control of uterine contractility can be altered by P4 and Es. The response of the uterus to adrenergic stimulation, whether it be induced directly by catecholamines or by sympathetic nerve stimulation, can be increased by Es or decreased by P4 (Neshien, 1972, 1974; Miller and Marshall, 1985). In rabbits ovariectomy is followed by a decrease in the norepinephrine content (Bodkin and Harper, 1973; Kennedy and Marshall, 1977; Sjoberg, 1962) and turnover rate (Kennedy and Marshall, 1977) in the uterus. The norepinephrine content and turnover rate are thought to be indications of adrenergic innervation. Bell and Malcolm (1978) proposed that the loss of adrenergic innervation in the pregnant human uterus was caused by P4 secreted by the placenta. In contrast, the norepinephrine content of the guines pig uterus is higher during anestrus, when the circulating P4 level is higher than the estrogen level, than during estrus when the uterus is subject to estrogen predominence (Thorbert, Alm and Rosengren, 1978). Despite these differences it is clear that P4 and Es can alter the sympathetic nervous control of uterine contractility. In addition, the adrenoreceptor population can be altered by P4 and Es. In the rabbit, Es increase and P4 decrease the number of α-adrenoreceptors (Williams and Lefkowitz, 1977; Roberts, Insel, Goldfien and Goldfien, 1977). The effect is limited to α2-adrenoreceptors (Hoffman et al, 1981) and there is no effect of P4 or Es on β-adrenoreceptors (Roberts et al, 1977). In sheep Rexroad (1981) demonstrated increased binding to α-adrenoreceptors during the luteal phase and following P4 treatment to ovariectomized ewes. Binding to β-adrenoreceptors was increased early in the reproductive cycle and

following E2 treatment to ovariectomized ewes. In rats, Knall et al (1977) found no effect of the estrus cycle on adrenergic receptor levels but did show an increased binding to β-adrenoreceptors following E2 treatment to ovariectomized rats. In humans, E2 increases binding to α2 and β1 adrenoreceptors but has no effect on α1 on β2 adrenoreceptors. The effect of E2 can be counteracted by P4 (Bottari et al, 1983, 1985).

1.7.4 Gap Junctions

Garfield and his collaborators have reported the appearance of gap junctions between myometrial smooth muscle cells of the rat, guinea pig, sheep and human shortly before, during or immediately after delivery of the fetus at normal term (Garfield, Sims and Daniel, 1977; Garfield, Sims, Kannon and Daniel, 1978; Garfield, Rabideau, Challis and Daniel, 1979; Garfield, Kannan and Daniel, 1980). Gap junctions were not found or were present only with a low frequency in non-pregnant animals or at any other time during pregnancy (Garfield et al, 1978). Garfield et al (1979) have proposed that gap junctions are essential for normal labour and delivery because they permit the synchronization of uterine activity. In the rat, estrogen stimulates the formation of gap junctions whereas P4 in the presence of estrogen inhibits their formation (Garfield et al, 1979, 1980). In sheep, the appearance of gap junctions have been correlated with increasing E:P4 ratio (Garfield et al, 1979).

1.7.5 Uterine Blood Flow

Changes in uterine blood flow have been linked to changes in the concentration of Es in the maternal plasma. Uterine blood flow is highest during estrus (Greiss and Anderson, 1969) and can be increased by exogenous Es (Huckabee, Crenshaw, Curet, Mann and Barron, 1970; Greiss and Anderson, 1970; Killam, Rosenfeld, Battaglia, Makowski and Meschia, 1973). Uterine blood flow also shows a great increase towards the end of gestation (Rosenfeld, Morris, Makowski, Meschia and Battaglia, 1974) when the concentration of Es is elevated, and can be increased by exogenous Es during the last 50 days of pregnancy (Rosenfeld, Morris, Battaglia, Makowski and Meschia, 1976). The hormonal control of uterine blood flow is selective, with Es increasing blood flow in favour of the myometrium and P4 favouring increased flow to the caruncles (Rosenfeld et al, 1976; Anderson, Hackshaw, Still and Greiss, 1977).

CHAPTER 2

RATIONALE

During pregnancy and at parturition, the influence of P4 and Es on the myometrium, endometrium and fetal membranes may be important with respect to the physiological mechanisms involved in the control of myometrial activity. The effects of P4 and Es may be mediated through alterations in PG production, gap junction formation, and intracellular and plasmalemmal protein synthesis. Changes in the concentrations of P4 and Es in intrauterine tissues throughout pregnancy and at parturition have been inferred from changes in the concentrations of these steroids in the maternal and fetal plasmas. As described in the literature review, the concentrations of P4 and Es in the maternal and fetal plasmas have been well documented and change in response to changes in their production by the placenta. Thus, the predominant influence affecting concentrations of P4 and Es in the intrauterine tissues has been assumed to be endocrine; ie, via the circulation. It has not been proven whether or not the plasma concentrations are the sole factor influencing levels of P4 and Es in the intrauterine tissues. It would be important to know whether the changes in the concentrations of P4 and Es that occur in the fetal and maternal plasma throughout pregnancy and at parturition also occur in the intrauterine tissues.

Steroids in the fetal fluids may be available to the fetal membranes. The concentrations of Es in the fetal fluids have been measured throughout gestation and around parturition (Carnegie and Robertson, 1978; Challis and Patrick, 1981) and compared to those in

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the maternal and fetal plasmas (Challis and Patrick, 1981). Similar studies on P4 are lacking. I will describe the concentrations of P4 in the amniotic and allantoic fluids throughout pregnancy and at parturition and compare then to those measured in the maternal and fetal plasma. I will discuss the findings in relation to the earlier finding for Es.

The patterns of changes in the concentrations of P4 and Es in the intrauterine tissues have not been reported. A few studies have measured concentrations of P4 and Es in the myometrium although the number of samples studied at any particular gestational age were small. I will describe the concentrations of P4, E1 and E2β in the myometrium, endometrium, chorion and amnion obtained from pregnant sheep at different gestational ages and in labour.

In addition to steroids reaching the intrauterine tissues via the circulation and fetal fluids, local concentrations within the tissues may be increased by local production or decreased by local metabolism. I have performed experiments to determine the ability of intrauterine tissues to synthesize and/or metabolize P4 and Es. Samples of myometrium, endometrium, chorion, allantois and amnion were obtained from pregnant sheep at different gestational ages and in labour and the ability of these tissues to synthesize and/or metabolize P4 and Es was analyzed in homogenate preparations and/or dispersed cells.

SECTION II

EXPERIMENTAL DATA

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CHAPTER 3

GENERAL TECHNIQUES AND MATERIALS

3.1 Introduction

The following anatomical description of the pregnant sheep uterus is summarized from several excellent reviews (Amoroso, 1952; Boyd and Hamilton, 1952; Steven, 1975; Wintour, Laurence and Lingwood, 1986). The growing fetus is surrounded by several layers of maternal and fetal tissues. The uterine wall (maternal) consists of the smooth muscle of the myometrium and the underlying stroma and epithelial surface of the endometrium. The fetal chorion lies adjacent to the entire surface of the endometrium. The endometrial mucosa of the non-pregnant ewe possesses about 100 caruncles which are localized thickenings of sub-epithelial dense connective tissue projecting into the uterine lumen. During pregnancy the fetal chorion invades these areas of endometrium forming villus proliferations known collectively as a cotyledon. These structures comprise the sheep placenta. Within the chorion lie the amniotic and allantoic fluid compartments.

The cotyledonary placenta has been classified as a syndesmochorial placenta based on the type of fetal and maternal tissues juxtaposed in the cotyledons (Amoroso, 1952). In this type of placenta, the chorionic epithelium is in contact with endometrial connective tissue, the endometrial epithelium having been denuded at implantation. More recently, Wooding (1980) has shown with electron microscopic studies that an endometrial epithelial layer is present

in the placenta. Separating the fetal and maternal circulations are, therefore, fetal endothelium, fetal connective tissue, chorionic epithelium, maternal epithelium, maternal connective tissue and maternal endothelium. The fetal component of the placenta develops as villus projections of fetal ectoderm which later contain cores of mesoderm and branches of allantoic vessels.

The chorion is derived from extra embryonic ectoderm and mesoderm. The 12 day old blastocyst consists of an inner cell mass (embryonic) at the pole of a bilaminar arrangement of extra embryonic ectoderm and endoderm. A layer of mesoderm forms in between the ectoderm and endoderm forming the chorion (ectoderm and mesoderm) and the yolk sac (endoderm and mesoderm). The extra embryonic coelom forms as a cavitation in the mesoderm through the entire extent of the blastocyst wall, separating the chorion from the yolk sac.

The amnion forms from an elaboration of ectodermal tissue over the inner cell mass. It expands as a fluid filled cavity to occupy space in the extra-embryonic coelom. It comes in contact with the chorion forming the chorioamniotic membrane.

The allantois develops from a diverticulum in the hind gut consisting of endoderm and mesoderm. During the fetal period of growth, the allantoic cavity is continuous with the cranial pole of the fetal bladder via the urachus in the umbilical cord. With the amniotic cavity the allantoic expands as a fluid filled cavity to occupy the entire extra-embryonic coelom. The allantoic mesoderm comes in contact with the chorionic mesoderm and contributes to the vascularization between chorion and allantois (chorioallantoic membrane).

3.2 Collection of Plasmas, Fetal Fluids and Tissues

3.2.1 Anesthetized Animals

Samples of fetal and maternal blood, amniotic and allantoic fluid, (AMF and ALF, respectively) and intrauterine tissues were obtained from anesthetized animals at different gestational ages. Gestational age was based upon single insemination dates with a raddled ram and was confirmed by fetal crown-rump length and weight which were compared to Barcroft's (1946) published figures at the time of sacrifice. Anesthesia was induced with nembutal and was maintained by using a 50:50 nitrous oxide-oxygen mixture and 2-3% halothane with a flow rate of 2-3 L/min. The uterus was exposed through an abdominal midline incision (Plate 1). Samples of maternal blood were obtained simultaneously from the maternal jugular vein and a main uterine vein draining the pregnant uterine horn. An incision was made through the myometrium (MYO) to the endometrium (ENDO) (Plate 2) and the MYO was peeled off (Plate 3) and collected. A second incision was made through the ENDO to the intercotyledonary chorion (CHOR) and the ENDO was peeled off (Plate 4) and collected. A greater surface area of chorion was exposed (Plate 5). A third incision was made in the CHOR to the underlying amnion (AMN) and allantois (ALL) and the CHOR was peeled off and collected. The amniotic and allantoic cavities were better exposed (Plate 6, 7). The continuity between the allantoic cavity and the urachus, and the umbilical vessels are shown in Plate 8. Samples of ALF and AMF were collected by needle puncture. Samples of AMN and ALL were collected. Several cotyledons were dissected from adjoining intrauterine tissues and collected. Samples of blood were collected from the umbilical artery and the umbilical vein by needle puncture. The animals were killed with intravenous euthanol.

Plates 1-4: Collection of tissues from the pregnant sheep uterus (M, myometrium; E, endometrium; C, chorion).

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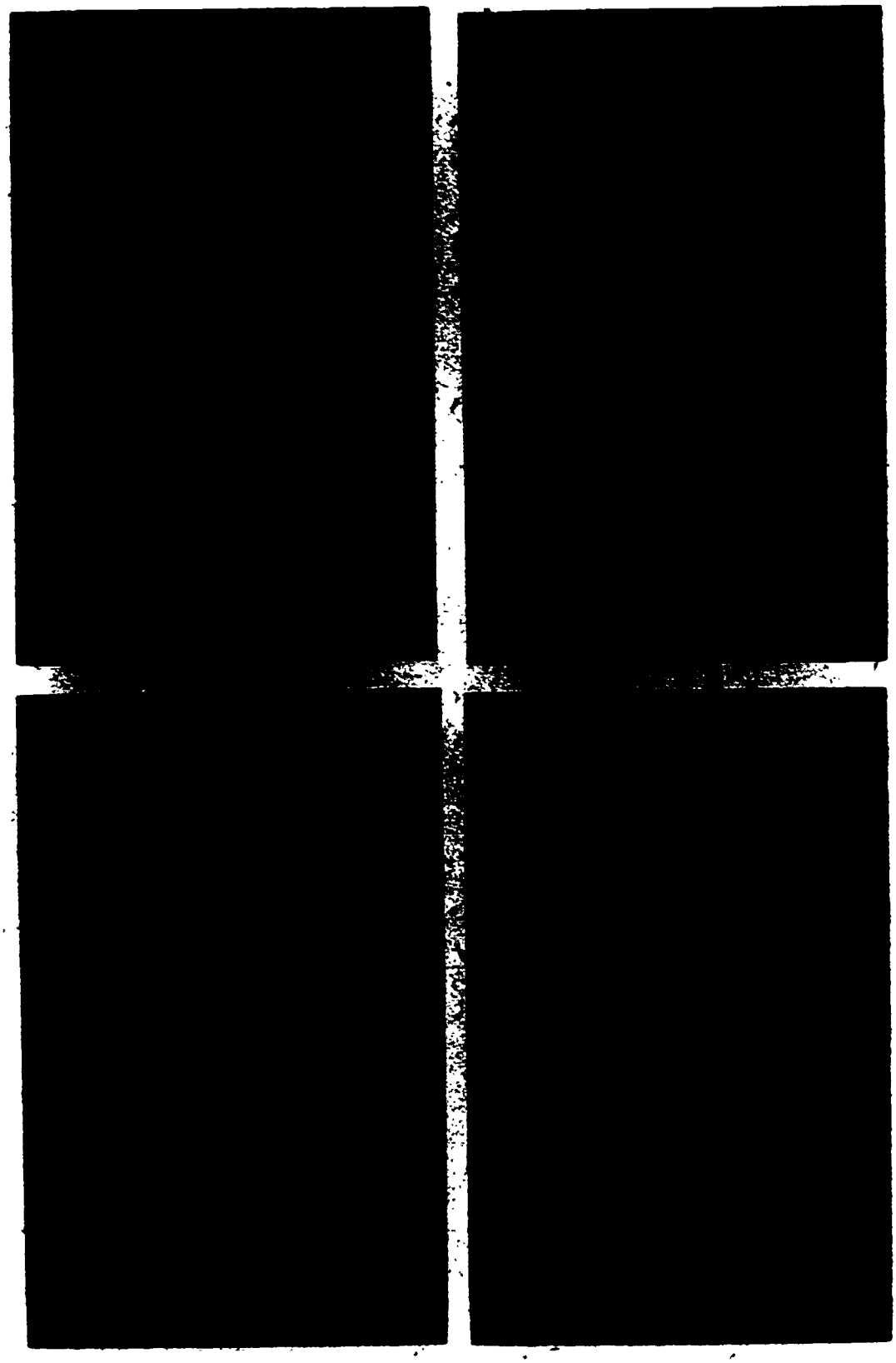
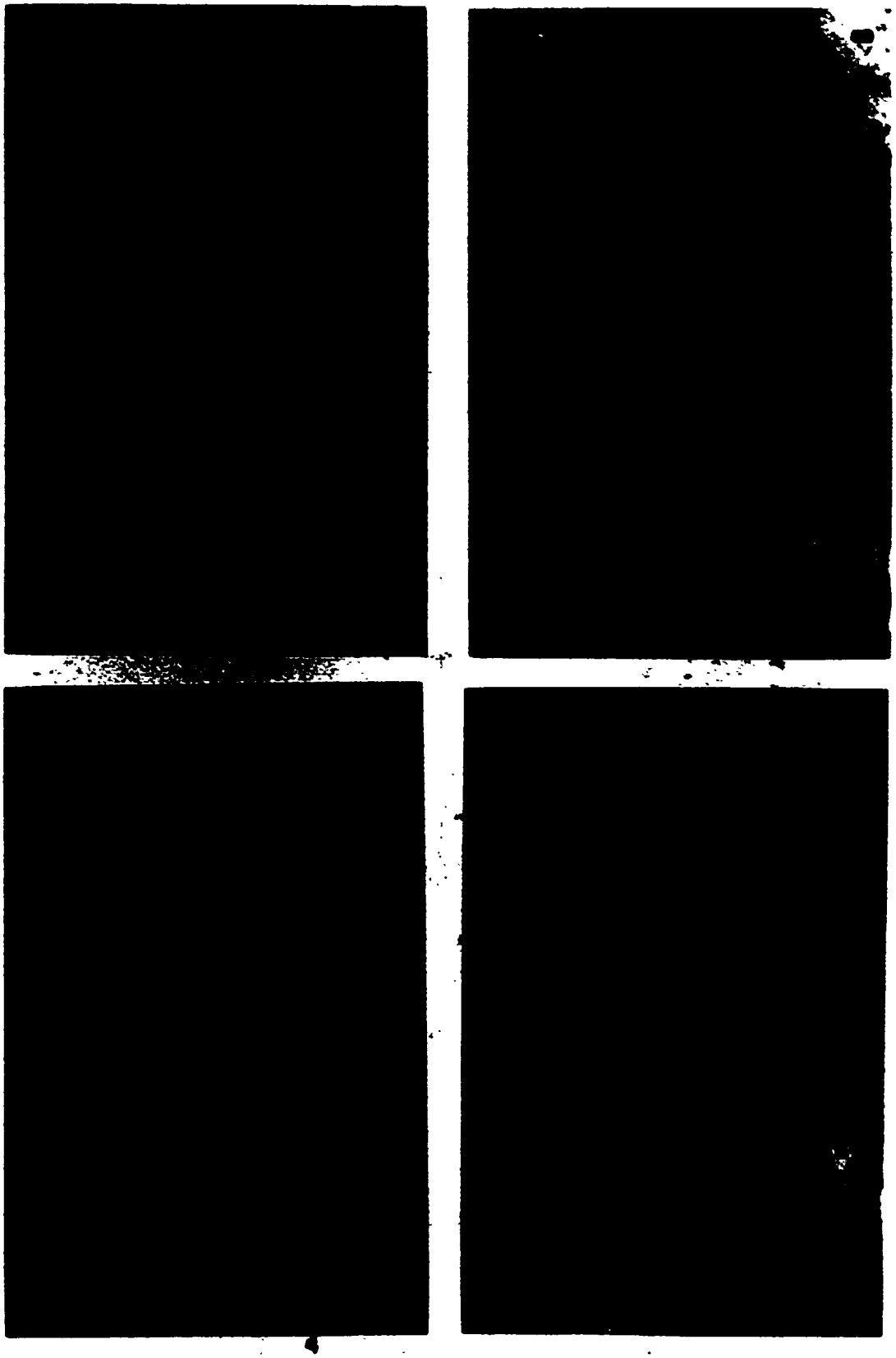


Plates 5-8: Collection of tissues from pregnant sheep uterus (C, chorion; AM, amnion; AL, Allantois; a, umbilical artery; v, umbilical vein; U, urachus)



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3.2.2 Conscious Animals

Some sheep were prepared for chronic studies as described previously (Manchester, Challis, Maclennan, Patrick and Workewych, 1979). Catheters were introduced into the fetal carotid artery and jugular vein. One or two catheters were introduced into the amniotic cavity and one catheter was introduced into the allantoic cavity. The maternal femoral artery and femoral vein were also catheterized. Surgery was performed between days 115-130 and at least 5 days elapsed after surgery before sampling was commenced. Samples of maternal and fetal bloods and fetal fluids were obtained over the last two weeks of pregnancy.

3.2.3 Labour

Animals destined to proceed to labour had a free floating amniotic fluid catheter connected to a pressure transducer (Statham) for continuous recordings of intra-amniotic fluid pressure on a polygraph recorder (Grass polygraph D.C. Driver Amplifier, Model 7DAC). Labour was defined as successive elevations of pressure measuring 30 mmHg for 2 min. occuring every 5 min. It has been established previously that once this pattern of uterine activity had started, lambing would normally occur within 12 hours (Lye, Sprague, Mitchell and Challis, 1983). Labour occurred in our population of sheep between day 140 and day 150.

3.2.4 Storage of Samples

Bloods were collected into heparinized syringes. Bloods and fetal fluids from unconscious ewes were transferred to tubes and frozen immediately in an acetone-solid carbon dioxide bath. Blood

and fetal fluids from conscious ewes were collected into chilled tubes, centrifuged at 4°C for 15 min. at 1800g and the plasma and supernatant fractions from the fetal fluids stored until analysis. Samples of each tissue to be analyzed for the concentrations of steroids or the subcellular enzymatic activities were frozen immediately after collection in a solid CO_2/ acetone bath. Tissues for cell dispersion were collected into saline and maintained on ice.

All frozen samples were stored at -20°C.

3.3 Materials

The following materials were purchased from the companies indicated:

In vivo experiments:

ACTH (Synacthen, $ACTH_{1-24}$)	Ciba-Geigy Co. Ardsly, New York
Antibiotics - Pen-Di-Strep	Rogur STB Inc. Montreal, Quebec
- Crystapen	Glaxo Canada Ltd. Toronto, Ontario
catheters (Bolab V4, V10)	Bolab Inc. Lake Havsu City, Arizona
heparin	Allen and Hanburys Toronto, Ontario
pentothol	Abbot Laboratories Montreal, Quebec

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saline	Travenol Laboratories Inc. Malton, Ontario
In vitro experiments:	
absolute alcohol	Commercial Alcohols Ltd. Scarborough, Ontario
bovine serum albumin	Sigma Chemical Co. St. Louis, Missouri
collagenase	Sigma Chemical Co. Boehringer Mannheim Canada Dorval, Quebec
dextran T70	Pharmacia Fine Chemicals HB Uppsula, Sweden
dry chemicals	Fisher Scientific Co. Fair Lawn, New Jersey J.T. Baker Chemical Co. Phillipsburg, New Jersey
β-mercaptoethanol	Sigma Chemicals Co.
organic solvents	Fisher Scientific Co. J.T. Baker Chemical Co.
PPO (2,5 diphenyloxazole)	Canadian Scientific Products London, Ontario
radioactive steroids	New England Nuclear Corp. Boston, Masschusettes
steroids	Sigma Chemicals Co. Steraloids Wilton, New Hampshire
TLC plates - Anasil GF	Analabs

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North Haven, Connecticut

- Watman LKGDF

Terochem Laboratories Ltd.

Mississauga, Ontario

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CHAPTER 4

CONCENTRATIONS OF PROGESTERONE IN FETAL FLUIDS AND MATERNAL AND FETAL BLOOD

4.1 Introduction

Challis et al (1981) measured progesterone (P4) in the amniotic fluid (AMF) and allantoic fluid (ALF) during late pregnancy. However, the changes in the concentrations of P4 and any relation to variations in fetal and maternal plasma concentrations of P4 during pregnancy have not been established. This chapter describes the concentrations of P4 in the AMF and ALF of pregnant sheep and compares the patterns of change throughout pregnancy to those in the maternal and fetal plasmas.

4.2 Materials and Methods

4.2.1 Acute Experiments

Thirty-three sheep of known gestational age were used. Four stages of pregnancy were studied: day 50 (nine animals), day 100 (six animals), day 130 (nine animals) and days 145-147 (term; nine animals). Samples of blood from maternal jugular vein, uterine vein, umbilical artery, umbilical vein, and samples of AMF and ALF were collected from anesthetized pregnant ewes. All fluids and plasmas were not always collected from a single animal.

4.2.2 Chronic Experiments

Sixteen sheep were prepared for serial sampling of maternal and fetal plasmas and AMF and ALF. Samples of fetal arterial blood (2ml), maternal arterial blood (5ml) and AMF (5ml) were collected

between 0900-1100h at intervals of 1-2 days. Insufficient ALF samples could be collected for analysis from chronically catheterized animals.

4.2.2 Radioimmunoassay

The concentrations of P4 were measured by radioimmunoassay (Erickson, Challis and Ryan, 1974) using an antiserum (kindly donated by Dr. B.J.A. Furr, ICL Ltd., Macclesfield, England) whose cross reactivities have been described previously (Glickman, Carson and Challis, 1979; see Appendix 2). The recovery of P4 added to Krebs-Ringer solution at concentrations between 0.125 - 1.0ng/ml was described by the relationship y=0.001+1.03x, where y was the amount of P4 recovered and x was the amount of P4 added. The intraassay coefficient of variation over this concentration range was 12.5%, and the interassay coefficient of variation was 13.6%. The specificity of the antibody was assessed further by measuring the [P4] in 6-12 random samples of maternal and fetal plasma, AMF and ALF either directly after extraction with petroleum ether (boiling point, 40-60C) or after extraction and purification using micro-celite chromatography (Challis, Davies and Ryan, 1973). Correction was made for methodological losses. The regression and correlation coefficients were as follows: maternal plasma (n=12 samples), y=0.65 + 1.029x and r=0.986; fetal plasma (n=12), y=0.008 + 0.909x and r=0.962; AMF (n=6), y=0.147 + .763x and r=0.986; and ALF (n=12), y=0.082 + 0.929x and r=0.913, where y is the amount of P4 measured without chromatography, x is the amount of P4 measured after micro-celite chromatography, and r is the correlation coefficient. These results indicate that some factor is interferring with the estimation

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Therefore, the concentrations of P4 in the AMF are probably over-estimated.

In a further sample of each fluid, P4 was extracted using petroleum ether, and to the dried extract were added 1200 DPM [^{3}H]-progesterone (2,4,6,7-^{3}H(N)-progesterone, ^{3}H-P4; SA 90-115Ci/mmole), purified previously by thin layer chromatography (TLC) in the solvent system benzene-ethyl acetate (3:2, vol/vol). The samples were dried, redissolved in 1.0ml isooctane, and applied to micro-celite columns (Challis et al, 1973). The concentrations of P4, determined by RIA, and radioactivity, determined by liquid scintillation counting, in successive 0.5ml eluate fractions were measured. Figure 2 shows for each type of sample the cochromatographic profile of the authentic ^{3}H-P4 with the material measured by RIA. The similarity of the specific activities in the peak fractions of the different samples is a further indication of the specificity of the RIA measurements.

4.2.3 Analyses of Results

Results are presented as the mean ± SEM. The effect of gestational age on mean concentrations of P4 was assessed with one way analysis of variance. The data were transformed logarithmically when Bartlett's test revealed heterogeneity of variance. Comparisons between multiple mean hormone concentrations were made using Duncan's analysis. Comparison between the means of two groups or between pairs of samples was made using the Mann-Whitney U test or the Wilcoxon paired sample test, respectively (Zar, 1974).

4.3 Results

FIGURE 2. Elution profiles from micro-celite columns of added [^{3}H]-progesterone and endogenous radioimmunoassayable progesterone in samples of fetal plasma, maternal plasma, AMF, and ALF from sheep in late pregnancy. Successive 0.5 ml isooctane eluates were assayed for [^{3}H]-progesterone by scintillation counting and for progesterone mass by RIA. The specific activities (counts per min/pg) through the major peak samples are indicated. The first eluate was collected after the elution of the first 1.0ml corresponding to the application of sample.

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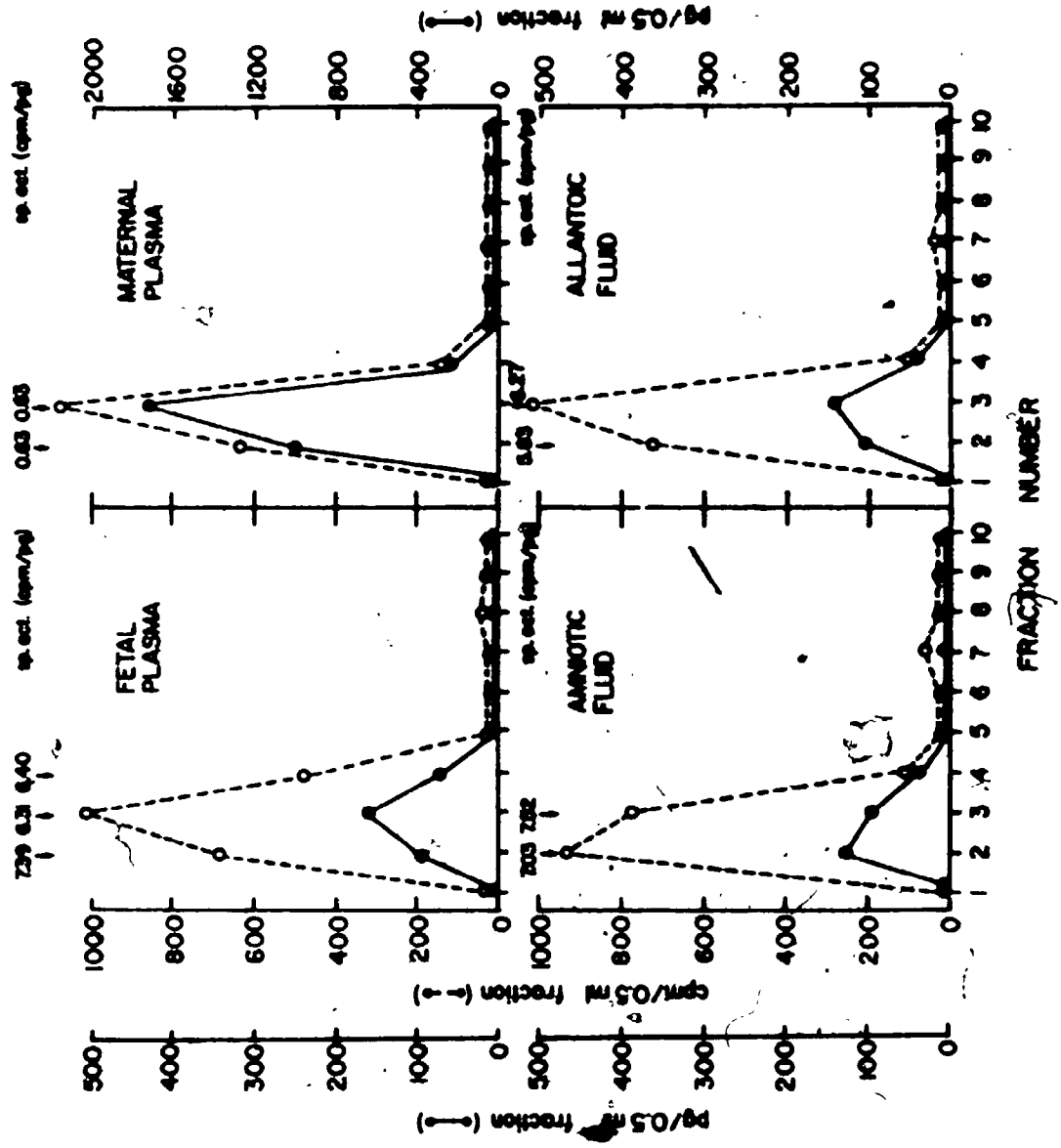

MATERNAL PLASMA
ALLANTOIC FLUID
FETAL PLASMA
AMNIOTIC FLUID
sp. act. (cpm/pg)
pg/0.5 ml fraction (—●—)
cpm/0.5 ml fraction (- -●- -)
FRACTION NUMBER

4.3.1 Acute Experiments

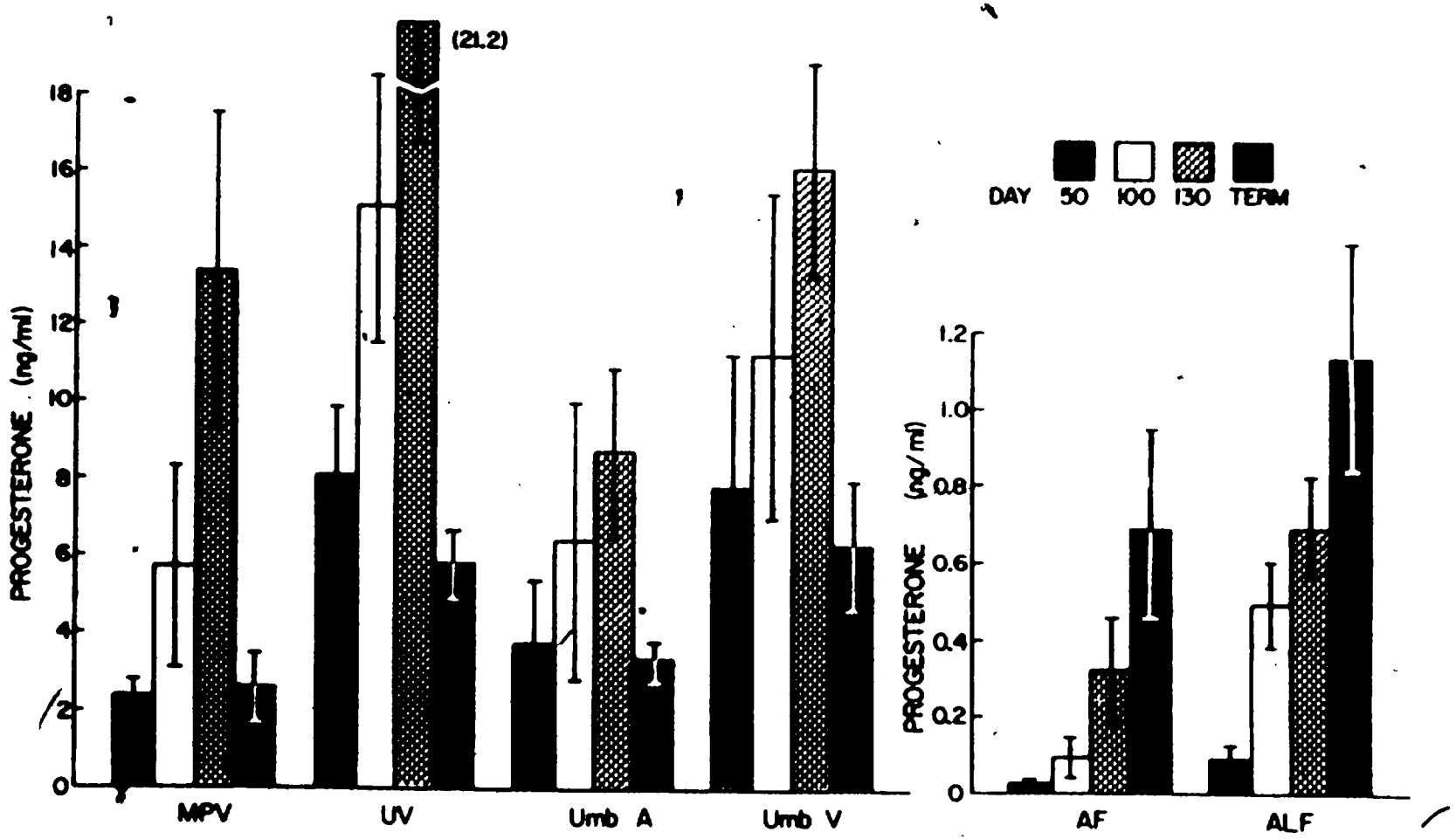
The mean [P4] in AMF and ALF rose progressively throughout pregnancy (Fig. 3). In both fluids the average concentrations at term were significantly higher than those measured on days 50 and day 100 (both $P< 0.05$), although the values at term were not significantly different from the concentrations on day 130 ($P>0.05$).

The change in the [P4] in AMF and ALF showed a rise similar to that of the [P4] in the maternal peripheral, uterine venous, umbilical arterial, and umbilical venous blood samples between days 50-130 (Fig. 3). However, while the mean [P4] in the fetal fluids remained elevated near term, the average P4 values decreased in the blood samples taken at this time.

4.3.2 Chronic Experiments

To examine the uniformity of the [P4] in the AMF and to determine whether single samples of AMF would provide representative information in later studies, I measured P4 in 39 paired samples of AMF and found good correlation between the [P4] in samples taken from the two catheters (A and B). The relation could be expressed by the equation $y=0.013 + 0.92x$, ($r=0.95$), where y and x are the concentrations (in nanograms per ml) in samples taken from catheters A and B, respectively. In chronically catheterized pregnant sheep, the mean [P4] in AMF rose from less than 300 pg/ml 2 weeks before parturition to 866± 265 (SEM; n=6) pg/ml on the day of delivery ($P<0.05$; Fig. 4). The [P4] in AMF in the chronic studies were similar to those found in acute experiments at comparable times of gestation [day 130: chronic, 247 ± 74 pg/ml (n=8); acute 323 ± 140 pg/ml (n=8)] and at term [chronic, 669 ± 162 pg/ml (n=12); acute 697 ± 268 pg/ml

FIGURE 3. Mean concentration (±SEM; n=4-9 determinations) of progesterone in maternal jugular venous blood (MPV), uterine vein (UV), umbilical artery (Umb A), umbilical vein (Umb V), AF, and ALF on day 50, day 100, day 130 and at term in pregnancy.


(21.2)
PROGESTERONE (ng/ml)
0
2
4
6
8
10
12
14
16
18
MPV
UV
Umb A
Umb V
DAY 50 100 130 TERM
PROGESTERONE (ng/ml)
0
0.2
0.4
0.6
0.8
1.0
1.2
AF
ALF

60

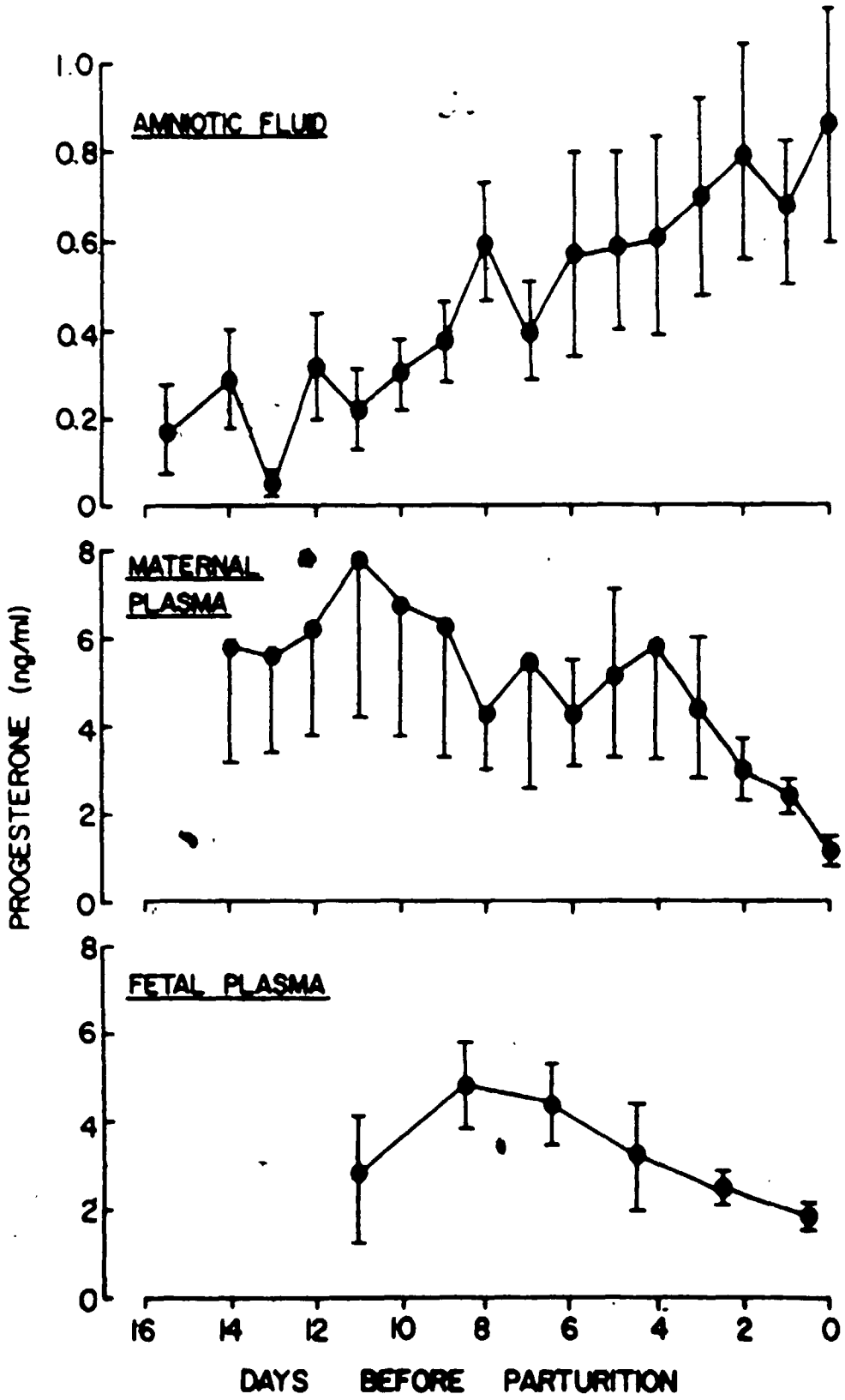
(n=6)]. In contrast to the pattern in AMF, the mean [P4] in maternal plasma decreased significantly during the 5 days before birth ($P<0.01$). In fetal blood, the [P4] measured within 24 hours of birth (1.95 ± 0.27 ng/ml; n=8) were significantly lower than those found on days 6 and 7 or days 8 and 9 pre-partum [4.41 ± 0.95 ng/ml (n=6); 4.89 ± 0.84 ng/ml (n=7), respectively; both $P<0.05$].

4.4 Discussion

I have shown that P4 is present in the AMF and ALF of pregnant sheep. These results confirm the earlier finding of Challis et al (1981). In both fetal fluid compartments, P4 values increased during late pregnancy. The mean concentrations of P4 in maternal and fetal plasma were generally higher than those in the fetal fluids and, in contrast, decreased before parturition. The fetal membranes might, therefore, be exposed to an increasing [P4] at parturition.

In AMF, it is possible that the increase in the [P4] reflects a decrease in fluid volume during late pregnancy (Malan, Malan and Curson, 1937) relative to the secretion of P4 into the fluid compartment. This argument, however, would not explain the observations made earlier in pregnancy when the volume of AMF is increasing. The volume of ALF increases throughout pregnancy, such that near term it is twice that of the AMF (Malan et al, 1937). It seems unlikely, therefore that alterations of ALF volume would account for the changes in the [P4]. The clearance of P4 from the AMF and ALF has not been measured. It is possible that a decrease in the clearance rate of P4 from AMF and ALF or an increase in the addition of P4 via fetal urine accounts for the rise in concentration that were observed.

FIGURE 4. Concentration of progesterone in relation to day of parturition (day 0) in samples of AMF, maternal plasma, and fetal plasma from chronically catheterized sheep. Each value is the mean ± SEM for 3-13 observations in AMF and 3-8 observations in maternal and fetal plasma.


AMNIOTIC FLUID
MATERNAL PLASMA
FETAL PLASMA
PROGESTERONE (ng/ml)
1.0
0.8
0.6
0.4
0.2
0
8
6
4
2
0
16
14
12
10
8
6
4
2
0
DAYS BEFORE PARTURITION

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The origin of P4 in the fetal fluids and the mechanisms of its clearance from the fluids have not been established.

A similar rise in the [P4] in AMF has been reported during late pregnancy in the rhesus monkey (Challis, Hartley, Johnson, Patrick, Robinson and Thorburn, 1977). In this species, the possibility exists that the fetal adrenal contributes to the levels observed (Challis et al, 1977; Novy, 1977). In women, the [P4] in the AMF decreases slightly in late gestation (Johansson and Johansson, 1971) during a time when the maternal plasma [P4] remains elevated (Tulchinsky et al, 1972). Gibb, Lavoie and Roux (1978) have demonstrated the conversion of pregnenolone to P4 in fetal membranes. Thus the fetal membranes may be a source of P4 in the fetal fluids. In subsequent chapters this possibility is explored in the pregnant sheep.

Investigators (Carnegie and Robertson, 1978; Challis and Patrick, 1981) have also measured estrogens (Es) in AMF and ALF. Carnegie and Robertson (1978) first detected Es in both the AMF and the ALF on day 30. The concentration of estrone sulphate [E1-S] in both fluids peaked around day 50 at values much higher than those in the fetal or maternal plasmas. The [E1-S] in the ALF declined sharply between day 50 and day 60 whereas that in the AMF declined gradually between day 50 and day 100. The [E1-S] in the ALF, but not the AMF peaked again by day 100. In the ALF, the pattern of changes in the [E1] was similar to that of the [E1-S] but at much lower values. The [E1] in the AMF remained low throughout gestation. The pattern of changes in the concentration of estradiol sulphate [E2-S] was similar to that of the [E1-S] in the ALF throughout pregnancy and in the AMF up to day 90. In the AMF the values fluctuated without

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discernable pattern between day 90 and day 140. The [E2] in both fluid compartments remained low throughout gestation. Challis and Patrick (1981) compared the [E1] and the [E2β] in the AMF and ALF at several different gestational ages to those in the maternal and fetal plasmas. Their findings confirmed those of Carnegie and Robertson (1978) and showed the [E1] and the [E2β] in the AMF and ALF throughout pregnancy to change independently from those in maternal and fetal plasmas. During the last few days of pregnancy, however, they reported a rise in the concentrations of both E1 and E2β in the AMF occuring concurrently with a rise in the fetal and maternal plasmas.

In summary, P4 and Es are present in the ALF and AMF and their concentrations change either concurrently with, in the absence of, or opposite to changes in concentrations in the maternal and fetal plasmas. It is uncertain whether changes in the concentrations of P4 and Es in the maternal plasma, fetal plasma, or fetal fluids reflect changes in the intrauterine tissues. The following chapters report concentrations of P4, E1 and E2β in the myometrium, endometrium, chorion and amnion throughout pregnancy and at the time of labour.

CHAPTER 5

CONCENTRATIONS OF PROGESTERONE IN INTRAUTERINE TISSUES

5.1 Introduction

Liggins et al (1972) found a close similarity between the concentrations of progesterone (P4:ng) per milliter of peripheral plasma and per gram of myometrium (MYO) in ewes in late pregnancy. This finding would suggest that the concentration of P4 ([P4]) in the MYO falls in those sheep which deliver at term. Liggins et al (1972) did report a decrease in the [P4] in the MYO of sheep induced to deliver by an intra-fetal infusion of dexamethasone. However, these authors failed to inhibit induced premature labour with P4 and in these experiments the [P4] in the MYO of animals in labour showed no fall in values compared with normal controls. Rawlings and Ward (1976) found a similar pattern of change in the [P4] in the MYO as that found in the plasma (Bassett et al, 1969); however, they did not detect a fall in the [P4] in the MYO prior to parturition. Since these authors measured P4 in the MYO of only two animals on the day of parturition they may have missed any significant change. In addition, measurements of P4 in the endometrium (ENDO) and fetal membranes (chorion, CHOR; amnion, AMN) have not been reported previously. Since the production and release of prostaglandins by these tissue may be controlled by P4 (Thorburn and Challis, 1979),

it would be important to know the effect of gestational age and labour on the endogenous concentrations of P4.

This chapter describes the [P4] in the MYO, ENDO, CHOR, and AMN of non-labouring animals at different gestational ages and of labouring animals. Labour either occurred spontaneously at term or was induced prematurely by the pulsatile administration of ACTH to the fetus (Lye et al, 1983). This study was performed to determine if any changes occur in the [P4] in the intrauterine tissues that might be involved in the triggering mechanisms of parturition.

5.2 Materials and Methods

5.2.1 Animals

Fifty-five sheep of known gestational age were used in this study. In the first experiment samples of MYO, ENDO, CHOR, and AMN were obtained from pregnant ewes on day 50-60 [MYO, n=11; ENDO, n=4; CHOR, n=7; AMN, n=7], day 100 [MYO, n=4; ENDO, n=4; CHOR, n=3; AMN, n=3], and days 130-135 [MYO, n=9; ENDO, n=9; CHOR, n=10; AMN, n=10] and at spontaneous labour at term [days 140-150; MYO, n=11; ENDO, n=9; CHOR, n=9; AMN, n=7]. In the second experiment, ACTH (66 ng/min for 15 min every 2h) in saline or saline alone was administered to the fetus beginning on day 128 of gestation. Samples of tissue were collected from the animals treated with ACTH after induction of labour (~100 hrs)(MYO, n=6; ENDO, n=4; CHOR, n=5; AMN, n=5) and from the animals treated with saline after 100 hrs (MYO, n=8; ENDO, n=4; CHOR, n=5; AMN, n=5). All of the different tissues were not collected from each animal because of difficulties with tissue separation and identification.

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5.2.2 Concentrations of Progesterone

The [P4] in the tissues was measured by radioimmunoassay (RIA) using an antiserum (kindly donated by Dr. D.T. Armstrong, MRC Group in Reproductive Biology, London, Canada) whose cross reactivities are listed in Table 1. One gram or less of tissue was thawed, minced, and homogenized in distilled water. Homogenates were extracted with petroleum ether (5vol). [^{3}H]-progesterone (1200 DPM 2,4,6,7-^{3}H(N)-progesterone, ^{3}H-P4; specific activity, 90-115 Ci/mmole) was added to each homogenate prior to extraction. From several samples, an aliquot of the organic phase was taken for determination of radioactivity and immunoreactive-P4 (IR-P4) and the remainder was dried, resuspended in 1.0 ml isoctane and applied to micro-celite columns. The IR-P4 and radioactivity in successive 0.5 ml eluate fractions in isooctane from a representative sample of each tissue are shown in Figure 5. The specific activities in the peak fractions are shown and compared to that in the aliquot measured directly after extraction. There was reasonable agreement between the specific activities in the successive fractions through the P4 elution peak, although these were generally higher than the specific activity measured directly in the sample before application to the celite columns (see Figure Legend). The major cross reactants, 5α-and 5β-pregnan-3, 20-dione elute with peak retention volumes 1.0ml less than the P4 peak. The constancy of the specific activities through the P4 elution peak indicates that the amounts of the major cross reactants, if present in the sample, were not high enough to contribute significantly to the levels of P4 that were measured. Listed in Table 2 are the regressions and correlation coefficients when X is the amount of P4 measured directly after extraction and Y is the amount of P4

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TABLE 1. Cross-reactivities of C21, C19, and C18 steroids with the progesterone antiserum

	Percent Cross-Reactivity (X)
Pregn-4-en-3,20-dione	100.0
5β-pregnan-3,20-dione	32.0
5α-pregnan-3,20-dione	21.0
5β-pregnan-3α-ol-20-one	0.5>x>0.1
Pregn-4-en-21-ol-3,20-dione	0.5>x>0.1
Pregn-4-en-11β,21-diol-3,20-dione	0.5>x>0.1
Pregn-4-en-17α-ol-3,20-dione	0.5>x>0.1
Pregn-5-en-3β-ol-20-one	0.5>x>0.1
Pregn-4-en-20α-ol-3-one	0.5>x>0.1
Androst-4-en-3,17-dione	0.5>x>0.1
Androst-4-en-17β-ol-3-one	0.1>x>0.05
Pregn-4-en-11β,17α,21-triol-3,20-dione	0.05>x>0.01
Androst-5en-3β-ol-20-one	0.01>x>0.005
Pregn-5-en-3β,17α-diol-20-one	0.01>x>0.005
Estra-1,3,5(10)-trien-3,17β-diol	0.005>x>0.001
5β-pregnan-3α,17α,20α-triol	0.005>x>0.001
Preg-5-en-3β,20α-diol	0.005>x>0.001
Estra-1,3,5(10)-trien-3-ol-17-one	0.005>x>0.001
5β-pregnan-3α,20α-diol	0.005>x>0.001
Pregn-5en-17α,20α,3β-triol	0.001>X
Cholesterol	0.001>X

FIGURE 5. Elution profiles from micro-celite columns of progesterone from amnion (AMN), chorion (CHOR), endometrium (ENDO) and myometrium (MYO) of late pregnant sheep. The amount of progesterone mass, determined by RIA (●) and of authentic [^{3}H]-progesterone, (○), added to the sample before chromatography, is shown. Values refer to the specific activity of progesterone through the major peaks or prior to chromatography shown in parenthesis.

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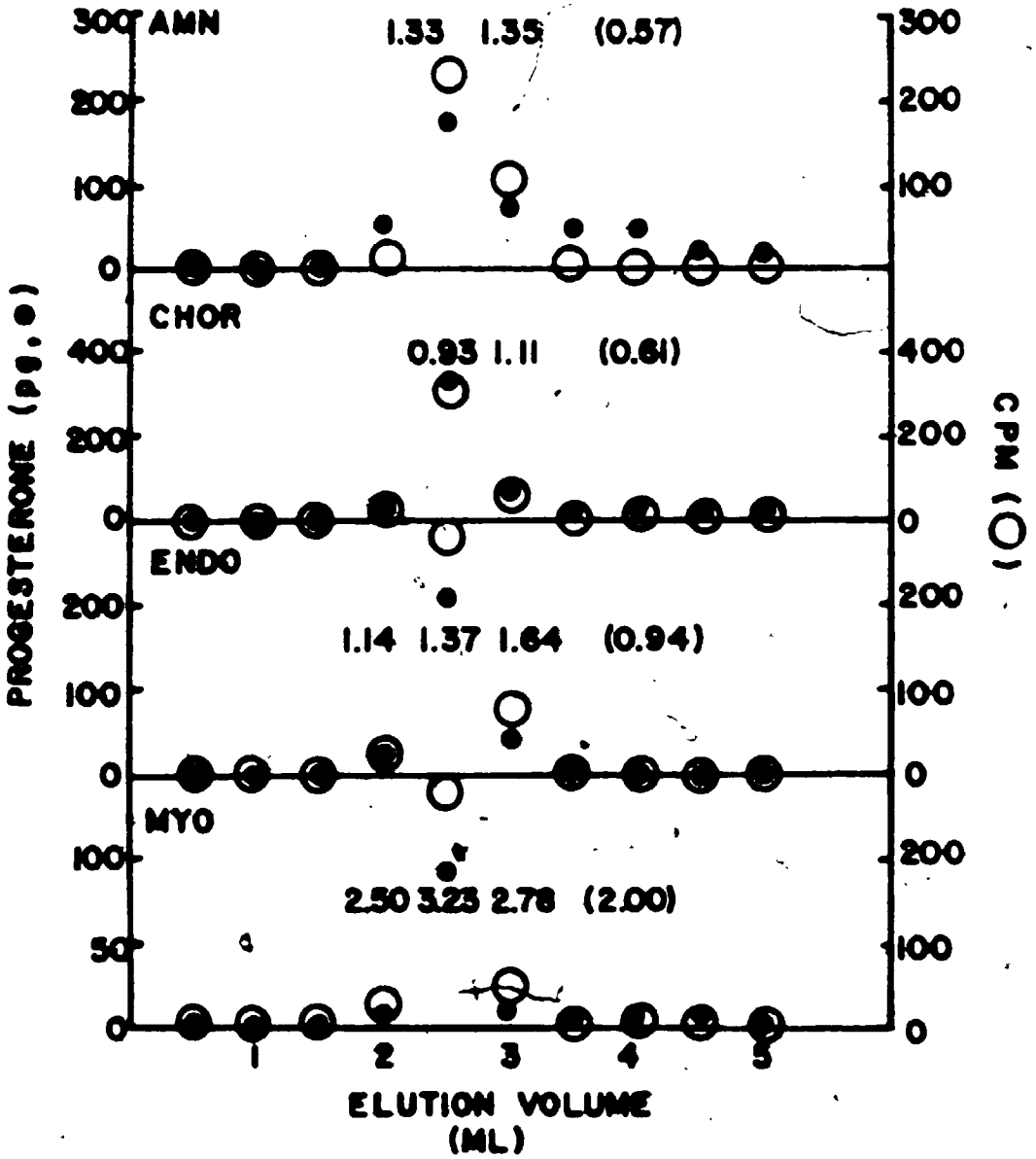

AMN
1.33 1.35 (0.57)
CHOR
0.83 1.11 (0.61)
ENDO
1.14 1.37 1.64 (0.94)
MYO
2.50 3.23 2.78 (2.00)
PROGESTERONE (pg.●)
CPM (○)
ELUTION VOLUME (ML)

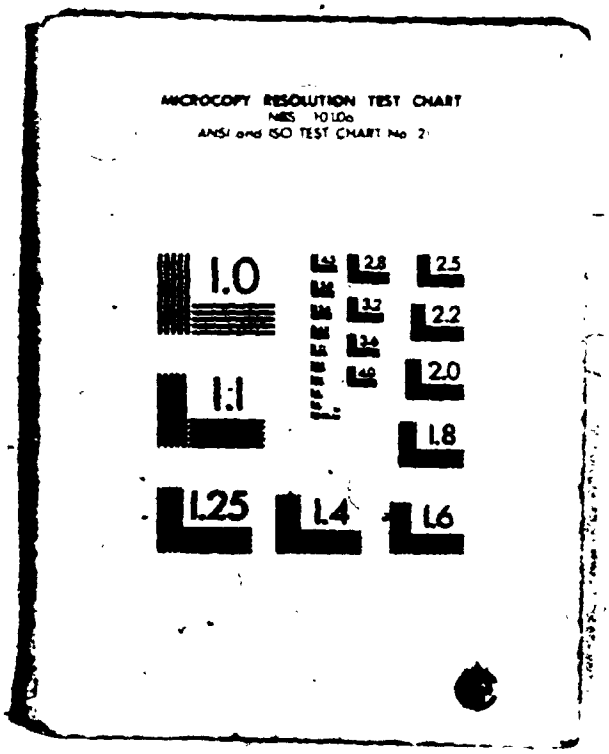

MICROCOPY RESOLUTION TEST CHART
NBS 1010a
ANSI and ISO TEST CHART No 2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.4
3.6
2.5
2.2
2.0
1.8

TABLE 2. Validation * of progesterone assay

	Endogenous Post Extraction vs Post Extraction and Purification	Exogenous Added vs Recovered
Amnion regression	y = 0.48x + 11.2	y'=0.88x' + 12.5
r	0.732	0.984
n	17	21
Chorion regression	y = 0.85x + 42.0	y'=0.90x' - 4.8
r	0.732	0.973
n	14	16
Endometrium regression	y = 0.94x - 33.4	y'=1.01x' - 28.3
r	0.929	0.951
n	6	6
Myometrium regression	y = 1.23x - 76.1	y'=0.85x' + 26.6
r	0.974	0.966
n	8	14

* Regressions and correlation coefficients (r). When x is the amount of P4 measured in the organic phase after extraction, y is the amount of P4 after extraction and purification by micro-celite chromatography; x' is the amount of exogenous P4 added to homogenates and y' is the amount of P4 recovered. The range of x is as follows: 60-350pg (amnion); 98-1560 (chorion); 196-570 (endometrium) and 94-440 (myometrium). x' ranged from 62-500 for all tissues.

n = number of samples

measured after extraction and purification. Values for X and Y were corrected for recovery. Also listed in Table 2 are the same parameters when X is the amount of P4 added to an homogenate and Y is the amount recovered by extraction. Although the RIA can estimate precisely exogenous authentic P4 added to each homogenate as evidenced by the good agreement between the amount of P4 added and the amount of P4 recovered, it does not estimate specifically the endogenous P4 within the tissues as evidenced by the poor agreement between the amount of P4 and its specific activity measured after extraction and those measured after extraction and purification. Thus the material in the organic phase after extraction of every sample was applied to micro-celite columns and P4 measured in the eluate fractions with retension times corresponding to authentic P4. The inter- and intra-assay coefficients of variation were 12.9% and 12.5% respectively. The concentrations are expressed as means (pg/mg protein) ± SEM. Protein concentrations were measured by the method of Lowry, Rosenbrough, Barr and Randall (1951) using bovine serum albumin as standard. No correction was made for the contribution of the plasma levels of P4 to the tissue levels (see Discussion).

As a further validation of the RIA used in this study, P4 in several samples of AMN (n=3), CHOR (3), ENDO (3) and MYO (4) was purified by micro-celite chromatography and comparison was made between the levels estimated using the antibody described above (x) and a second antibody (y) with different cross reactivities for 5α- and 5β-pregnan-3; 20-dione (11% and 7% cross-reaction respectively). Over a range of 300-3400 pg the regression equation was $y=1.13x-101.8$ pg with a correlation coefficient of 0.997. The similarity between

the values measured with the two different antibodies argues further in support of the authenticity of my P4 estimations.

5.2.3 Analysis of Results

Comparisons between group means were made using the Mann Whitney U test.

5.3 Results

The mean [P4] in the AMN and CHOR rose throughout pregnancy (Fig. 6). In both tissues the concentrations on day 130-135 (both $P<0.025$) and at term (AMN and CHOR, $P<0.01$ and $P<0.025$, respectively) were significantly higher than the concentration on day 50-60. There was no significant ($P>0.05$) difference between the concentrations at term and those on day 130-135 and day 100. In the ENDO (Fig. 7) the [P4] rose between day 50-60 and day 130-135. The concentrations on day 100 and day 130-135 were significantly ($P<0.05$ and $P<0.005$, respectively) higher than those on day 50-60. The concentrations fell between day 130-135 and term. The concentrations at term were significantly lower than those on day 130-135 ($P<0.001$) and day 100 ($P<0.05$) but were significantly ($P<0.05$) higher than those on day 50-60. In the MYO (Fig. 7) the [P4] rose significantly ($P<0.025$) between day 50-60 and day 100 but fell significantly ($P<0.01$) between day 100 and day 130-135 to a value significantly ($P<0.05$) higher than those on day 50-60. The concentration fell significantly ($P<0.005$) between day 130-135 and term.

The [P4] in each intrauterine tissue obtained from animals in premature labour induced by intrafetal ACTH (Fig. 8) was not significantly different from the [P4] in the respective tissue obtained from the saline controls. In the ENDO, the [P4] was significantly

FIGURE 6 The concentration of progesterone in amnion and chorion from sheep at different times of pregnancy Values are mean ± SEM for the number of animals (samples) indicated. SL, spontaneous labour

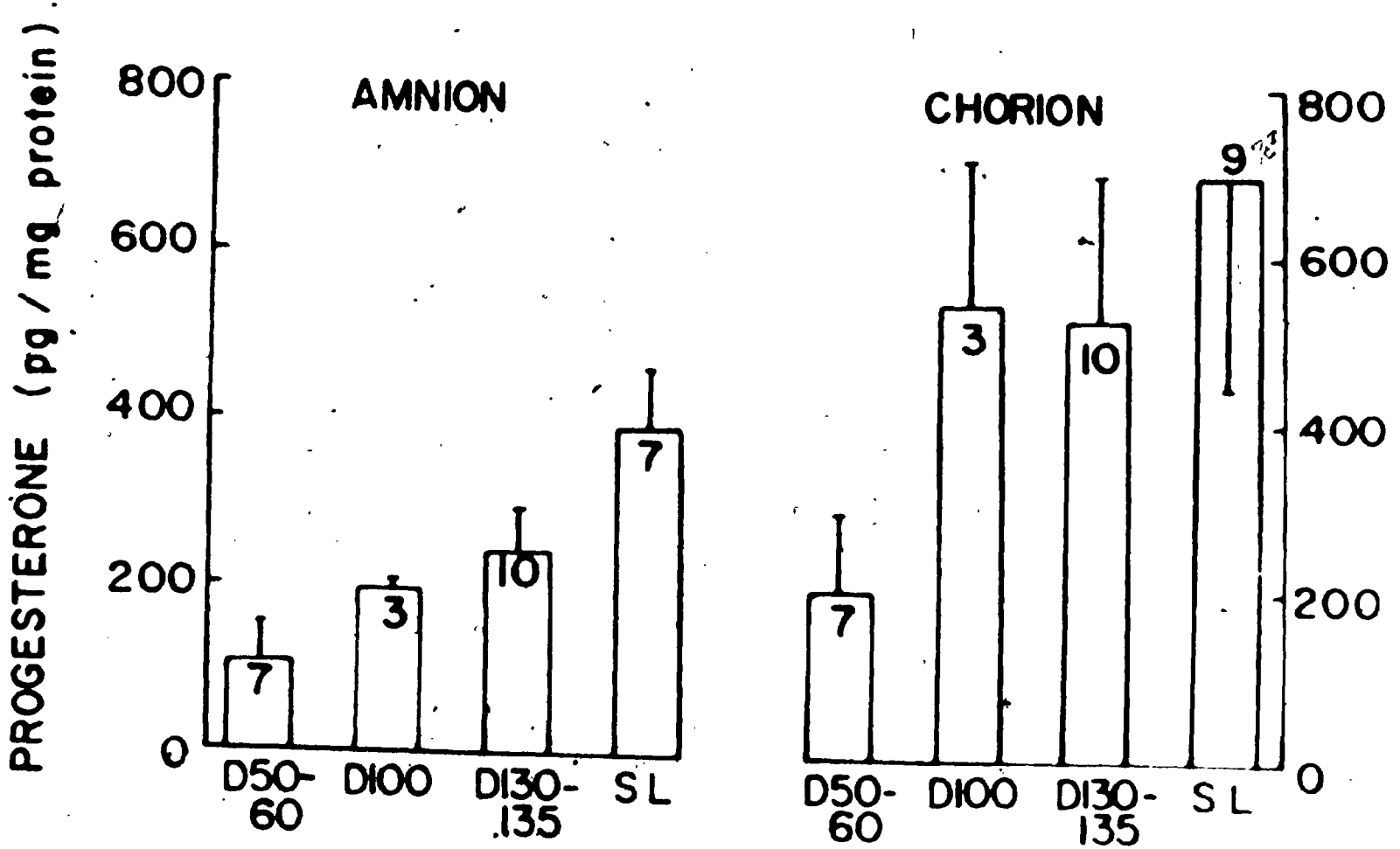

PROGESTERONE (pg / mg protein)
AMNION
CHORION
800
600
400
200
0
7
3
10
7
7
3
10
9
D50-60
D100
D130-135
SL

FIGURE 7. The concentration of progesterone in endometrium (ENDO) and myometrium (MYO) from sheep at different times of pregnancy. Values are mean ± SEM for the number of animals (samples) indicated.

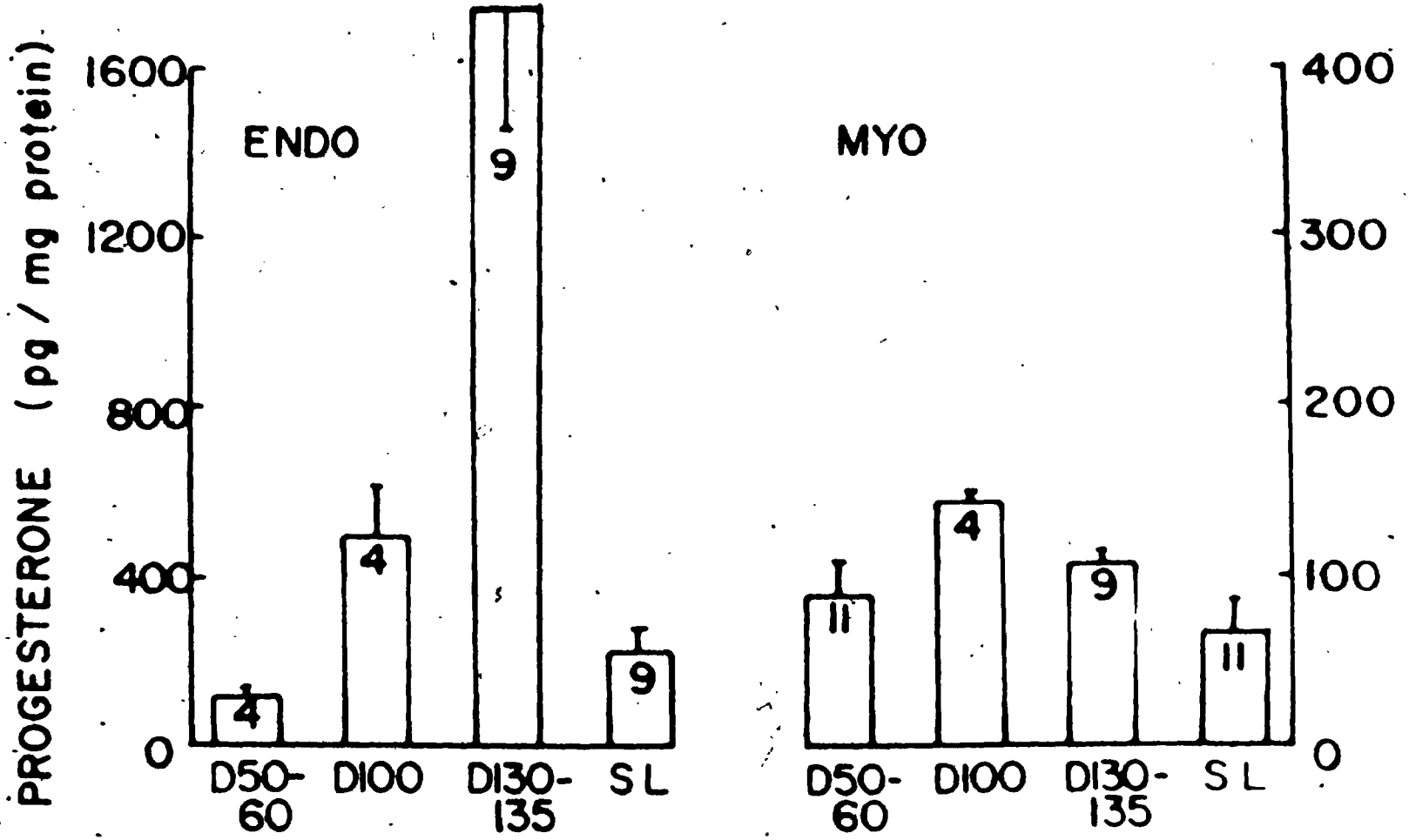
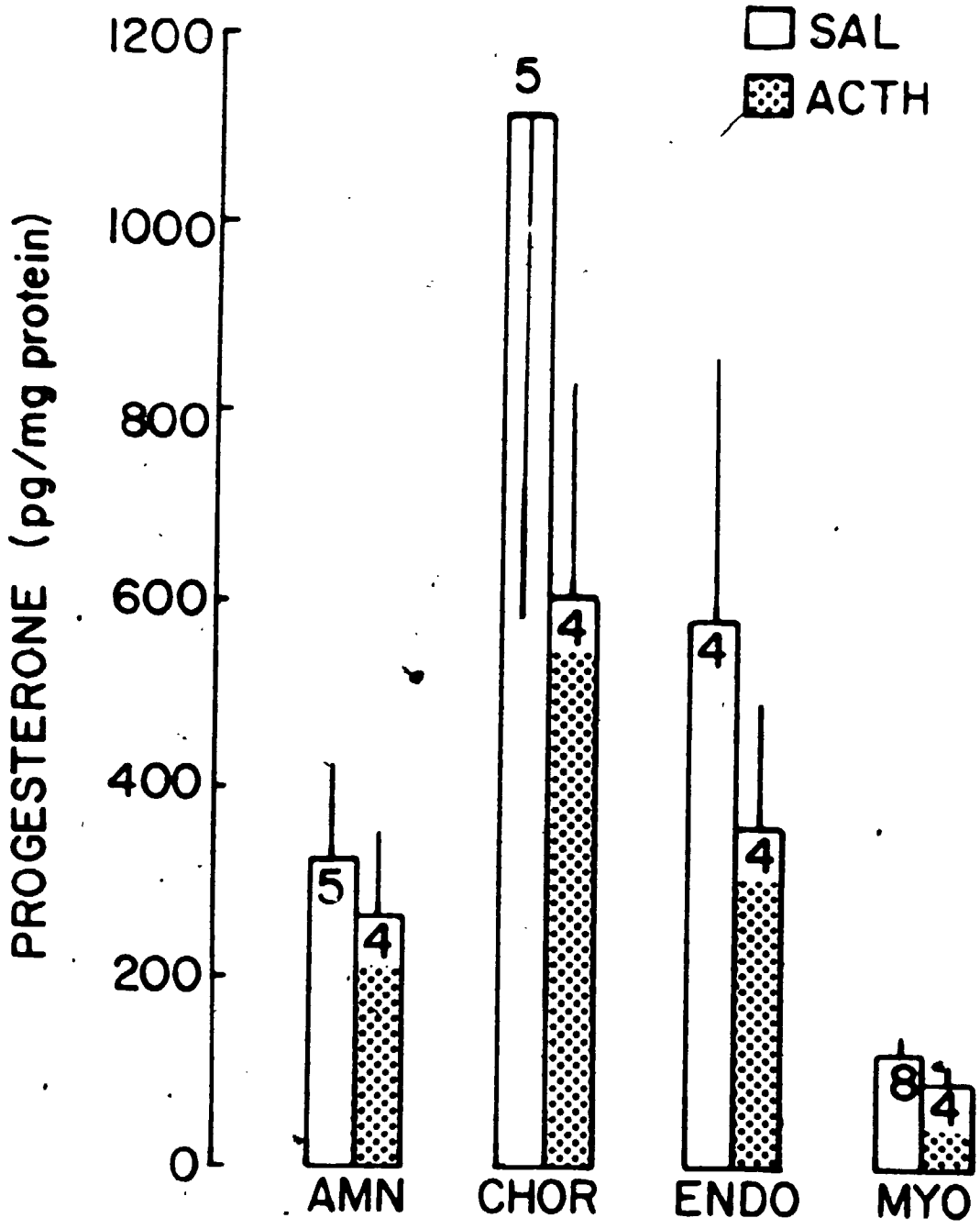

PROGESTERONE (pg / mg protein)
ENDO
MYO
1600
1200
800
400
0
400
300
200
100
0
4
4
9
9
11
4
9
11
D50-60
D100
D130-135
SL

FIGURE 8. The concentration of progesterone in amnion (AMN), chorion (CHOR), endometrium (ENDO) and myometrium (MYO) of d 131 pregnant sheep pretreated with saline (100h) or ACTH (~100h until active labour). Values are mean ± SEM for the number of animals indicated in each group.


SAL
ACTH
PROGESTERONE (pg/mg protein)
1200
1000
800
600
400
200
0
5
4
5
4
4
4
8
4
AMN
CHOR
ENDO
MYO

lower ($P<0.05$) in the saline controls compared to unoperated animals of similar gestational age and [P4] in ACTH-induced labour was not significantly different ($P>0.05$) from that in spontaneous labour at term.

5.4 Discussion

I have described the [P4] in the intrauterine tissues at different times during pregnancy and in labour. The changes in the [P4] throughout pregnancy are not similar in all tissues. The [P4] in all tissues reach maximum levels in the latter half of pregnancy, however, the times maximum concentrations are achieved and maintained differ. The highest [P4] in the AMN was measured in spontaneous labour at term. The [P4] in the AMF also rose throughout pregnancy to maximum values at term (see Chapter 4). In the other fetal membranes analysed, the CHOR, maximum levels were measured on day 100 and maintained through until spontaneous labour and as such did not mirror changes in the AMF at term. ACTH treatment did not alter the [P4] in either the AMN or CHOR. Thus, labour was not associated with lower levels of P4 in the fetal membranes and, as I have shown in Chapter 4, in the fetal fluids as well. In the ENDO the changes in the [P4] mirror those in the maternal plasma (see Chapter 4), rising throughout pregnancy to a maximum at day 130-135 and falling markedly to lower levels at term. The [P4] in the MYO fell in the latter half of pregnancy, starting on day 100, to lower levels at normal parturition but the fall in the myometrial [P4] between day 130-135 and spontaneous labour at term was not as marked as that in the ENDO. However, levels in both tissues obtained from animals in labour induced prematurely by intrafetal ACTH were similar to those obtained.

from the non-labouring saline controls suggesting that a fall in the levels of P4 in the MYO and ENDO is not a prerequisite for the onset of what I have defined as labour. It is important to stress the fact that the animals in spontaneous labour at term or ACTH-induced labour and the saline control animals had been surgically treated prior to the collection of tissues. The surgical procedure may have contributed to lowering the levels of P4 in the ENDO of these animals. Despite the lower levels of P4 in the ENDO of saline control animals compared to those in unoperated animals of comparable gestational age, there was no evidence of increased intra-amniotic pressure. These results support the conclusion of Liggins et al, (1972) that a fall in the concentration of P4 in the MYO is not a major factor controlling the timing of the onset of labour and extends it to include the ENDO, AMN and CHOR.

The contribution of plasma P4 within capillaries to tissue concentrations is probably minimal. The amount of protein measured in homogenates of CHOR, ENDO, and MYO was estimated to be between 5 and 10% of wet tissue weight. The amounts of P4 in the ENDO and MYO per gram wet weight were ten and twofold, respectively, that per ml of maternal plasma in late pregnancy. The amount of P4 in the CHOR per gram wet weight was fiftyfold that per ml of fetal plasma.

The lack of parallelism between the concentrations of P4 in the intrauterine tissues and those in the plasma and fetal fluids, described above, suggests the existence of other factors influencing tissue levels in sheep. In other species, a local effect of the placenta on the [P4] in the myometrium (rat: Csapo, 1969b; Csapo and Weist, 1969; human: Runnebaum and Zanner, 1969) and fetal membranes (human: Pulkkenen and Enkola, 1972) has been demonstrated. In my

experiments, tissues were taken from intercotyledonary areas but no relationship to distance from cotyledons was considered. Schwarz, Milewich, Johnston, Porter and MacDonald (1976) described a progesterone binding substance in human fetal membranes that appeared during the last few days of pregnancy. These authors argued that the binding of P4 at the level of the fetal membranes could produce a local withdrawal of P4 No such substance has been described in the sheep fetal membranes and no further characterization of this substance in the human fetal membranes has appeared in the literature

An alternate mechanism that might control the [P4] in intrauterine tissues is that of local synthesis and/or metabolism The presence in intrauterine tissue of enzymes involved in the production and metabolism of P4 and Es was investigated and the results are described in a later chapter.

CHAPTER 6

CONCENTRATIONS OF ESTROGENS IN INTRAUTERINE TISSUES

6.1 Introduction

Rawlings and Ward (1976) showed that the concentrations of total unconjugated estrogens (Es) in the myometrium (MYO) of two pregnant ewes on the day of parturition were elevated over the values measured daily during the last week of pregnancy. Analysis of more samples is required to assess the significance of this change. It would also be important to know the specific Es that contribute to the reported changes and whether or not similar changes occur in the endometrium (ENDO) and fetal membranes.

This chapter describes the concentrations of estrone (E1) and estradiol-17β (E2β) in the MYO, ENDO, chorion (CHOR) and amnion (AMN) obtained from pregnant ewes at different times in gestation and at the time of labour which was either spontaneous at term or induced prematurely in late pregnancy by the pulsatile administration of ACTH to the fetus. These studies were performed to determine if any changes occur in the levels of specific Es in the intrauterine tissues that might be involved in the triggering processes of parturition.

6.2 Materials and Methods

6.2.1 Animals

Fifty-three sheep of known gestational age were used in this study. Tissues were collected from the same groups of animals as in the previous chapter (day 50-60: MYO, n=10; ENDO, n=8; CHOR, n=9; AMN, n=8; day 100: MYO, n=4; ENDO, n=5; CHOR, n=3; AMN, n=5; day 130-135: MYO, n=8; ENDO, n=7; CHOR, n=6; AMN, n=5; term: MYO, n=7; ENDO, n=7; CHOR, n=4; AMN, n=6; sal: MYO, n=8; ENDO, n=7; CHOR, n=6; AMN, n=5; ACTH: MYO, n=8; ENDO, n=4; CHOR, n=5; AMN, n=7). In addition, samples of MYO (n=6) were obtained after 72 hours of intrafetal ACTH at a time when labour activity was not present. For the same reasons as stated in Chapter 5, not all of the different tissues were obtained from each animal.

6.2.2 Concentrations of Estrogens

Concentrations of estrone (E1) and estradiol-17β (E2β) were measured using radioimmunoassays (RIA) (Challis and Patrick, 1981; see Appendix 3 for cross reactivities). Tissues were prepared as described in the previous chapter. Homogenates were extracted with 5 vol. diethyl ether. Values in the organic phase were expressed as means (pg/mg protein) ± SEM. The concentrations of protein were determined as described in the previous chapter.

6.2.3 RIA Validation

One-two thousand CPM of [^{3}H]-estrone (^{3}H-E1, specific acticity, 90-95 Ci/mmole), or [^{3}H]-estradiol-17β (^{3}H-E2β, specific activity 90-95 Ci/mmole) were added to several homogenates prior to extraction.

An aliquot of the organic phase was taken for measurements of immunoreactive -E1, (IR-E1) or immunoreactive -E2β (IR-E2β) and radioactivity. The remainder of the organic phase was dried, resuspended in isooctane and applied to micro-celite columns (Challis and Patrick, 1981). E1 and E2β were eluted from the column with 15% and 25% ethyl acetate, respectively, in isooctane. Figure 9 and 10 show representative profiles of ^{3}H-E1 with IR-E1 and ^{3}H-E2β with IR-E2β, respectively, measured in successive 0.5 ml eluates. Also shown are the specific activities of the peaks and that measured prior to purification. The amount of E1 coeluting with ^{3}H-E1 averaged 85%, 92%, 103%, and 101% of the unpurified values in the AMN, CHOR, ENDO and MYO, respectively. Similarly the amounts of E2β averaged 83%, 83%, 83% and 89% of the unpurified values.

In Table 3 are the regression and correlation coefficients when X represents the amount of exogenous estrogen added to homogenates and Y represents the amount of estrogen recovered. There was good agreement between the values added to AMN, CHOR and MYO and those recovered. When considered together these analyses indicate that the RIA for E1 and E2β, estimated precisely and specifically authentic E1 and E2β, respectively, in the different intrauterine tissues. The relatively smaller slope of the regression equation of E1 recovery from ENDO suggests that some factor was lowering the precision of E1 estimation in this tissue. The inter- and intra-assay coefficients of variation for the E1 assays were 14.9% and 7.9%, and for the E2β assays were 9.8% and 9.4% respectively.

6.2.4 Statistical Analysis

FIGURE 9. Elution profiles from micro-celite columns of estrone from amnion (AMN), chorion (CHOR), endometrium (ENDO), and myometrium (MYO) of late pregnant sheep. Estrone was eluted with 15% ethylacetate in isooctane. The amount of estrone mass, determined by RIA (●) and of authentic [^{3}H]-estrone (O), added to the sample before chromatography are shown. Values are specific activities through the major peaks or prior to chromatography, as shown in parentheses.

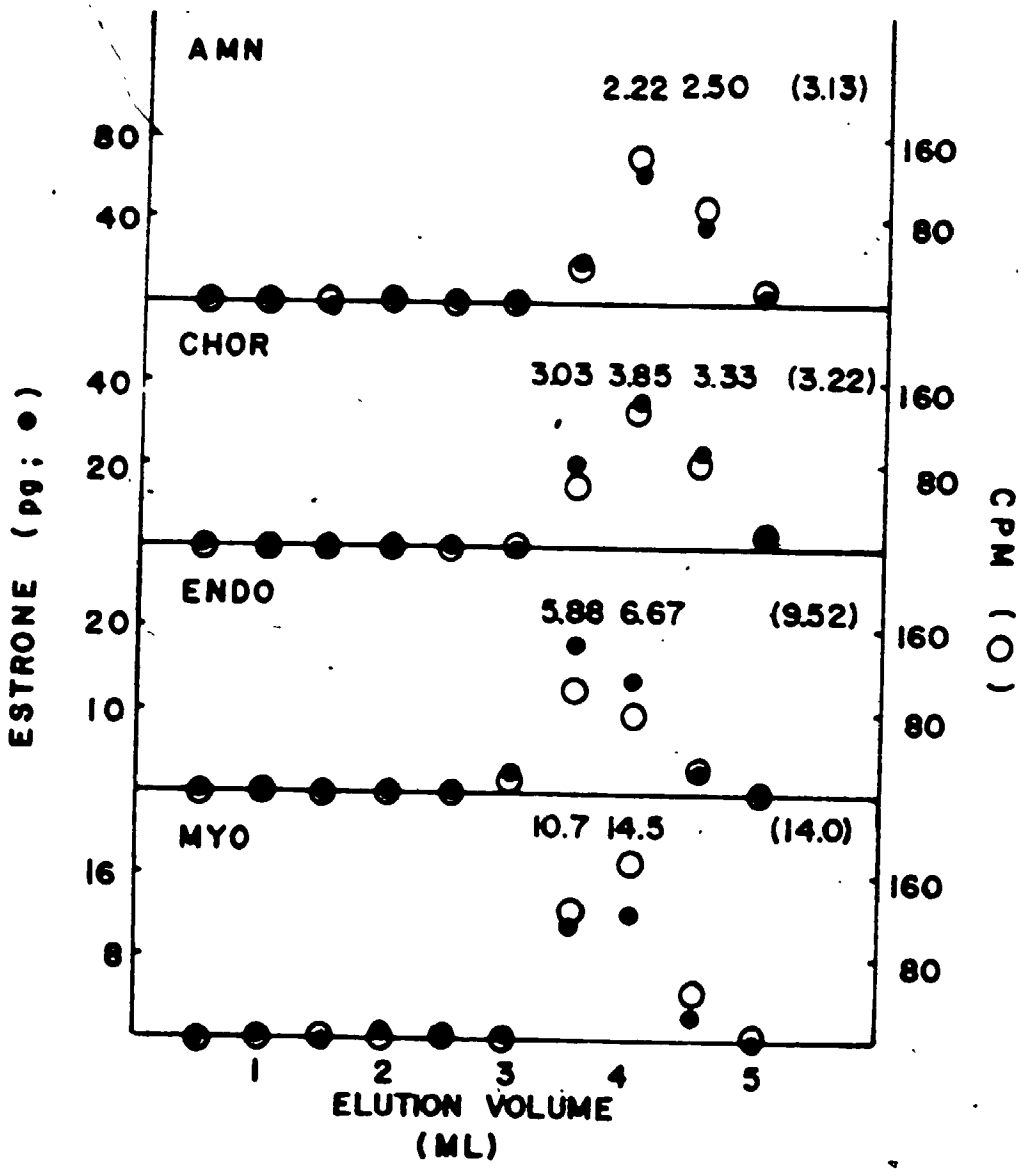

AMN
2.22 2.50 (3.13)
80
40
160
80
CHOR
3.03 3.85 3.33 (3.22)
40
20
160
80
ENDO
5.88 6.67 (9.52)
20
10
160
80
MYO
10.7 14.5 (14.0)
16
8
160
80
ESTRONE (pg; ●)
CPM (○)
1
2
3
4
5
ELUTION VOLUME
(ML)

FIGURE 10. Elution profiles from micro-celite columns of estradiol-17β from amnion (AMN), chorion (CHOR), endometrium (ENDO), and myometrium (MYO) at late pregnant sheep. Estradiol-17β was eluted with 25% ethylacetate in isooctane. The amount of estradiol-17β mass, determined by RIA (●) and of authentic [^{3}H]-estradiol-17β (○), added to the sample before chromatography, are shown. Values are specific activities through the major peaks or prior to chromatography as shown in parentheses.

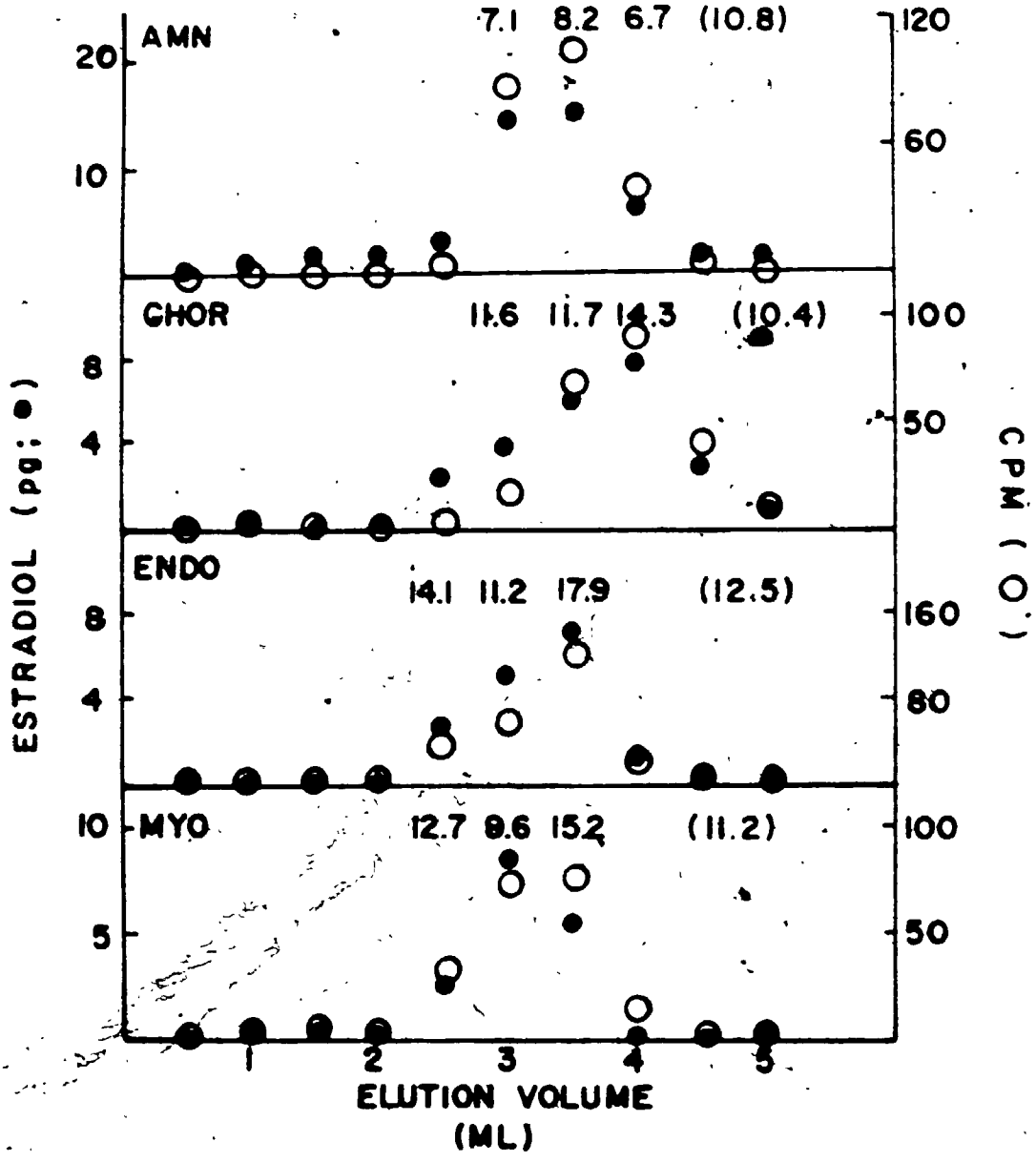

AMN
7.1 8.2 6.7 (10.8)
20
10
120
60
CHOR
11.6 11.7 14.3 (10.4)
8
4
100
50
ENDO
14.1 11.2 17.9 (12.5)
8
4
160
80
MYO
12.7 9.6 15.2 (11.2)
10
5
100
50
ESTRADIOL (pg; ●)
CPM (○)
1
2
3
4
5
ELUTION VOLUME
(ML)

TABLE 3. Regression equations and correlation coefficients describing recovery of exogenous estrone and estradiol added to homogenates of intrauterine tissues.

	Estrone	Estradiol-17β
Amnion	y = .91x - 6.5 r = .969	y = .90x - 4.8 r = .995
Chorion	y = .86x - 2.2 r = .998	y = .96x - 17.8 r = .995
Endometrium	y = .73x + 2.6 r = .987	y = .80x + 4.0 r = .982
Myometrium	y = .88x - 7.1 r = .971	y = .81x + 1.3 r = .984

x = amount of exogenous estrogen added, 62-500pg
y = amount of estrogen recovered
r = correlation coefficient
number of samples = 16

Comparisons between group means were made using the Mann Whitney - U test.

6.3 Results

The concentrations of E1 ([E1]) and E2β ([E2β]) in the intrauterine tissues are shown in figures 11 to 14. In the AMN, there was no significant (P>0.05) change in the [E1] (Fig. 11) between any of the times studied. The values of E2β on day 50-60 were extremely variable and not significantly (P>0.05) different from those at any of the other times studied. The [E2β] fell significantly (P<0.01) between day 100 and day 130-135 but rose significantly (P<0.05) between day 130-135 and term. The ratio of E1:E2β (Table 4) increased significantly (P<0.01) between day 100 and day 130-135 but did not change significantly (P>0.05) between day 130-135 and term.

In the CHOR, the [E1] (Fig. 12) at term was not significantly different from those on day 100 and day 130-135. The [E1] on day 50 was significantly lower than those on day 100 (P<0.025), day 130-135 (P<0.01), and term (P<0.025). The [E2β] did not change significantly between any of the times studied. The ratio of E1:E2β increased significantly (P<0.05) between day 50-60 and day 100 and fell significantly (P<0.025) between day 130-135 and term.

In the ENDO, the [E1] (Fig. 13) rose between day 130-135 and term. The [E1] at term was significantly higher than those on day 130-135 (P<0.025), day 100 (P<0.005) and on day 50-60 (P<0.005). Comparisons between other times did not show significant differences. The [E2β] rose between day 100 and term. The [E2β] at term (P<0.005) and day 130-135 (P<0.01) were significantly higher than that on day

100 although the [E2β] at term was not significantly different from those on day 130-135 and day 50. The ratio of E1:E2β increased significantly ($P<0.025$) between day 130-135 and term.

In the MYO, the [E1] (Fig. 14) rose between day 130-135 and term. The [E1] at term was significantly higher than those on day 130-135 ($P<0.001$), day 100 ($P<0.01$) and day 50-60 ($P<0.001$). Comparisons between other times did not show significant differences. The [E2β] was elevated on day 100. The [E2β] on day 100 was significantly higher than those on day 50-60 ($P<0.05$) and day 130-135 ($P<0.05$). The [E2β] rose between day 130-135 and term. The [E2β] at term was significantly different from those on day 130-135 ($P<0.001$), day 100 ($P<0.01$) and day 50-60 ($P<0.001$). The ratio of E1:E2β increased significantly ($P<0.025$) between day 100 and day 130-135 but did not change significantly between day 130-135 and term.

The concentrations of E1 and E2β in the ENDO, CHOR and AMN (Fig. 15) obtained from animals in premature labour induced by intrafetal ACTH were not significantly different from the concentrations in the respective tissues obtained from saline controls. The concentrations of E1 and E2β in the MYO (Fig. 16) were significantly (both $P<0.001$) elevated over control values. The mean concentrations of E1 and E2β after 72 hours of ACTH administration, were elevated over those of the saline controls but the differences were only significant at $0.10>P>0.05$.

The ratios of P4:E2β in each intrauterine tissue at each time studied are shown in Table 5. The ratios of P4:E2β in the AMN and CHOR did not change significantly between any of the times studied. The ratios in the ENDO and MYO fell between days 130-135 and term. The ratios at term were significantly lower than those on day 130-135

FIGURE 11. The concentration of estrone and estradiol-17β in amnion from sheep at different times in pregnancy. Values are mean ± SEM. The number of samples (animals) at each stage of gestation is indicated.

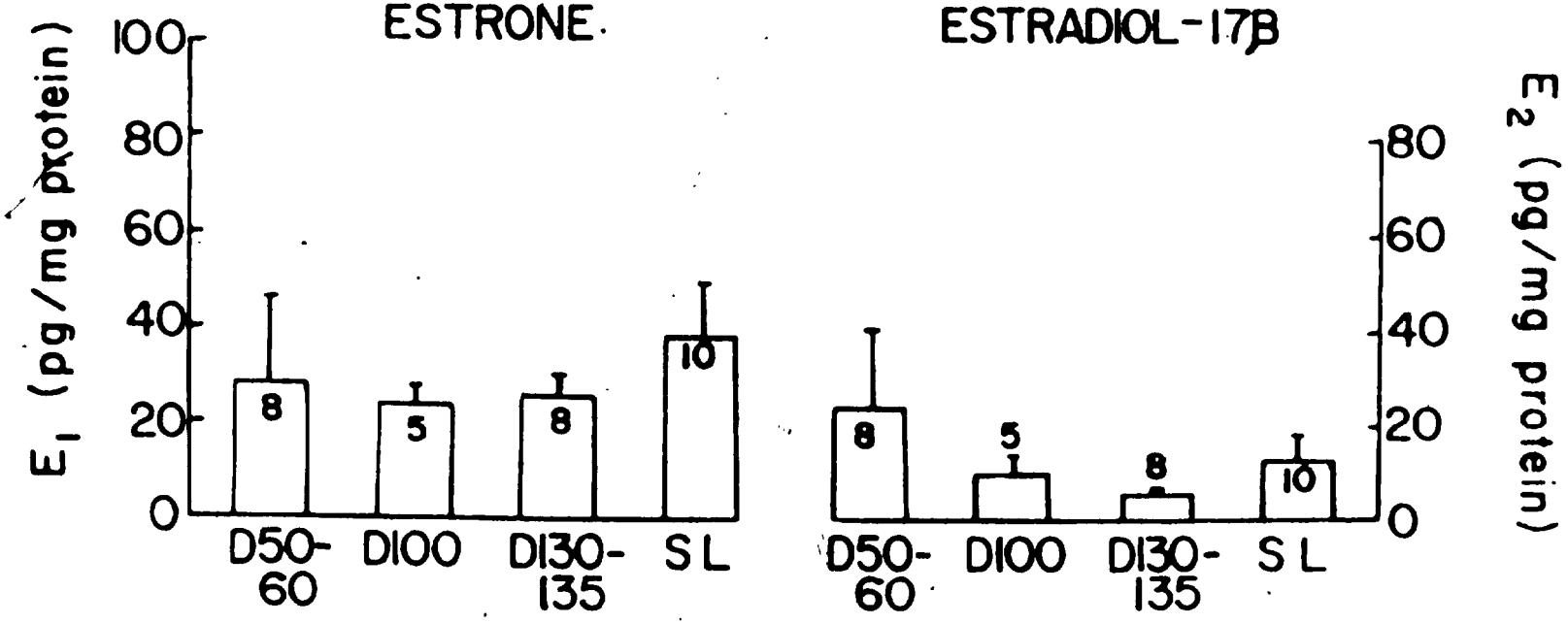

ESTRONE
ESTRADIOL-17β
E1 (pg/mg protein)
E2 (pg/mg protein)
100
80
60
40
20
0
8
5
8
10
D50-60
D100
D130-135
SL

FIGURE 12. The concentration of estrone and estradiol-17β in chorion from sheep at different times in pregnancy. Values are mean ± SEM. The number of samples (animals) at each stage of gestation is indicated.

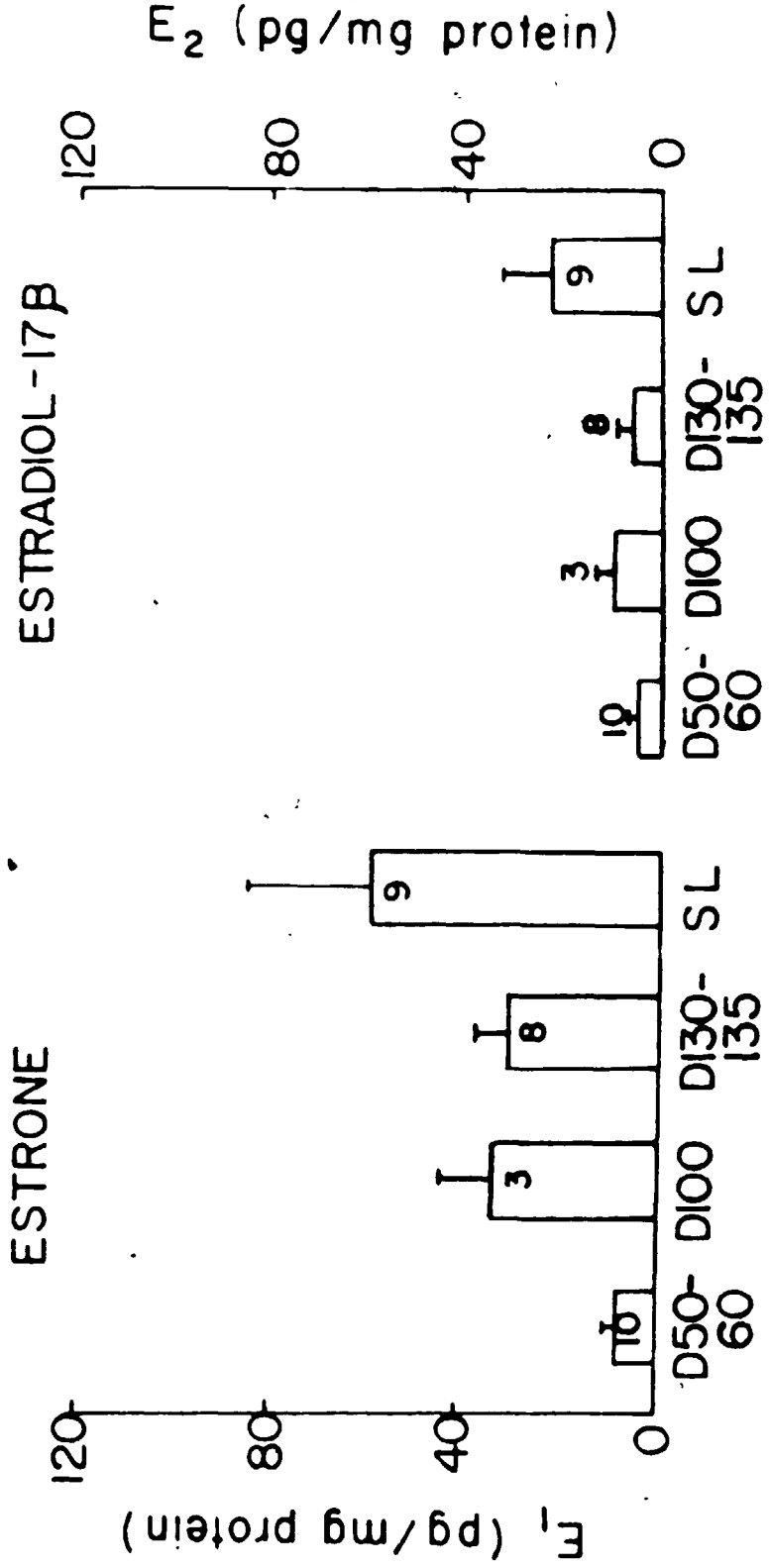

E2 (pg/mg protein)
ESTRADIOL-17β
120
80
40
0
10
3
8
9
D50-60
D100
D130-135
SL
E1 (pg/mg protein)
ESTRONE

FIGURE 13. The concentration of estrone and estradiol-17β in endometrium from sheep at different times in pregnancy. Values are mean ± SEM. The number of samples (animals) at each stage of gestation is indicated.

E2 (pg/mg protein)
ESTRADIOL-17β
30
20
10
0
11
15
5
8
D50-60
D100
D130-135
SL
E1 (pg/mg protein)
ESTRONE

FIGURE 14. The concentration of estrone and estradiol-17β in myometrium from sheep at different times in pregnancy. Values are mean ± SEM. The number of samples (animals) at each stage of gestation is indicated.

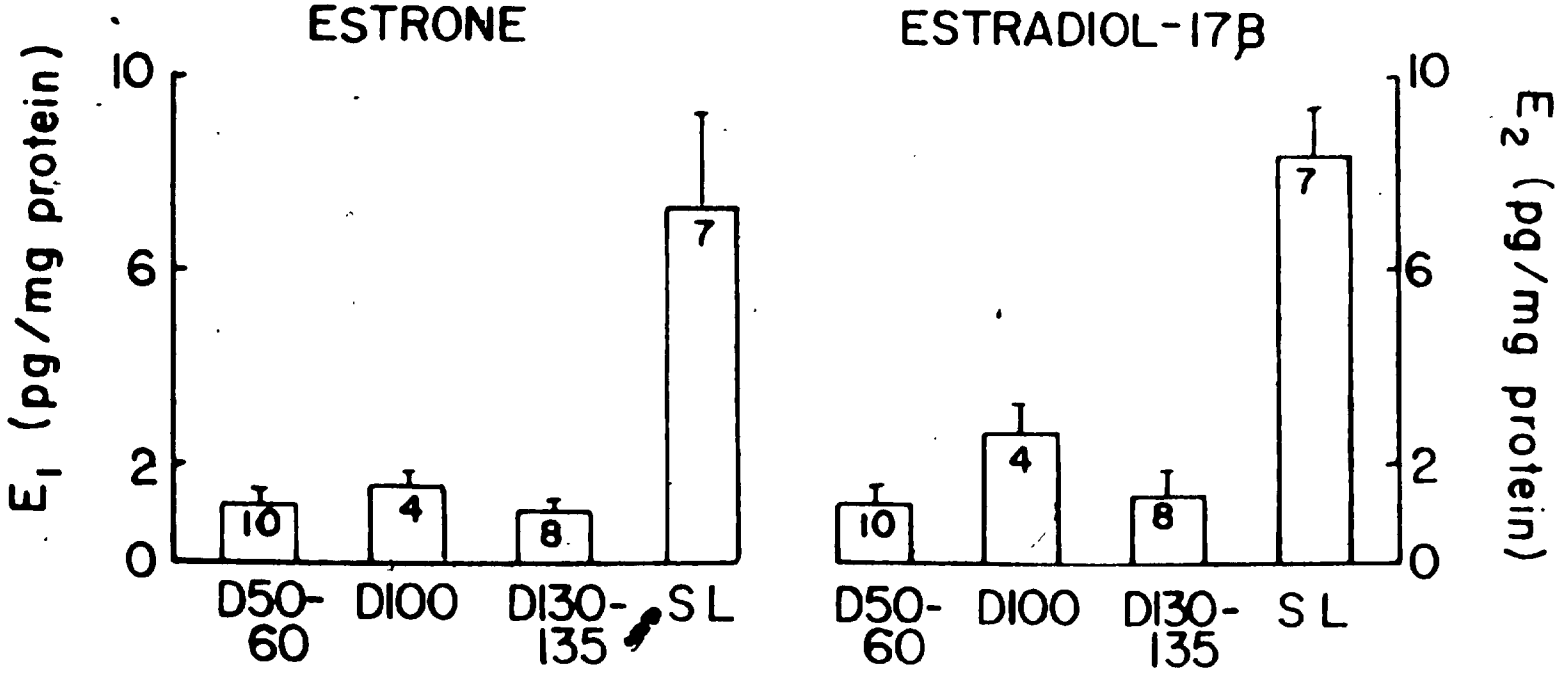

ESTRONE
ESTRADIOL-17β
E_1 (pg/mg protein)
E_2 (pg/mg protein)
10
6
2
0
10
4
8
7
D50-60
D100
D130-135
SL

FIGURE 15. The concentration of estrone (upper panel) and estradiol-17β (lower panel) in amnion (AMN), chorion (CHOR) and endometrium (ENDO) taken from day 131 pregnant sheep after 100h *in vivo* pretreatment with saline or ACTH. Values are mean ± SEM for a number of animals indicated in each group.

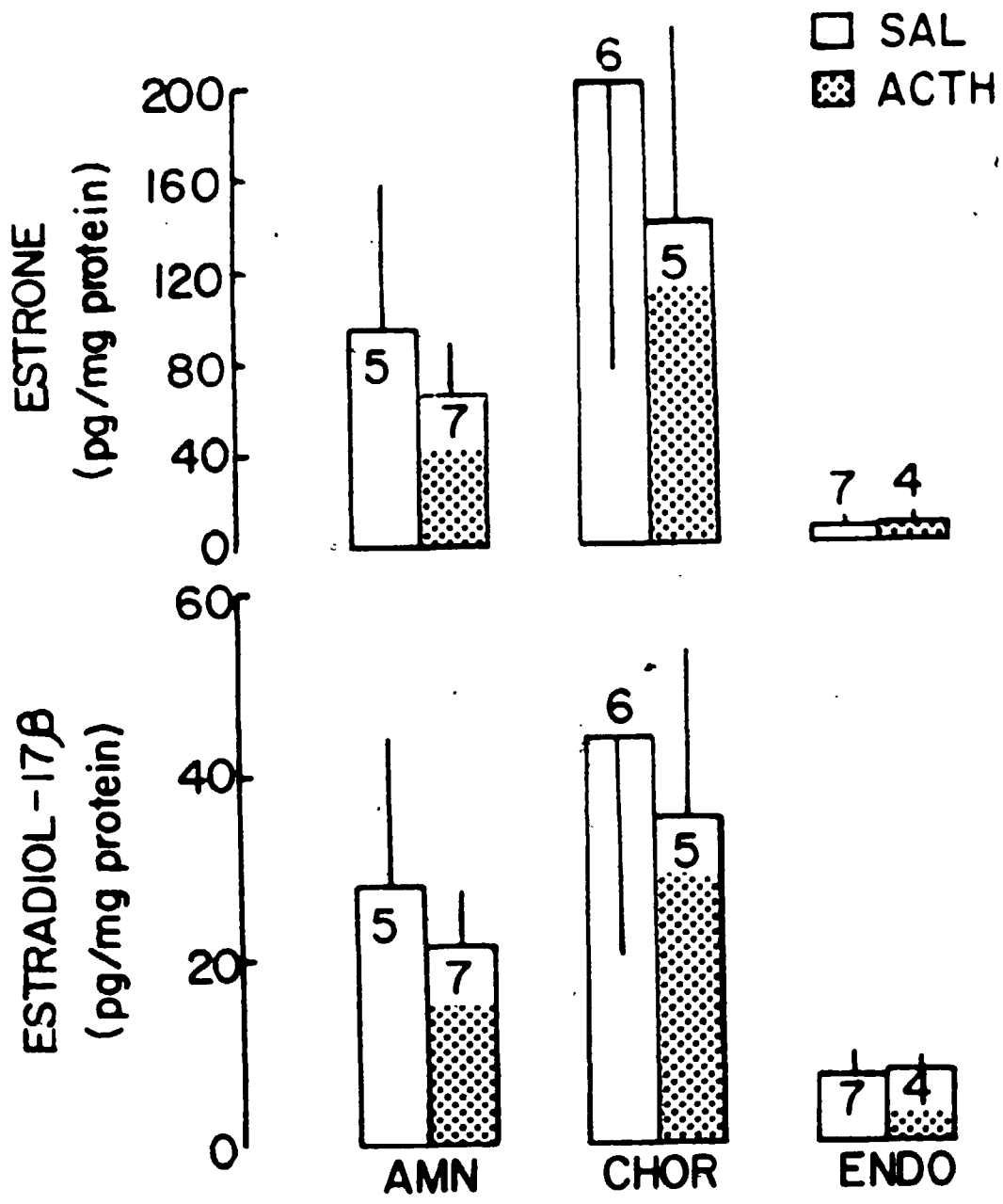
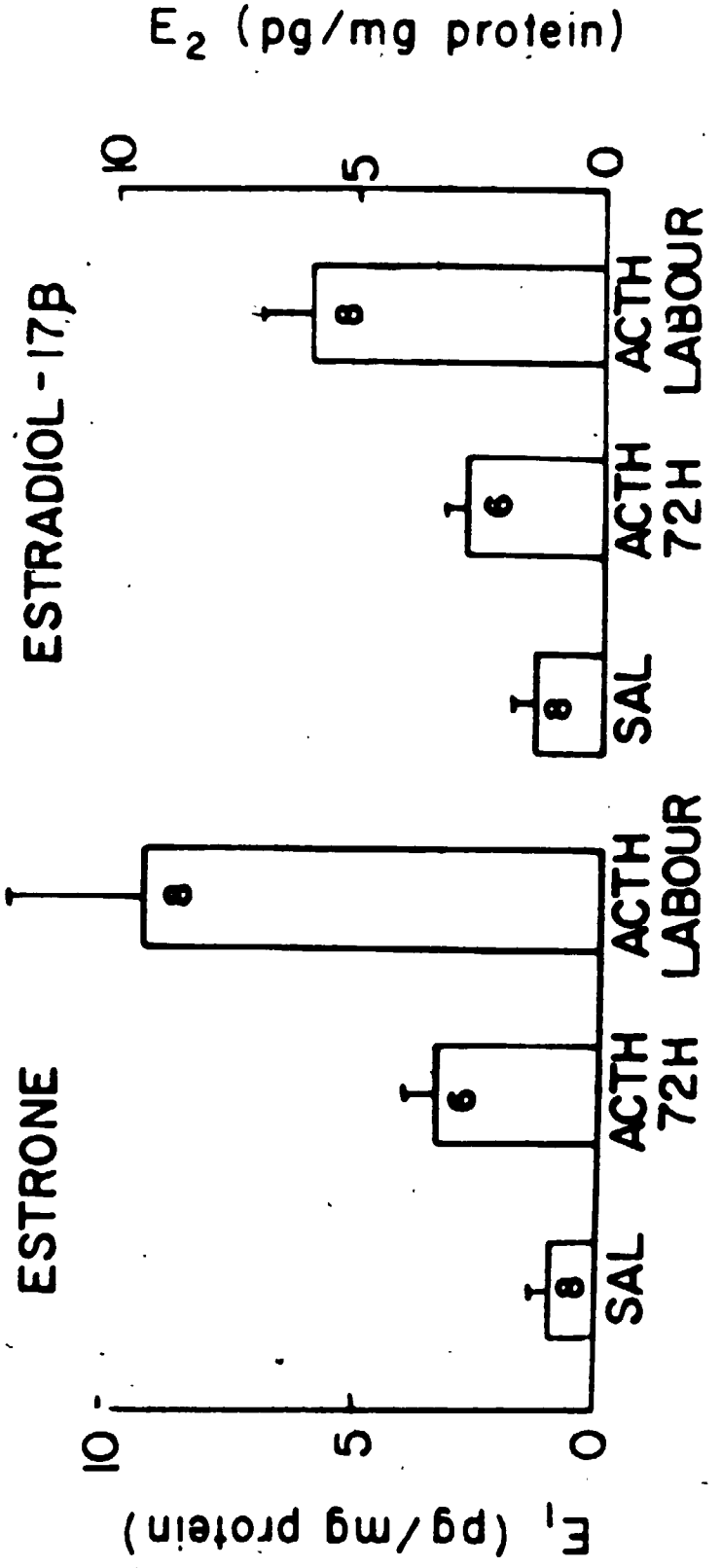

SAL
ACTH
ESTRONE
(pg/mg protein)
200
160
120
80
40
0
ESTRADIOL-17β
(pg/mg protein)
60
40
20
0
5
7
6
5
7
4
5
7
6
5
7
4
AMN
CHOR
ENDO

FIGURE 16. The concentration of estrone and estradiol-17β in the myometrium of day 130-131 pregnant sheep pretreated with saline (100h), ACTH (72h) or ACTH (~100h to active labour) *in vivo*. Values are mean ± SEM for the number of animals indicated in each group.


E2 (pg/mg protein)
ESTRADIOL-17β
ESTRONE
E1 (pg/mg protein)
10
5
0
SAL
ACTH 72H
ACTH LABOUR
8
6

105

TABLE 4. The ratio* of estrone to estradiol-17β in intrauterine tissues at different times during pregnancy

Stage of Pregnancy	Amnion	Chorion	Endometrium	Myometrium
d50-60	$2.04\pm0.45_{a}$ (8)	$1.82\pm0.33_{a}$ (10)	$1.24\pm0.40_{ab}$ (8)	$0.99\pm0.18_{ab}$ (10)
d100	$2.56\pm0.31_{a}$ (5)	$4.86\pm1.74_{b}$ (3)	$1.38\pm0.47_{ab}$ (5)	$0.62\pm0.13_{a}$ (4)
d130-135	$5.46\pm0.63_{b}$ (13)	$4.29\pm0.70_{b}$ (14)	$0.79\pm0.20_{a}$ (15)	$1.45\pm0.35_{b}$ (8)
Term	$3.69\pm0.60_{ab}$ (10)	$2.57\pm0.75_{a}$ (9)	$2.20\pm0.55_{b}$ (11)	$0.98\pm0.24_{ab}$ (7)

* Values are mean±SEM. The number of samples at each time is indicated in parenthesis.

Means with different subscripts are significantly different at P values described in text.

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TABLE 5. The ratio* of progesterone to estradiol-17β in intrauterine tissues at different times during pregnancy in sheep

Days of Pregnancy	Amnion	Chorion	Endometrium	Myometrium
d50-60	23.4± 5.7	48.4± 10.8	111.0± 25.1	102.1±30.1
d100	22.9± 4.6	151.6±122.3	272.6± 91.8	64.5±17.7
d130-135	71.0±14.4	75.8± 15.6	262.1±102.7	102.3±22.5
Term	49.6±17.6	54.6± 16.3	26.4± 8.6**	16.0± 8.2**

* All values are mean±SEM for 3-10 observations

** Mean is significantly different than those at earlier gestational ages (P values described in text).

TABLE 6. The ratio of progesterone to estradiol-17β in intrauterine tissues of sheep during ACTH-induced labour compared to saline controls

Treatment	Amnion	Chorion	Endometrium	Myometrium
Saline	32.3±18.9	185.0±130.2	148.6±107.1	102.3±22.5
ACTH	18.1± 9.9*	24.2± 6.5	74.1± 44.7	17.5± 4.8**

* All values are mean±SEM for 3-8 observations

** Mean is significantly different than that of saline treated animals at $P<0.005$.

(both P<0.005) day 100 (P<0.005 and P<0.05) and day 50-60 (P<0.01 and P<0.005, respectively) in the ENDO and MYO. There was no significant difference in the ratio of P4:E2β in AMN, CHOR, or ENDO between tissues collected after saline treatment or during ACTH administration (Table 6). However, there was a significant decrease in the P4:E2β ratio in MYO collected at the time of ACTH-induced labour, compared to tissue collected from saline treated control animals.

6.4 Discussion

I have described patterns of changes for the concentrations of E1 and E2 throughout pregnancy in the AMN, CHOR, ENDO, and MYO of pregnant sheep. The concentrations of E1 and E2β were elevated in the MYO of animals in spontaneous labour at term and in the MYO of animals in premature labour induced by the intrafetal administration of ACTH in late pregnancy. These findings confirm and extend those of Rawlings and Ward (1976). The [E1] was elevated in the ENDO of animals in spontaneous labour, but not in the animals in premature labour. The mean [E2β] was elevated in the ENDO of animals in spontaneous labour although this change was not statistically significant. In addition, the [E2β] in the ENDO of animals in premature labour were similar to those of the saline controls. The ENDO and MYO are exposed to the sharp rise in the concentrations of E1 and E2β in the maternal peripheral plasma during the last few days of pregnancy (see literature review); however, only in the MYO do the levels change as markedly as they do in the maternal plasma. The declining P4 levels (see literature review) and the rising estrogen levels in the maternal plasma in late pregnancy coexist with a marked

decline in the P4:E2 ratio in both the ENDO and MYO. In the MYO the decline resulted from a rise in the [E2β], whereas in the ENDO a fall in the [P4] was responsible.

In late pregnancy, the ratio of E1:E2 in the MYO and ENDO is five to ten fold lower than the values reported for this ratio in the maternal plasma (Challis and Patrick, 1981). These findings suggest that factors other than the circulatory concentrations of E1 and E2β influence the concentration of Es in the ENDO and MYO. Rossier and Pierrepoint (1974a) provided evidence for the presence of 17α- and 17β-hydroxysteroid dehydrogenase and estrogen sulphotransferase and sulphatase activity in the ENDO of pregnant sheep. These findings raise the possibility that the MYO and ENDO can influence the local levels of specific estrogens during pregnancy.

The concentrations of Es in the AMN on day 50-60 were extremely variable. At this time, the concentration of conjugated and unconjugated Es in the AMF and ALF are falling from very high values (Carnegie and Robertson, 1978). Measurements made at various stages of a similar decline in the AMN may have accounted for this variability. The mean concentrations of E1 and E2β in the AMN rose between day 130-135 and term but only the change in the [E2β] was significant. Challis and Patrick (1981) found similar changes in the AMF during the last few weeks of pregnancy.

The concentrations of Es in the CHOR were independent of changes in the umbilical venous plasma (Challis and Patrick, 1981) and fetal fluids (Carnegie and Robertson, 1978; Challis and Patrick, 1981) and did not change significantly between day 130-135 and term. As was found for P4, the concentrations of E1 and E2β in the CHOR appear to be influenced by factors other than the concentrations in the fetal

circulation and fetal fluids. The concentrations of Es in the CHOR were also substantially higher than those in the ENDO and MYO. The predominant estrogen in the fetal fluids and fetal plasma is E1-S (see literature review) which may be available to the fetal membranes for further metabolism to E1 and E2β. These enzymatic capabilities were investigated in the fetal membranes as well as their ability to produce estrogens from C19 precursors. The results are described in a later chapter and compared to those in the ENDO and MYO.

CHAPTER 7

THE PRODUCTION OF PROGESTERONE BY SUBCELLULAR PREPARATIONS OF FETAL MEMBRANES

7.1 Introduction

The source of progesterone (P4) in the amniotic fluid (AMF) and allantoic fluid (ALF) is not known. Gibb et al (1978) have shown that homogenates of amnion and chorion obtained from women at term pregnancy possess 3β-hydroxysteroid dehydrogenase, Δ5-4 isomerase (3β-HSD) activity, and could convert pregnenolone (P5) to P4. This finding raises the possibility that the fetal membranes contribute to the P4 measured in AMF (Johansson and Johansson, 1971).

I have modified the assay system of Gibb et al (1978) in order to demonstrate that the chorion (CHOR) and amnion (AMN) of sheep possess 3β-HSD activity, to compare this activity in CHOR, AMN and placenta of sheep at different times during pregnancy, and to assess the relation with concentrations of P4 in the fetal fluids. I then determined the optimum conditions of 3β-HSD assay in CHOR and examined the subcellular distribution and changes in enzyme activity between early and late pregnancy. Finally, I determined the possible regulatory influences of other steroids on the conversion of P5 to P4.

7.2 Materials and Methods

7.2.1 Animals

Twenty-three sheep of known gestational age were used in this study. Three gestational age groups were studied: day 50 (nine animals), day 100 (six animals) and spontaneous labour at term (143-147, eight animals).

7.2.2 Tissue Preparation

Tissues were thawed on ice and homogenized in 0.05 M potassium phosphate buffer, pH 7.4 containing 0.25 M sucrose and 0.7 mM β-mercaptoethanol. Homogenates were centrifuged at 800g for 20 min. The 800g supernatants of several homogenates were centrifuged at 10,000g for 15 min. The 10,000g supernatant fractions were centrifuged at 105,000g for 90 min. The 10,000g and 105,000g pellets were resuspended in phosphate buffer (as above).

The fetal tissue in the cotyledons was separated manually from maternal tissue. Although I refer to maternal and fetal cotyledons throughout this chapter, I recognize that one is unlikely to obtain clean separation of these tissues. Furthermore, migration of fetal binucleate cells to the maternal syncytial layer (Wooding, 1980) renders my terminology somewhat arbitrary.

7.2.3 In Vitro Incubations

7- [^{3}H] pregnenolone (^{3}H-P5; specific activity 10-25 Ci/mmole) was purified by thin layer chromatography (TLC) in chloroform: diethyl ether (10:3, v/v; solvent system A). The subcellular fractions were incubated with 0.5-1.0 μCi ^{3}H-P5 with known concentrations of nonradioactive P5 in phosphate buffer (pH 5-9) or carbonate buffer (pH 8-11) with 1mM oxidized nicotinamide adenine

dinucleotide (NAD+). Incubations were performed at 37°C and terminated by the addition of 1-3 volumes of diethyl ether.

4-[14C]-progesterone (^{14}C-P4; 2500 DPM; specific activity 50-60 mCi/mmole), purified in system A, was added to each sample for assessment of recovery. Unconjugated steroids were extracted with diethyl ether. The organic phase was dried and the radioactive P4 was separated by TLC in system A using the ultraviolet absorption at 254nm to locate 20ug P4 which was added to each sample. After TLC in system A, P4 (RF, 0.65) was well separated from P5 (RF, 0.50), 17α-OH-pregnenolone (RF, 0.32), 17α-OH-progesterone (RF, 0.40) and 20α-dihydroprogesterone (RF, 0.40). The P4 band was eluted with 2ml ethyl acetate, dried, and acetylated overnight with 200 ul pyridine-acetic anhydride (1:1, v/v). After 18h at 22°C the excess solvent was evaporated. P4 was separated from the acetylated products by TLC in system A and eluted in 2ml ethyl acetate. The eluate was transferred directly to scintillation vials, dissolved in Scinti-Verse and radioactivity levels were determined in a liquid scintillation counter using a double-isotope program which gave efficiencies of 34% for ^{3}H and 61% for ^{14}C.

7.2.4 3β-HSD Assay Validation

For several samples of CHOR and AMN, the material cochromatographing with P4 in system A following acetylation was eluted with ethyl acetate, dried, and redissolved in 200 ul ethanol. An aliquot (50ul) was taken for liquid scintillation counting to determine the ^{3}H to ^{14}C ratio. Recrystallized P4 (15mg) was added to the samples, and P4 was recrystallized in the solvent systems indicated in Table

7. The ^{3}H to ^{14}C ratios in the crystals and mother liquors were determined.

7.2.5 Radioimmunoassay

P4 in the AMF was measured by radioimmunoassay as described in Chapter 4.

Unconjugated steroids in the 800g supernatant fractions were extracted with 5vol diethyl ether. The organic base was dried, redissolved in 1ml isooctane and applied to micro-celite columns (Abraham, Tulchinsky and Korennan, 1970). P4 was eluted in the first 2ml isooctane, P5 was eluted with 2 further ml isooctane; DHEA, E1 and E2 were eluted with 3.5ml 5%, 15% and 30% ethyl acetate, respectively. P4 (see Chapter 4), P5 (Inaba, Wiest and Niswender, 1979) DHEA (Challis, Manning, Martin, Murata and Socol, 1980) E1 and E2 (see chapter 6) in the respective eluates were measured by radioimmunoassay. Within-assay coefficients of variation were <12%.

In preliminary experiments we established that the endogenous concentration of P5, P4, DHEA, E1 and E2 in the 800g supernatant fractions were less than 1% of the substrate concentrations used in subsequent incubations.

7.2.6 Protein Analysis

Protein concentrations were measured by the method of Lowry et al (1951) using bovine serum albumin as standard.

7.2.7 Statistical Analysis

All results are presented as means ± SEM. The statistical tests used to examine difference between means are indicated in the text.

7.3 Results

7.3.1 Activity of 3β-HSD in 800g supernatants

Homogenates (800g supernatants) of CHOR, AMN and maternal and fetal cotyledons converted ^{3}H-P5 to ^{3}H-P4 at all times studied. Figures 17 and 18 show representative chromatograms of radioactive material (^{3}H and ^{14}C in 1cm bands of silica gel from the origin to the solvent front) extracted from incubations with AMN and CHOR (term; fresh and boiled) and chromatographed in system A. Similar profiles were obtained with all tissues at all times studied. Products cochromatographing with P5 and P4 only were detected in the system used. The activity was reduced by greater than 95% after boiling. To substantiate the radiothemical purity of the ^{3}H-P4 formed from ^{3}H-P5 by fetal membranes, I determined the ^{3}H:^{14}C ratios in the eluate of P4 after acetylation and in the recrystallizations in the solvent pairs indicated in Table 7. Good agreement was found between ^{3}H to 14C ratios after acetylation and in the crystals and mother liquors of repeated recrystallizations. For most samples the coefficient of variation around the mean ^{3}H to ^{14}C ratio of the postacetylation sample (successive crystals and mother liquors) ranged between 3.0 - 8.0%. The coefficients of variation for five repeat counts on the same sample were 2.8% (high recovery) and 8.8% (low recovery). The higher coefficients of variation for the samples from animal 2 in series B were attributed to low ^{3}H levels.

Conversions of P5 to P4 of less than 10% of the initial substrate levels were linear with respect to incubation duration (Fig. 19a) and protein concentrations (Fig. 19b) in the 800g supernatant fractions.

Activities in the four tissues at the three gestational ages were analysed using one-way analysis of variance. Data were transformed to logarithmic values to remove heterogeneity of variance.

FIGURE 17. Thin layer chromatograms of radioactivity after incubations of amnion homogenates with [^{3}H[-pregnenolone. Amnion was from a sheep at term. Authentic ^{14}C-progesterone was added prior to chromatography. Homogenates were either fresh (a) or boiled (b).

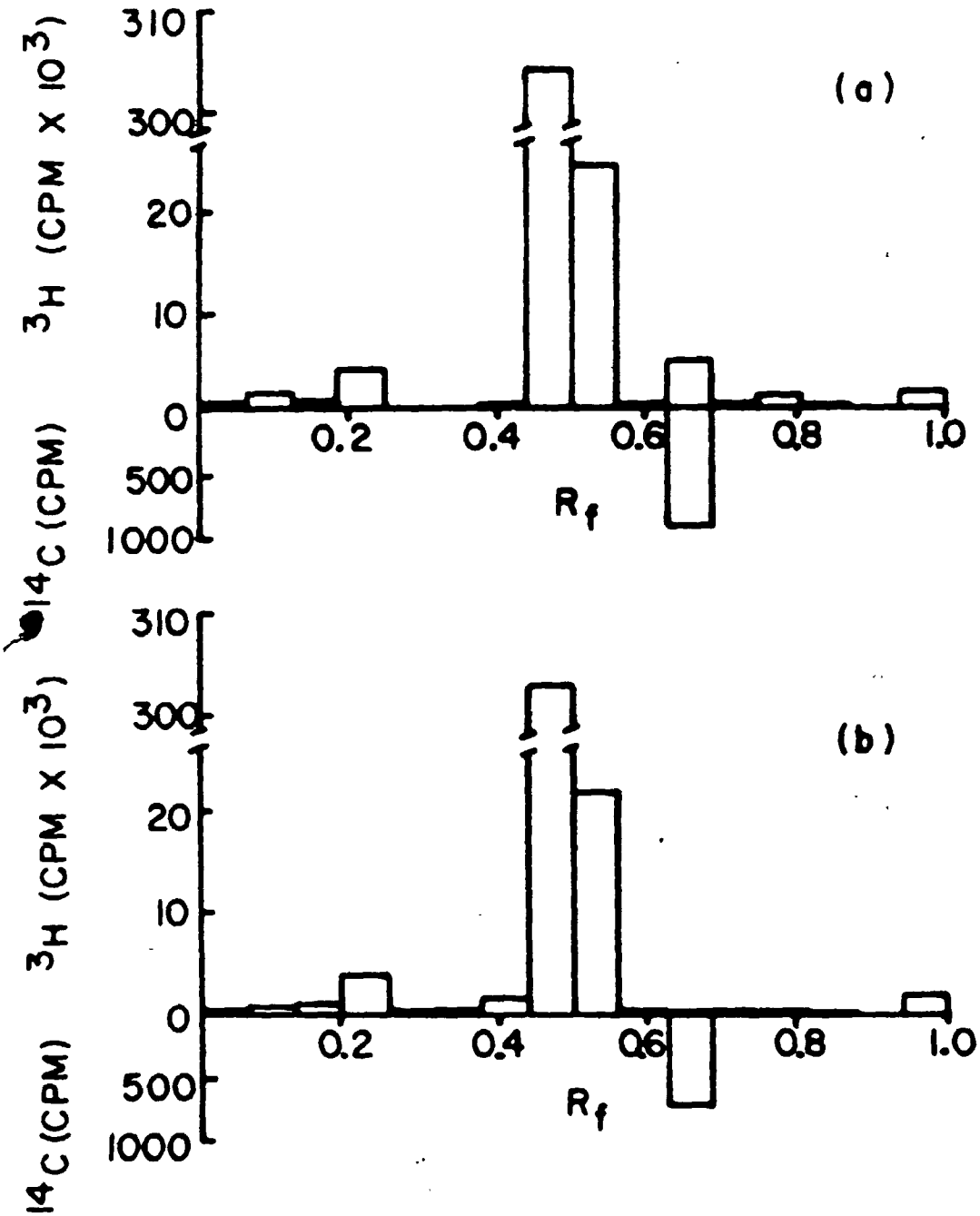

(a)
(b)
^3H (CPM X 10^3)
^{14}C (CPM)
310
300
20
10
0
500
1000
0.2
0.4
0.6
0.8
1.0
R_f

FIGURE 18. Thin layer chromatograms of radioactivity after incubations of chorion homogenates with [^{3}H]-pregnenolone. Chorion was from a sheep at term. Authentic ^{14}C-progesterone was added prior to chromatography. Homogenates were either fresh (a) or boiled (b).

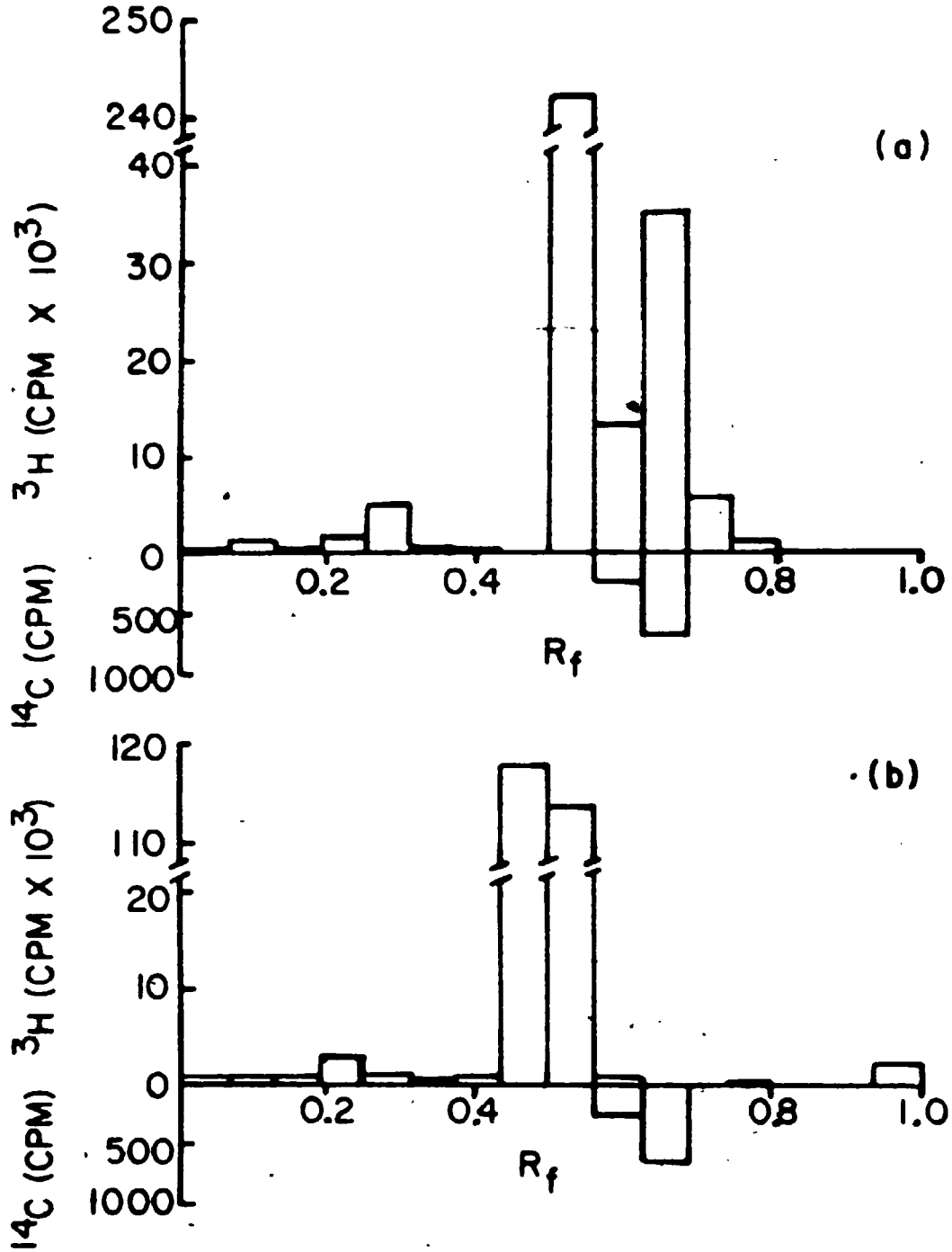
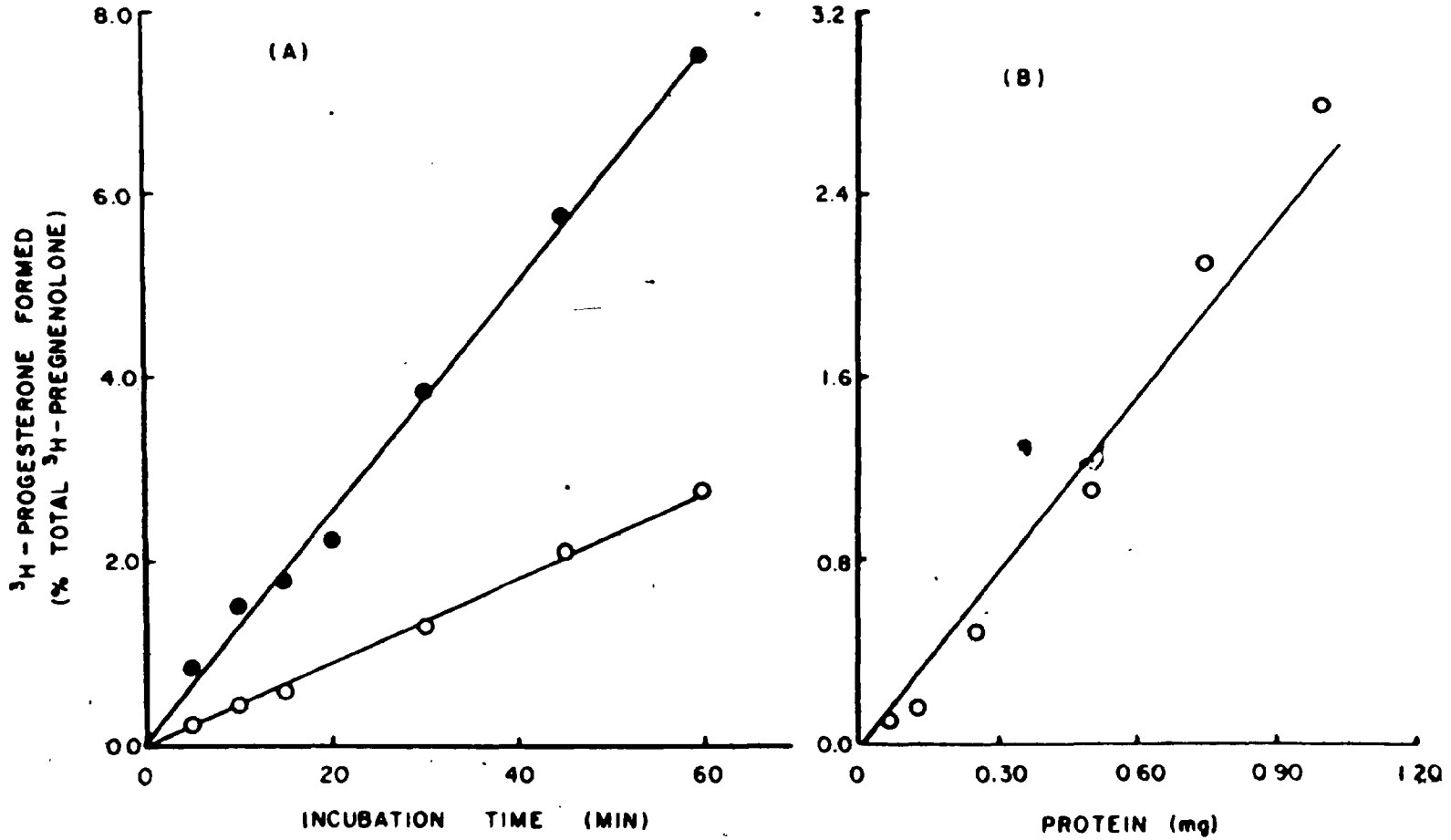

250
240
40
30
20
10
0
500
1000
^{3}H (CPM X 10^{3})
^{14}C (CPM)
0.2
0.4
0.8
1.0
R_f
(a)
120
110
20
10
0
500
1000
^{3}H (CPM X 10^{3})
^{14}C (CPM)
0.2
0.4
0.8
1.0
R_f
(b)

TABLE 7. Assessment of radiochemical purity: progesterone ^{3}H to ^{14}C ratios in successive recrystallizations

Sheep No.	Sample	^{3}H : ^{14}C ratio								
		Post acetylation	Recrystallization						Mean	COV
			X_1	ML_1	X_2	ML_2	X_3	ML_3		
Series A										
1	chorion	10.41	10.61	9.90	10.26	10.91			10.42	3.62
2	chorion	1.14	1.37	1.36	1.29	1.39			1.31	7.80
1	amnion	0.86	0.84	0.92	0.88	0.90			0.88	3.59
3	amnion	0.34	0.35	0.30	0.35	0.31			0.33	7.10
Series B										
1	chorion	13.85	15.23	12.98	14.63	14.12	15.51	15.08	14.49	6.20
2	chorion	1.31	1.28	1.35	1.48	1.21	1.47	1.82	1.41	14.31
1	amnion	1.05	1.06	1.05	0.99	1.15	1.07	1.15	1.07	5.37
2	amnion	0.25	0.28	0.41	0.26	0.30	0.33	0.38	0.32	19.25
3	amnion	0.43	0.47	0.45	0.46	0.49	0.56	0.59	0.49	12.10

Incubations were performed on the day of homogenization (Series A) or after 3 days at 4°C (Series B). ^{3}H:^{14}C ratios in samples of amnion and chorion from sheep are shown after the postacetylation TLC and in crystals (X) and mother liquors (ML) of successive recrystallizations. In Series A, there were two recrystallizations, both from ethanol water. In Series B samples, three recrystallizations from, successively, methanol-water, ethanol-water, and acetone-water were performed. The mean and coefficient of variation (COV) for five (Series A) or seven (Series B) ^{3}H to ^{14}C ratios are shown.

FIGURE 19. [^{3}H]-progesterone formed from incubations of chorion homogenates with [^{3}H]-pregnenolone. Incubations varied with respect to time (a) and protein concentration (b). Values are expressed as a percentage of total ^{3}H-pregnenolone. Open and closed symbols represent values from different animals.


(A)
8.0
6.0
4.0
2.0
0.0
0
20
40
60
INCUBATION TIME (MIN)
^{3}H - PROGESTERONE FORMED
(% TOTAL ^{3}H - PREGNENOLONE)
(B)
3.2
2.4
1.6
0.8
0.0
0
0.30
0 60
0 90
1 20
PROTEIN (mg)

Activity of 3β-HSD was dependent upon the gestational age of the tissue (Fig. 20). Comparisons between individual group means were made using Duncan's multiple range test. Activity in the fetal cotyledons at day 50 was significantly ($P<0.01$) greater than at day 100 or term. Activity in the chorion at term was significantly ($P<0.05$) greater than at days 50 and day 100. Activity in the amnion at day 100 was significantly ($P<0.01$) lower than at day 50 and term. At day 50, the mean activity in the fetal cotyledons was greater than that in the other three tissues. The mean activity in the amnion was lower than that in the other three tissues at the three times studied. At term the mean activity in the CHOR was similar to that in the fetal cotyledons.

7.3.2 Activity of 3β-HSD in Subcellular Preparations of Chorion

Incubations were performed at optimum pH 9.6 (Fig. 21). Conversions of less than 10% initial substrate were linearly dependent on the incubation time and concentration of protein (Fig. 22, a and b respectively). Activities in the 800g supernatant fraction, 10,000g pellet, 105,000g pellet and 105,000g supernatant fraction of chorion were compared on day 50 and term (Fig. 23). At both gestational ages, the specific activity was highest in the 10,000g pellet and lowest in the 105,000g supernatant. The specific activity in the 105,000g pellet was higher than that in the 800g pellet and 105,000g supernatant.

Preparations of 10,000g pellets of chorion at day 50 and term were incubated with several concentrations of pregnenolone. Eadie-Hofstee plots (Hoftsee, 1959) were drawn to determine Km and Vmax of 3β-HSD activity (slope and y intercept of regression line

FIGURE 20. Activity of 3β-hydroxysteroid dehydrogenase in 800g supernatent fractions of maternal cotyledons, fetal cotyledons, chorion and amnion obtained from pregnant sheep at D50, D100 and term. Incubations were performed at pH 7.4 with 1μg pregnenolone. Each bar represents mean ± SEM for 4-8 samples..

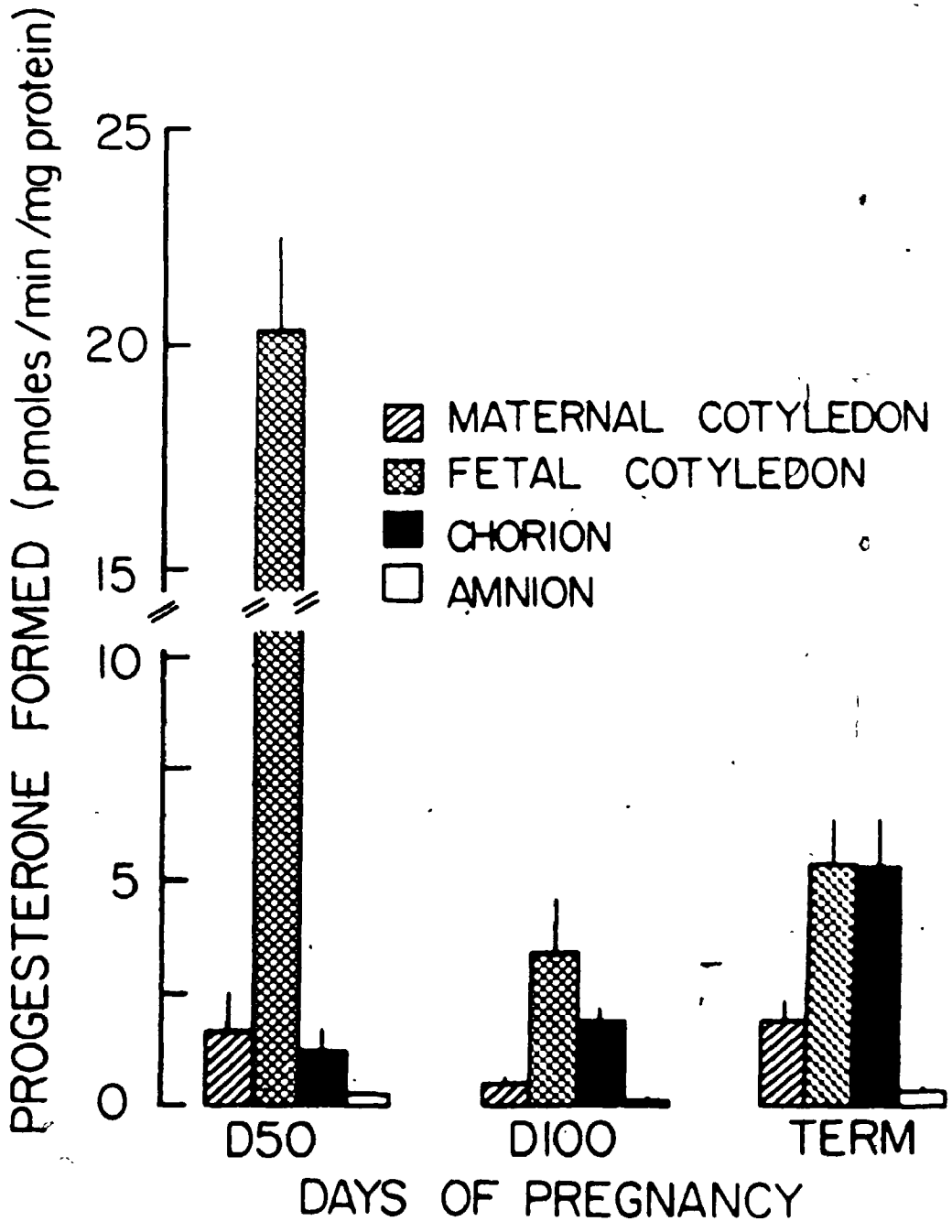

PROGESTERONE FORMED (pmoles/min/mg protein)
25
20
15
10
5
0
MATERNAL COTYLEDON
FETAL COTYLEDON
CHORION
AMNION
D50
D100
TERM
DAYS OF PREGNANCY

FIGURE 21. Activity of 3β-hydroxysteroid dehydrogenase in 105,000 g pellets of a representative sample of chorion incubated at different pH values in phosphate buffer (0.05 M; ○) and carbonate buffer 0.05 M (●). Incubations were performed with 100ng/L pregnenolone.

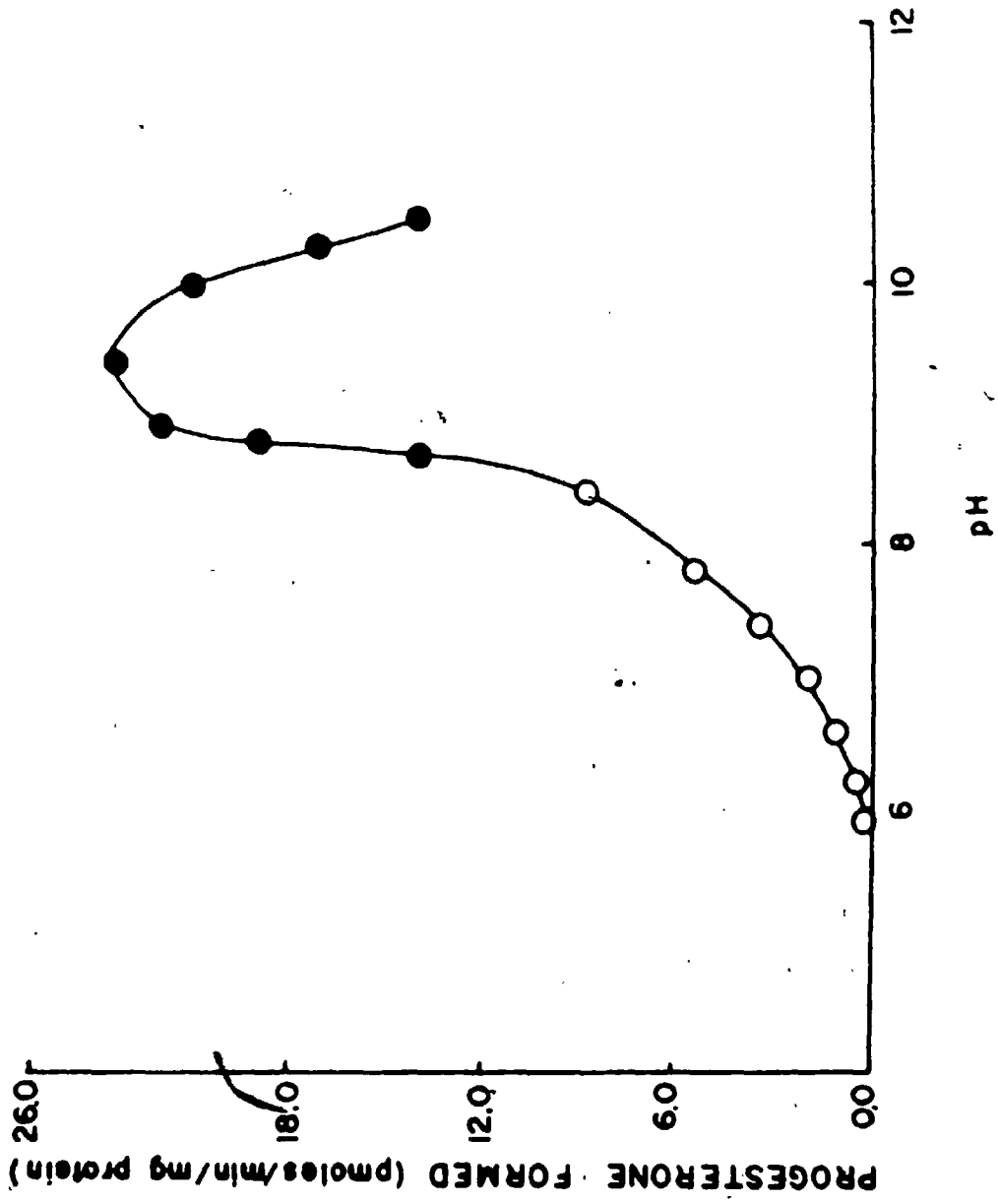

PROGESTERONE FORMED (pmoles/min/mg protein)
26.0
18.0
12.0
6.0
0.0
6
8
10
12
pH

FIGURE 22. [^{3}H]-progesterone formed from incubations of chorion 105,000 g pellet with [^{3}H]-pregnenolone. Incubations varied with respect to time (A) and protein concentration (B). Values are expressed as a percentage of total ^{3}H-pregnenolone. Open and closed symbols represent values from different animals.

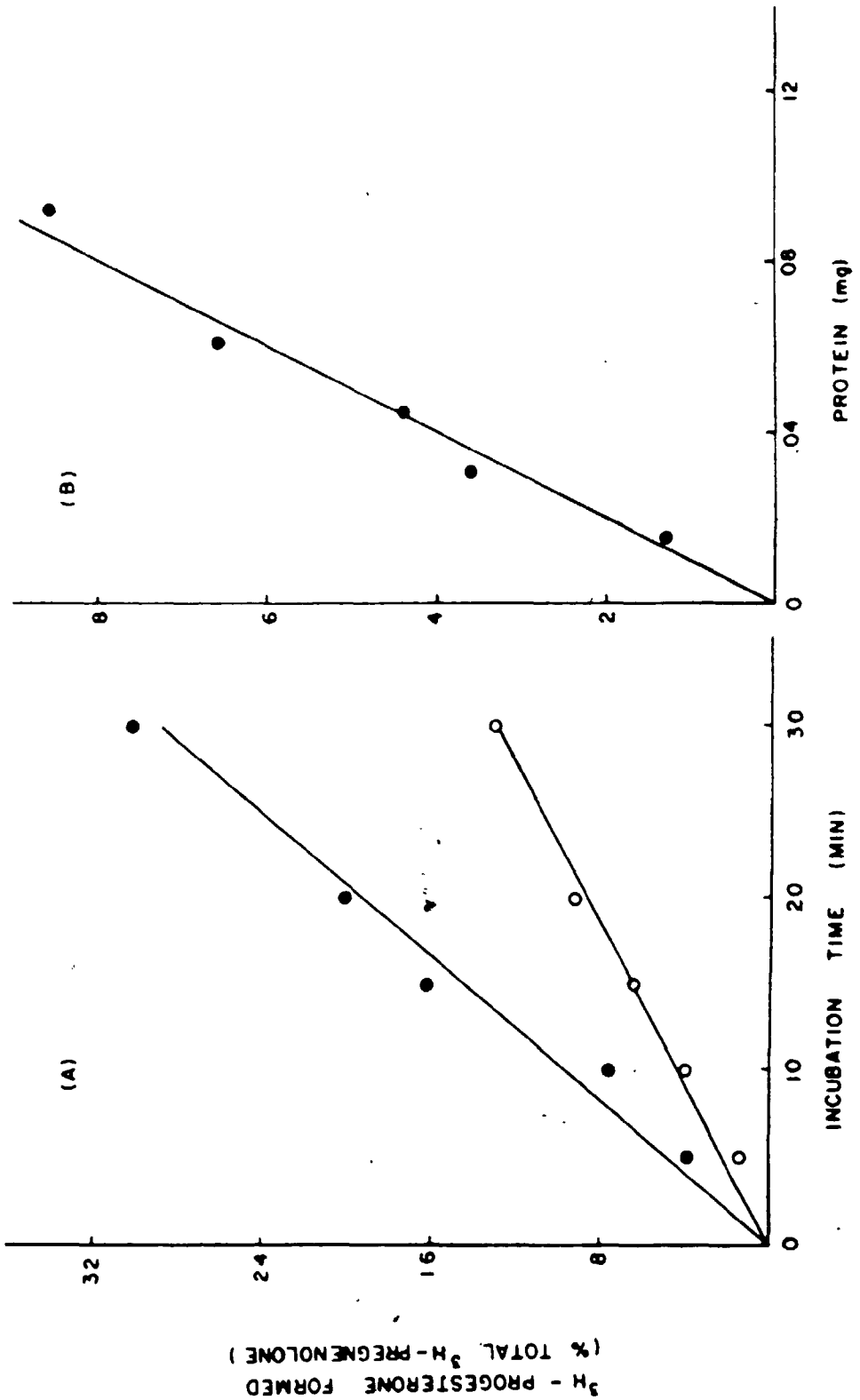
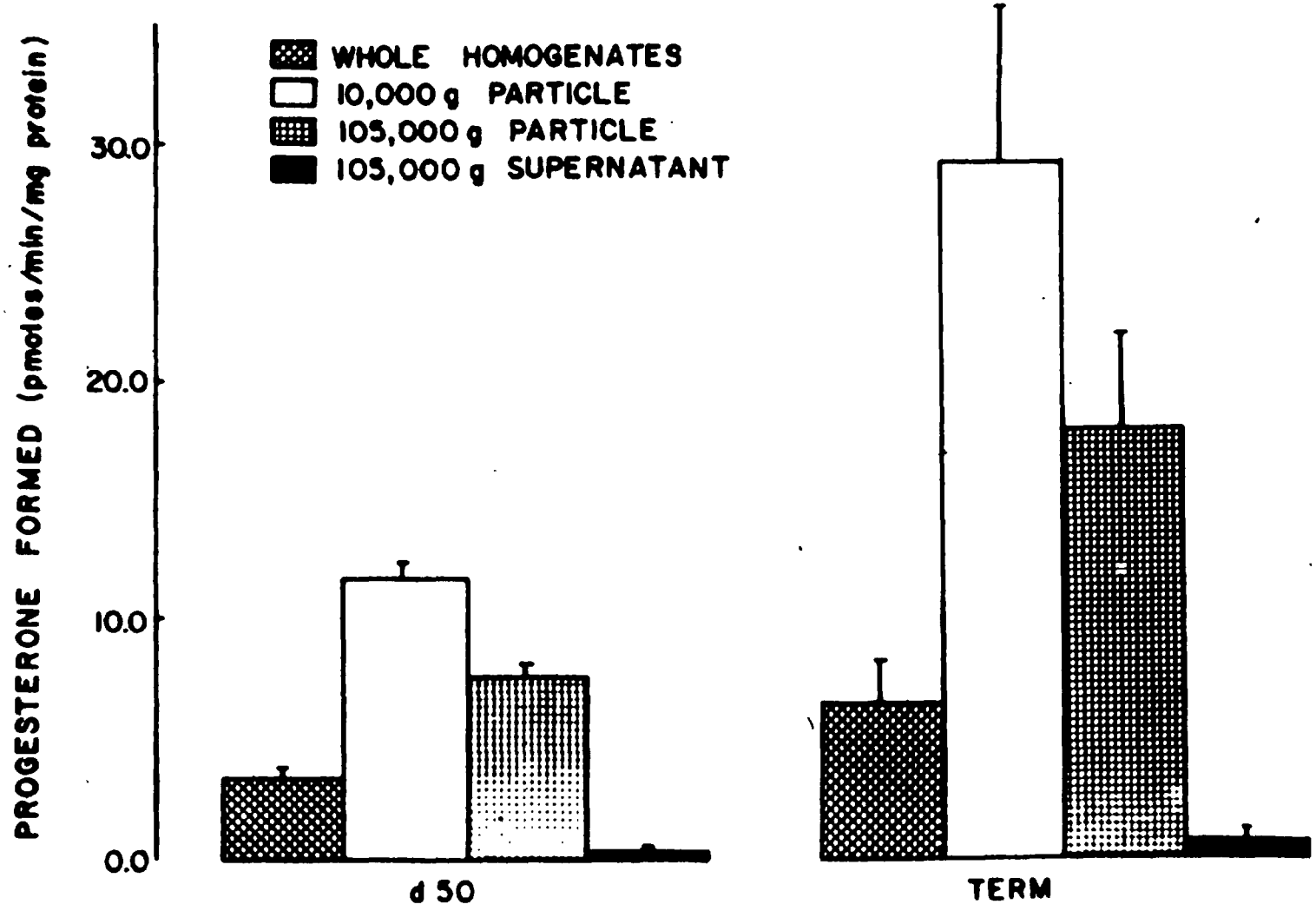

(A)
(B)
INCUBATION TIME (MIN)
PROTEIN (mg)
^3H - PROGESTERONE FORMED
(% TOTAL ^3H-PREGNENOLONE)

FIGURE 23. Activity of 3β-hydroxysteroid dehydrogenase (mean ± SEM; n=3-4) in subcellular fractions of chorion obtained from sheep at day 50 and term in pregnancy. Incubations were performed at pH 9.6 with 100ng/L pregnenolone.


WHOLE HOMOGENATES
10,000 g PARTICLE
105,000 g PARTICLE
105,000 g SUPERNATANT
PROGESTERONE FORMED (pmoles/min/mg protein)
30.0
20.0
10.0
0.0
d 50
TERM

respectively; see Fig. 24). The mean (±SEM) Km and Vmax at day 50 (n=6) were 56.8 ± 5.5 nM and 14.1 ± 3.1 pmole/min per mg protein respectively. The mean (±SEM) Km and Vmax at term (n=4) were 39.0 ± 8.7 nM and 62.7 ± 11.4 pmole/min per mg protein respectively. The Km values were not significantly different ($P>0.05$; student's t test); however, the Vmax at term was significantly ($P<0.01$) greater than that at day 50.

Preparations of 105,000g pellets of chorion at term were incubated with equimolar concentrations of P5 and one of several steroids. Some of the steroids inhibited the conversion of P5 to P4 (Fig. 25). E1 and DHEA were competitive inhibitors of this conversion. This was indicated by the lack of intersection of the plots (not shown) of the [P5]/pmoles P4 formed /min/mg protein vs the concentration of inhibitor (Dixon, 1953). Dixon plots (Dixon, 1953) were drawn to determine the inhibition constant, Ki. Figure 26 shows representative examples of Dixon plots for inhibition by E1 and DHEA. The mean Ki for inhibition by DHEA was 40.2 ± 14.7 nM (n=3). The mean Ki for inhibition by E1 was 19.6 ± 5.9 nM (n=3).

7.3.3 Concentration of Progesterone in Amniotic Fluid (AMF)

The mean (±SEM) concentration of P4 in AMF at day 50 (n=3), day 100 (n=4) and term (n=4) were 0.36 ± 0.11, 1.15 ± 0.20 ($P<0.01$ vs day 50) and 4.33 ± 1.69 nM ($P<0.01$ vs days 50 and 100; Duncan's multiple range test) respectively.

7.4 Discussion

I have demonstrated that the chorion and amnion obtained from sheep at day 50, day 100, and term possess 3β-HSD activity and are able to convert P5 to P4. The activity in the chorion was greater

FIGURE 24. Representative examples of Eadie Hofstee plots (Hofstee, 1959) of 3β-hydroxysteroid dehydrogenase activity in 10,000 g pellets of chorion obtained at day 50 and term. Incubations were performed at pH 9.6 with 30-1060 mmole pregnenolone/L. Y axis, V=mol progesterone formed x 10^{-12}/min per mg protein; x axis, V/pregnenolone concentration expressed as litres x10^{-4}/min per mg protein. Vmax = Y intercept =17.8x10^{-12} (day 50) and 31.4 x10^{-12} (term) mol progesterone formed/min per mg protein; Km = - slope = 49.7x10^{-9} (day 50) and 56.6x10^{-9} (term) mol/L.

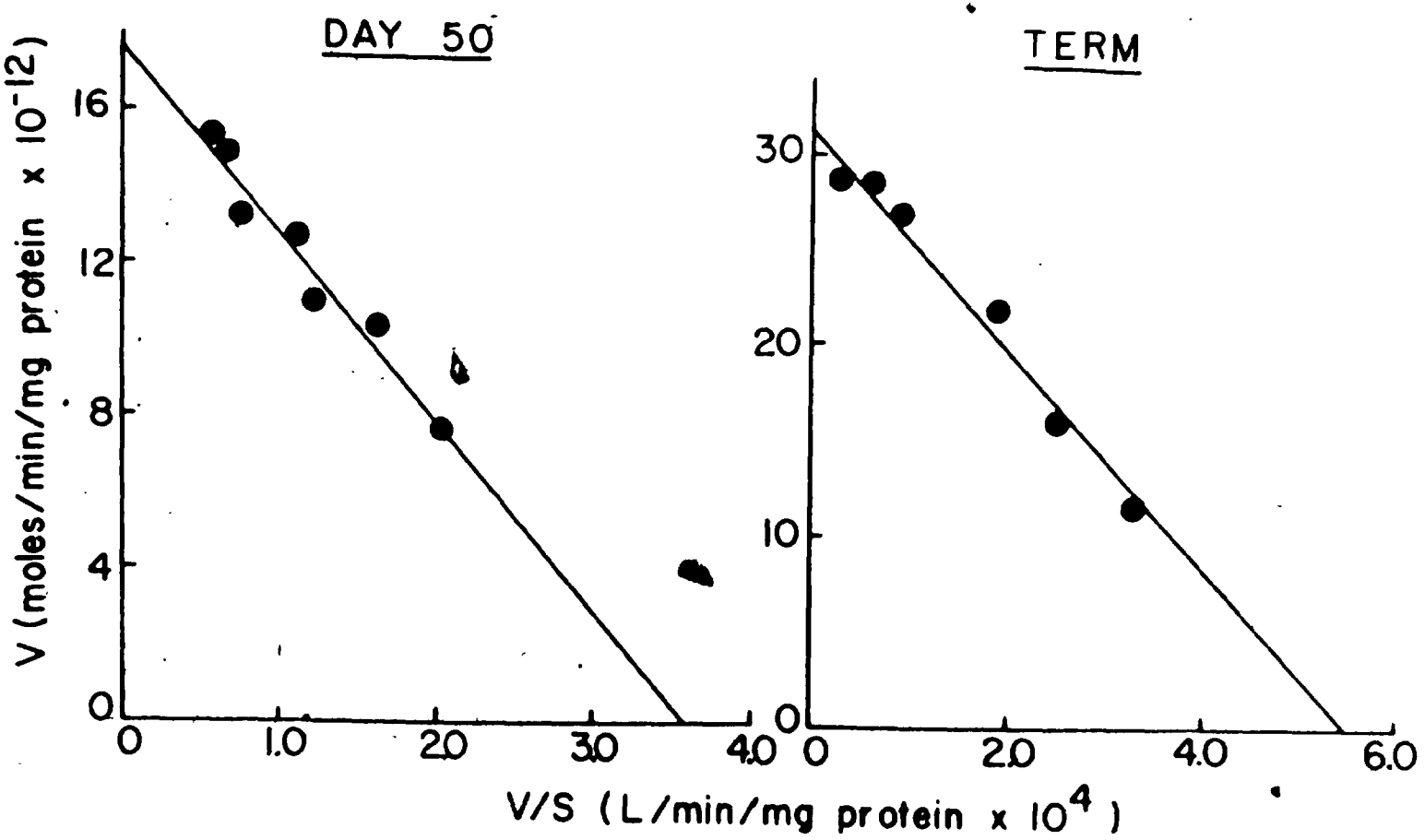

DAY 50
TERM
V (moles/min/mg protein x 10^{-12})
16
12
8
4
0
1.0
2.0
3.0
4.0
30
20
10
0
2.0
4.0
6.0
V/S (L/min/mg protein x 10^4)

FIGURE 25. Conversions of pregnenolone (Preg; P5) to progesterone (Prog; P4) by 105,000 g pellets of sheep chorion. Incubations were performed with equimolar concentrations (100nmol/L) of one of several C21, C19 and C18 steroids including P4, corticosterone (B), cortisol (F), dehydroepiandrosterone (DHA), testosterone (T), dihydrotestosterone (5αT), estrone (E1), estradiol-17β (E2) and estriol (E3). Results are expressed as a percentage of activity in the presence of substrate alone (mean ± SEM; n=4). one star = $P<0.05$, two stars=$P<0.01$ compared with pregnenolone (paired t-tests).

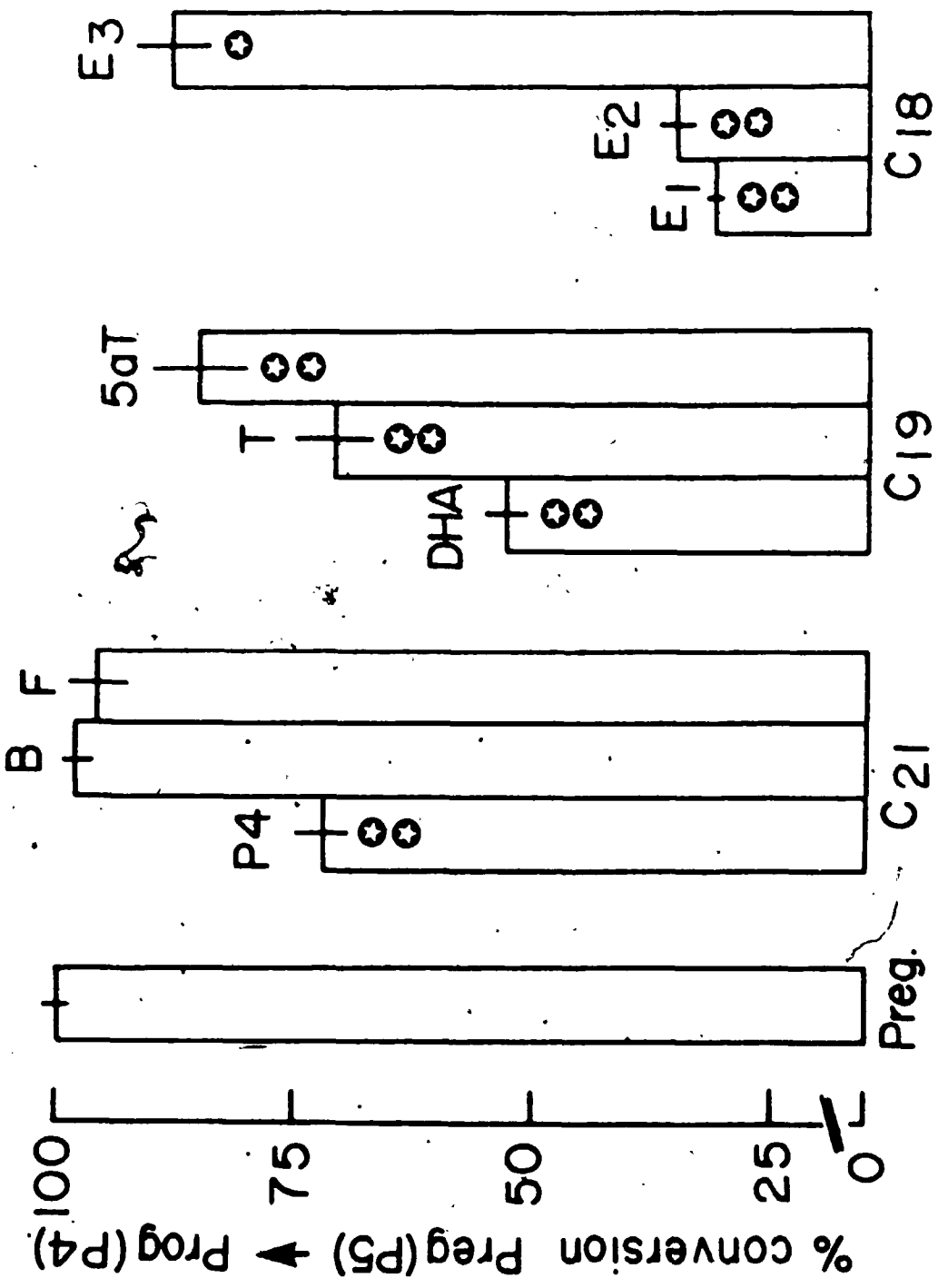
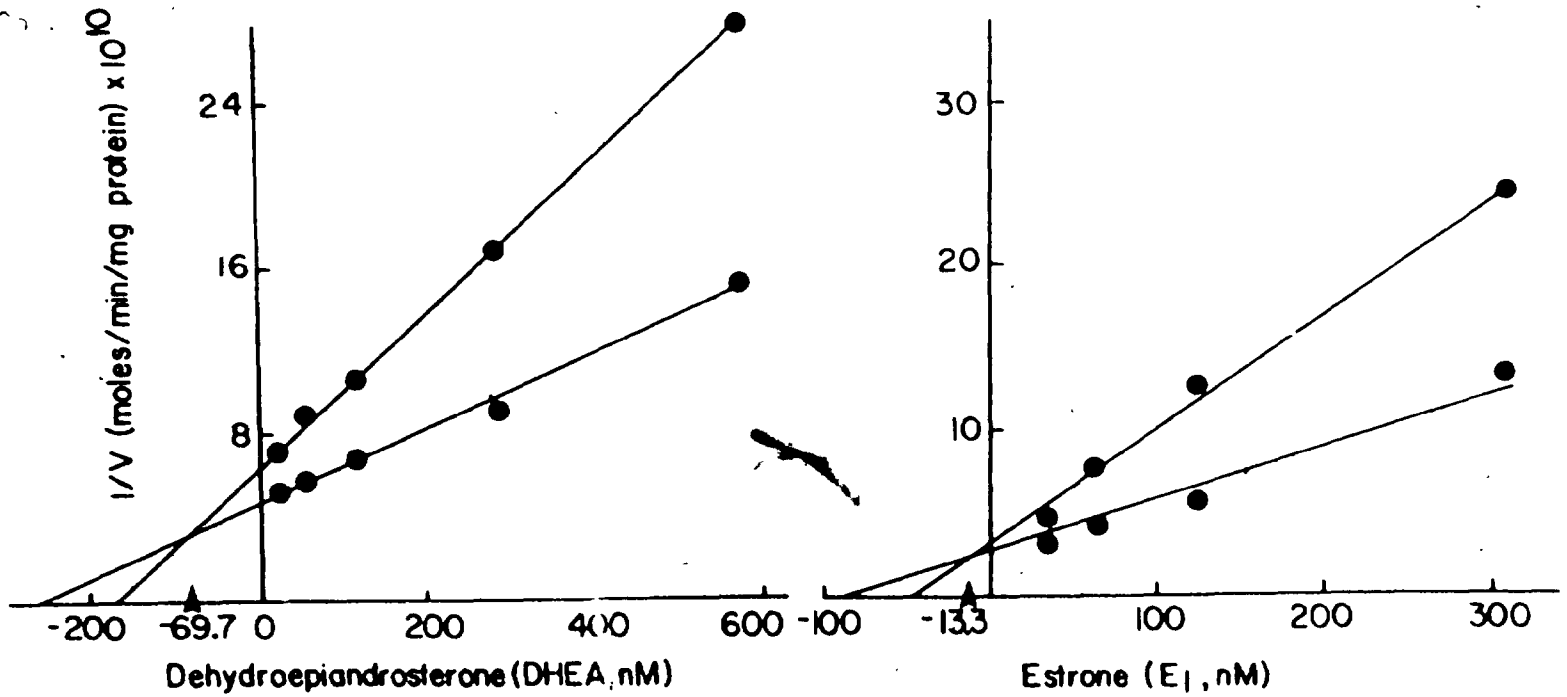

% conversion Preg(P5) → Prog(P4)
100
75
50
25
0
Preg.
C21
P4
B
F
C19
DHA
T
5αT
C18
E1
E2
E3

FIGURE 26. Representative examples of Dixon plots (Dixon, 1953) of dehydroepiandrosterone (left panel:DHEA) and estrone (right panel; E1) inhibition of the pregnenolone to progesterone conversion in 105,000g pellets (DHEA) and 10,000g pellets (E1) of chorion obtained from pregnant sheep at term. Incubations were performed at pH 9.6 with 105 and 263 mmol pregnenolone/L. V=mol progesterone formed $x10^{12}$/min per mg protein; K_i=69.7.7 mmol/L (DHEA) and 13.3 mmol/L (E1).


1/V (moles/min/mg protein) x 10^10
24
16
8
-200
-69.7
0
200
400
600
Dehydroepiandrosterone (DHEA, nM)
30
20
10
-100
-133
100
200
300
Estrone (E1, nM)

than that in the amnion at each of the three gestational ages. Gibb et al, (1978) have shown that human chorion and, to a lesser extent, amnion, obtained from women at term possess 3β-HSD. These findings raise the possibility that the fetal membranes are a source of P4 throughout much of pregnancy.

The 3β-HSD activity in the chorion was greater at term than at days 50 and 100. This increase in 3β-HSD activity occurs at a time when concentrations of P4 in the AMF and ALF are rising. Although transport of P4 across fetal membranes has not been investigated, it is possible that P4 produced by the chorion could reach the AMF and ALF. In the placenta cholesterol side-chain cleavage enzyme (CSCCE) is probably rate limiting to C21 steroid production (Anderson et al, 1975). It is not known whether CSCCE is rate-limiting in the fetal membranes or whether an increase in 3β-HSD activity alone could effect a rise in the levels of P4 in the AMF and ALF. In addition, there may be changes in the metabolism and clearance from the fetal fluids. Milewich, Gant, Schwarz, Chewn and MacDonald (1977) have reported a ten- twentyfold decrease in 20α-hydroxysteroid oxidoreductase (20α-HSD) activity and a two-sixfold decrease in 5α-reductase activity in human amnion and chorion laeve after 33 weeks gestation. Similar decreases in P4 metabolism might occur in the sheep to effect a rise in the levels of P4 in the fetal fluids. In subsequent chapters the production and metabolism of P4 by dispersed cells of fetal membranes of different gestational ages were investigated.

My initial attempts to investigate the relative importance of 3β-HSD activity in the fetal membranes were made by comparison to activity in the sheep placenta. Several investigators have described the placenta as the main source of P4 in the fetal and maternal

plasma after day 50 of pregnancy (Linzell and Heap, 1968; Ricketts and Flint, 1980). Anderson et al (1975) have reported that the fetal component of the cotyledon is the main site of production in the placenta. In my experiments, 3β-HSD activity in the fetal cotyledon was much greater than that in the maternal cotyledon; however, we recognize that there is probably some contamination of our cotyledon preparations after manual separation of fetal and maternal components (Wooding, 1980). Similar levels of 3β-HSD activity were measured in the fetal cotyledons and chorion at day 100 and term suggesting that the fetal membranes might be as important a site of progesterone synthesis as the placenta at these times. The decrease in 3β-HSD activity in the cotyledons between day 50 and day 100 was striking, though unexplained at the present time.

The sheep placenta contains 20αHSD activity, 5α-reductase and 3α-HSD (Ainsworth and Ryan, 1967). At term, or after dexamethasone treatment, placental 17α-hydroxylase is increased (Anderson et al, 1975). However, we did not detect radioactivity on the thin layer chromatograms other than that corresponding to P5 or P4 regardless of tissue and gestational age. It seems likely that our short incubation times did not allow P4 to accumulate and may explain the lack of 3α-, 17α-or 20α-products. As well, the predominance of NAD+ may have shifted the equilibrium in favour of dehydrogenated products.

In my experiments, the subcellular localization of 3β-HSD was particulate in nature and is similar to that found in the human placenta (Koide & Torres, 1965) and human fetal membranes (Gibb et al, 1978). The optimum pH 9.6 of 3β-HSD activity was similar to that reported by Ferre, Breviller, Cedard, Duchesne, Saintot, Descomps and

Crastes de Paulet (1975) using similar buffers to investigate 3β-HSD in human placenta. Kinetic analysis of particulate 3β-HSD activity revealed Km values at day 50 and term that were not significantly different. The Vmax at term was significantly greater than that on day 100, thus strengthening our finding of increased 3β-HSD activity in 800g supernatant fractions of chorion at term. Our values of Km are of the same order of magnitude (nM) as those reported by Gibb (1979).

Townsley (1975) and Gibb (1981) have reported the inhibitory effects of various steroids on human placental 3β-HSD activity. I have observed similar inhibitory effects of steroids on 3β-HSD in particulate fractions of chorion. Mitchell, Cruickshank, McLean and Challis (1982) have shown that steroids influence production of P4 from P5 by dispersed human placental cells and human fetal membranes Of the steroids analysed with the particulate preparations, E1 and E2 had the greatest inhibitory effects. DHEA, an alternative substrate for 3β-HSD, inhibited competitively the conversion of P5 to P4. E1 was also a competitive inhibitor of this conversion. Gibb (1981) has reported similar inhibition of human placental 3β-HSD by DHEA and E1 and has computed Ki values similar to those reported here. How E1, a structurally different steroid compared with DHEA and P5, acts as a competitive inhibitor of 3β-HSD is not known.

The finding that the fetal membranes can convert P5 to P4 supports the idea that the local concentrations of P4 in intrauterine tissues might be influenced by local synthesis. Estrogens are present in substantial amounts throughout pregnancy in the chorion. Local production of estrogens by the fetal membranes is described in

the next chapter The importance of estrogens in the production of P4 is not clear. Levels of P4 and estrogens in the chorion remain constant throughout most of the latter half of pregnancy, therefore, any inhibitory effect is probably chronic

CHAPTER 8

THE PRODUCTION AND METABOLISM OF PROGESTERONE AND ESTROGENS IN DISPERSED CELLS FROM INTRAUTERINE TISSUES

8.1 Introduction

The concentration of a steroid within a tissue is dependent on the tissue's intrinsic ability to produce and/or metabolize the steroid. Several steroid metabolizing enzymes have been described in the extra-placental intrauterine tissues of the pregnant sheep. Progesterone (P4) has been shown to be metabolized by 5α-reductase and 20α-hydroxysteroid dehydrogenase (20α-HSD) in the myometrium (MYO) (Rossier and Pierrepoint, 1974b). The conversion of estrone sulphate (E1-S) to estrone (E1) has been demonstrated in preparations of MYO (Rossier and Pierrepoint, 1974a) and endometrium (ENDO) (Dwyer and Robertson, 1980). The myometrial preparations also converted E1-S to estradiol-17β (E2) (Rossier and Pierrepoint, 1974a). These findings established the presence of estrone sulphatase activity in the MYO and ENDO and 17β-hydroxysteroid dehydrogenase (17β-HSD) activity in the MYO. I have demonstrated 3β-hydroxysteroid dehydrogenase-Δ5-4, isomerase (3β-HSD) activity in the chorion (CHOR) and amnion (AMN). The studies described above were performed using subcellular preparations.

In this chapter I examined the hypothesis that intact cells of AMN, CHOR, allantois (ALL), ENDO, and MYO can produce P4, E1 and

E2 from endogenous and exogenous precursors. I also determined the ability of these cells to take up and metabolize P4. Cells were dispersed from tissues obtained from animals in early and late pregnancy and from animals in labour occurring spontaneously at term. I measured their ability to produce progesterone from two potential precursors, pregnenolone (P5) and 20α-dihydroprogesterone (20α-DHP) and produce unconjugated estrogens from estrone sulphate (E1-S) and androstenedione (Δ4). These experiments were performed to determine if enzymes that produce and metabolize P4 and Es are in the cells of intrauterine tissues and are available to exogenous precursors and if changes in the net production of P4 and Es occur at parturition. P5 was measured in homogenates of AMN, CHOR, ENDO and MYO to assess availability of precursor within the intrauterine tissues.

8.2 Materials and Methods

8.2.1 Animals

Twenty-three sheep of known gestational age were used in this study. Three age groups were studied: day 50 (6 animals), day 130-135 (7 animals), and term (10 animals). Animals at term were in labour.

8.2.2 Cell Dispersion

Tissues were minced and incubated with 0.1% collagenase in Dulbecco's phosphate buffer at 37°C for 1hr. Cells were harvested by centrifugation at 500g for 20 min, washed in Krebs buffer (NaCl, 0.9%; KCl, 1.15%; $CaCl_2$-$6H_2O$, 1.22%; $MgSO_4$-$7H_2O$, 3.82%; K H_2PO_4, 2.11%; $NaHCO_3$, 1.30%) containing 0.2% glucose and 0.5% albumin (Krebs GA), reharvested, and resuspended in Krebs GA. Greater than 90% of

cells dispersed by this technique were viable as indicated by their ability to exclude the vital dye trypan-blue.

Since it has been shown that fetal red blood cells possess 20α-HSD and convert P4 to 20α-DHP (Nancarrow and Seamark, 1969), chorionic cells from 2 animals were separated from fetal RBC's contaminating the mixture harvested. Cells were layered onto discontinuous gradients of Percol (10, 20 and 40% volumes) and centrifuged at 500g for 20 min. Chorionic cells equilibrated in 10% and 20% Percoll whereas RBC pelleted in 40% Percoll.

Cell numbers were measured in a Coulter counter which was found to give similar values to those measured by counting on a hemocytometer.

8.2.3 Incubations

Cells (50,000 - 2,000,000 cells/ml) were incubated with or without substrate in Krebs GA for 4hr at 37°C. Substrates were also incubated in 1ml Krebs GA for 4h at 37°C without cells. All incubations were terminated by freezing at -20°C.

8.2.4 Production of Progesterone

P4 was measured in incubations using RIA as described in Chapter 5. The net production of P4 from endogenous substrate was calculated by subtracting P4 measured at T=0hr from that measured at T=4h. In addition P4 was measured following incubations of cells with 100ng/ml P5, 20α-DHP or P5 and 10ug/ml trilostane, an inhibitor of 3β-HSD (a gift from Sterling-Winthrop Research Institute, Rensselaer, New York). The amount of immunoreactive P4 in incubations of substrates without cells was subtracted from the amount of immunoreactive P4 in

incubations of substrates with cells. Production of P4 was expressed as pg/100,000 cells/4h. The authenticity of the P4 produced was verified by cochromatographing immunoreactive P4 (IR-P4) from pooled incubations of each tissue with purified ^{3}H-P4 using micro-celite columns (as described in Chapter 5).

8.2.5 Production of Estrogens

E1 and E2 were measured in incubations using RIA's as described in Chapter 6. The production of E1 and E2 from endogenous and exogenous substrate was calculated as described for P4. E1 and E2 were measured following incubations with 100 ng/ml E1-S (prepared and donated by Dr. R. Hobkirk, Dept. of Biochemistry, University of Western Ontario) and E1 was measured following incubations with 100 ng/ml Δ4. The authenticity of E1 produced from Δ4 was verified by cochromatographing IR-E1 from pooled incubations of each tissue with purified ^{3}H-E1 using micro-celite columns as described in Chapter 6.

In an additional experiment, cells were incubated with 100ng/ml P5, 20α-DHP or E1-S for 0 to 8h or with several concentrations (10 to 500ng/ml) of the same substrates for 4h. The net production of P4 or E1 was compared to that produced by cells dispersed from several cotyledons (fetal component).

8.2.6 Metabolism of Radioactive Substrates

Cells were incubated with [^{3}H]-pregnenolone (^{3}H-P5; 7-^{3}H(N)-pregnenolone; specific activity (SA) 10-25 Ci/mmole), [^{3}H]-progesterone (^{3}H-P4;2,4,6,7-^{3}H(N)-progesterone; SA, 90-115Ci/mmole), [^{3}H]-estrone sulphate (^{3}H-E1-S; SA 40-60 Ci/mmole) and [^{3}H]-androstenedione (^{3}H-Δ4;1,2,6,7-^{3}H(N)-androstenedione;SA,85 Ci/mmole). The purity of radioactive substrates was checked by thin layer chroma-

tography (TLC). Material from incubations with radioactive substrates was extracted with diethyl ether (5VOL). The organic phase was dried and resuspended in 40ul ethanol and applied to TLC plates. Radioactive material from incubations with ^{3}H-P5 and trilostane was developed in chloroform:diethyl ether (10:3 or 9:3, V:V) and that from incubations with ^{3}H-Δ4 and ^{3}H-E1-S was developed in cyclohexane: ethyl acetate (3:2, V:V). In both systems, plates with 40ug of authentic steroids were run simultaneously for determination of retention values (RF). One cm bands of silica gel from the origin to the solvent front were collected directly into scintillation vials containing 4ml toluene with 12% glacial acetic acid and 0.5gm% PPO and radioactivity levels were measured in a liquid scintillation counter.

8.2.7 Purification with High Pressure Liquid Chromatography

Incubations with ^{3}H-P4 were pooled with respect to tissue and gestational age and extracted and chromatographed as above. Radioactive material in the one cm bands of silica gel was extracted with 2ml ethyl acetate. The organic solvent was dried and the radioactive material resuspended in 200ul ethanol. An aliquot (50ul) was taken for measurement of radioactivity. The remaining material in the fractions corresponding to the peaks of radioactivity was purified using high pressure liquid chromatography (HPLC). Five thousand CPM [4-^{14}C]-progesterone (^{14}C-P4, specific activity, 50-60mCi/mmol) and 10ug of steroids with similar RF's as those of the radioactive peaks were added in ethanol to the fractions. The ethanol was dried and the steroid was resuspended in 0.5ml methanol. An aliquot (200ul) was taken for measurement of radioactivity and another aliquot (20ul)

was applied to the HPLC column and eluted with 60% methanol in distilled water. Elution of non-radioactive steroid was detected by monitoring U.V. absorbance at 240nm. Radioactivity was measured in 0.5ml fractions collected every minute.

The radiochemical purities of the tritiated labelled products were assessed by successive recrystallizations with authentic non-radioactive steroids. Specific activity of radioactivity in the crystals was expressed as either cpm/mg or $^{3}H:^{14}C$ ratios

8.2.8 Radioimmunoassay (RIA) of Pregnenolone

P5 was measured in homogenates of AMN, CHOR, ENDO and MYO (see Chapter 5 for preparation of tissues) using RIA (Inaba et al, 1979). Immunoreactive P5 (IR-P5) in several samples of each tissue was eluted from micro-celite columns (see Chapter 7). Purified ^{3}H-P5 (~1000 CPM) was added to each sample prior to extraction to monitor procedural losses. Comparisons were made between IR-P5 measured directly after extraction and that measured after purification on micro-celite columns. Tissues were collected throughout gestation; however, insufficient sample numbers made comparison between gestational ages inappropriate.

8.2.9 Analyses of Results

All results are presented in this chapter as mean ± SEM. Where appropriate, differences in steroid production or conversion at each gestational age were assessed by one way analysis of variance. The data were transformed logarithmically when Bartlett's test revealed heterogeneity of variance. Significant differences between mean values were sought using Duncan's multiple range test at $P<0.05$.

8.3 Results

8.3.1 Production of P4

There was no net production of P4 from endogenous substrate by any tissue at any gestational age. Only CHOR and ENDO converted P5 to P4 and they did so at each gestational age (Fig. 27). In the CHOR, the mean conversion at day 130 was two-fold greater than the value at term and 1.5-fold the value at day 50; however, these differences were not statistically significant ($P>.05$). In the ENDO the mean conversion at day 130 was about four-fold the values at day 50 and term; however, these differences were not statistically significant ($P>.05$). Insufficient amounts of P4 were produced from P5 to substantiate authenticity by purification on micro-celite columns; however, greater than 90% of the conversion of P5 to IR-P4 by CHOR and ENDO was inhibited by 10ug/ml trilostane (Fig. 28) suggesting that almost all IR-P4 was the product of 3β-HSD activity.

All tissues at all times converted 20α-DHP to P4 (Fig. 29). The mean conversions were about ten-fold those of P5 to P4. In the AMN and CHOR the mean conversions at term were about half the values on day 50 and day 130; however, these differences were not statistically significant ($P>0.05$). In the ALL, ENDO, and MYO, differences between the mean conversions at different gestational ages were not statistically significant ($P>.05$). At each time in pregnancy the mean conversions of 20α-DHP to P4 by AMN, CHOR and ENDO were similar, and were greater than the conversion by ALL and MYO. Table 8 lists the specific activities of the P4 measured directly after extraction and compares them to those measured after extraction and purification by micro-celite chromatography. There was good agreement between the specific activities of P4 produced from 20α-DHP by AMN, CHOR and ENDO

FIGURE 27. Conversion of pregnenolone to progesterone by dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO), and myometrium (MYO) from sheep at d50 and d130 in pregnancy and in spontaneous labour at term (SL). Incubations were performed with 100ng/ml pregnenolone and the results expressed as pg progesterone formed /100,000 cells/4h (mean ± SEM; n=5-11).

151

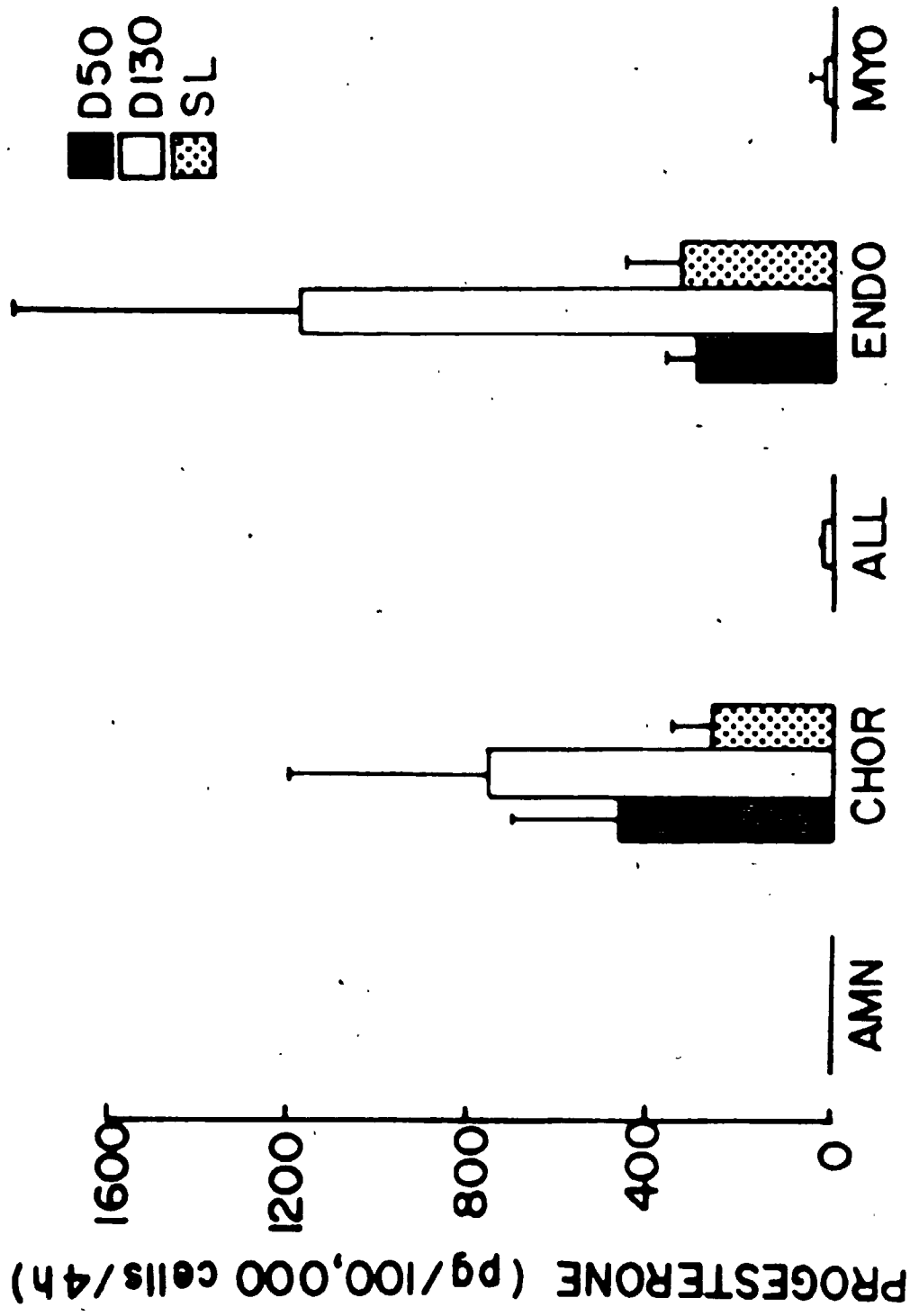

D50
D130
SL
MYO
ENDO
ALL
CHOR
AMN
1600
1200
800
400
0
PROGESTERONE (pg/100,000 cells/4h)

FIGURE 28. Conversion of pregnenolone to progesterone by dispersed cells of endometrium (ENDO; upper panel) and chorion (CHOR, lower panel) of sheep at d130 of pregnancy and in spontaneous labour at term (SL). Incubations were performed with 100ng/ml pregnenolone in the presence or absence of 1µg/ml trilostane. Results are expressed as pg progesterone formed /100,000 cells/4h (mean ± SEM; n=5).

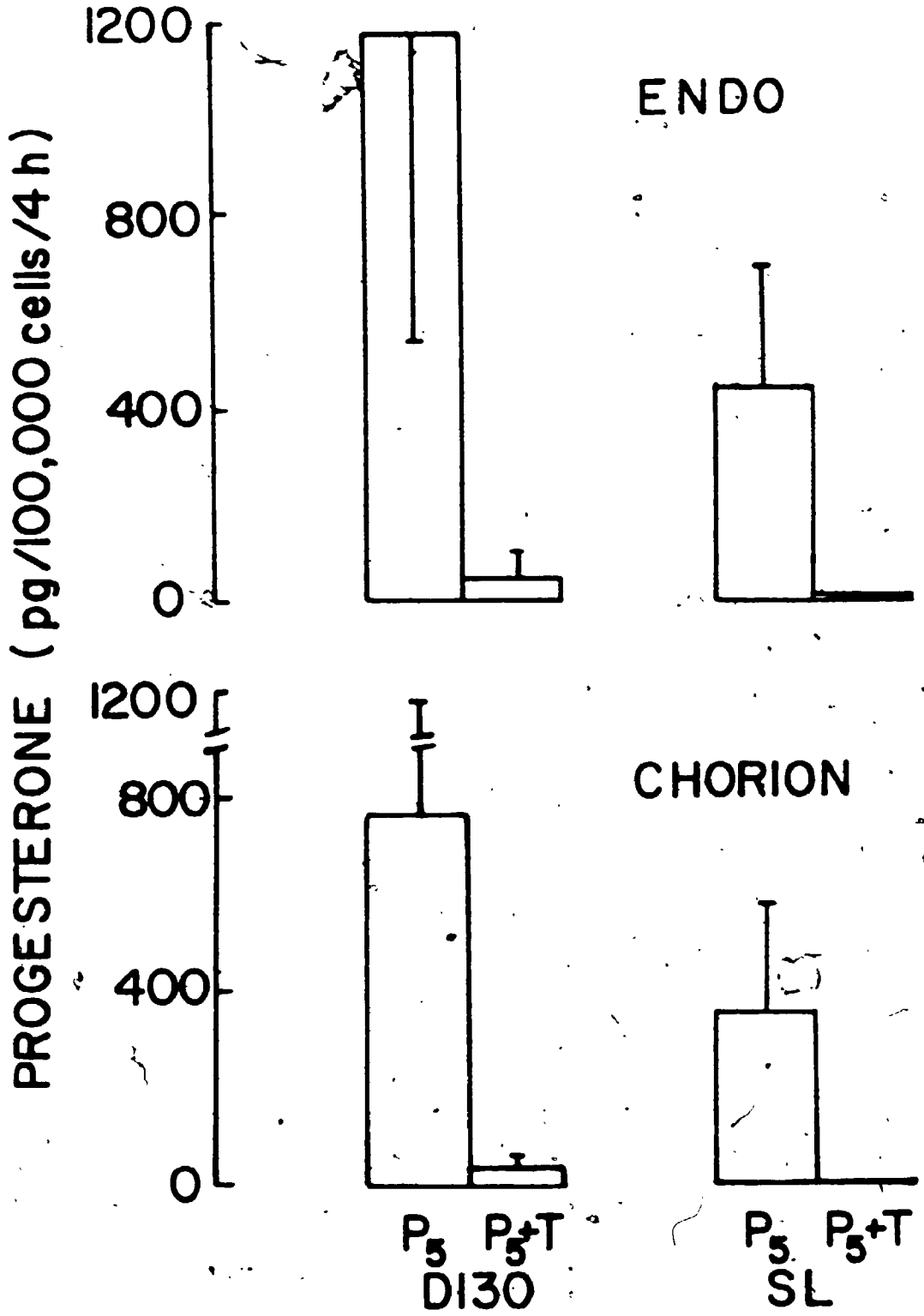
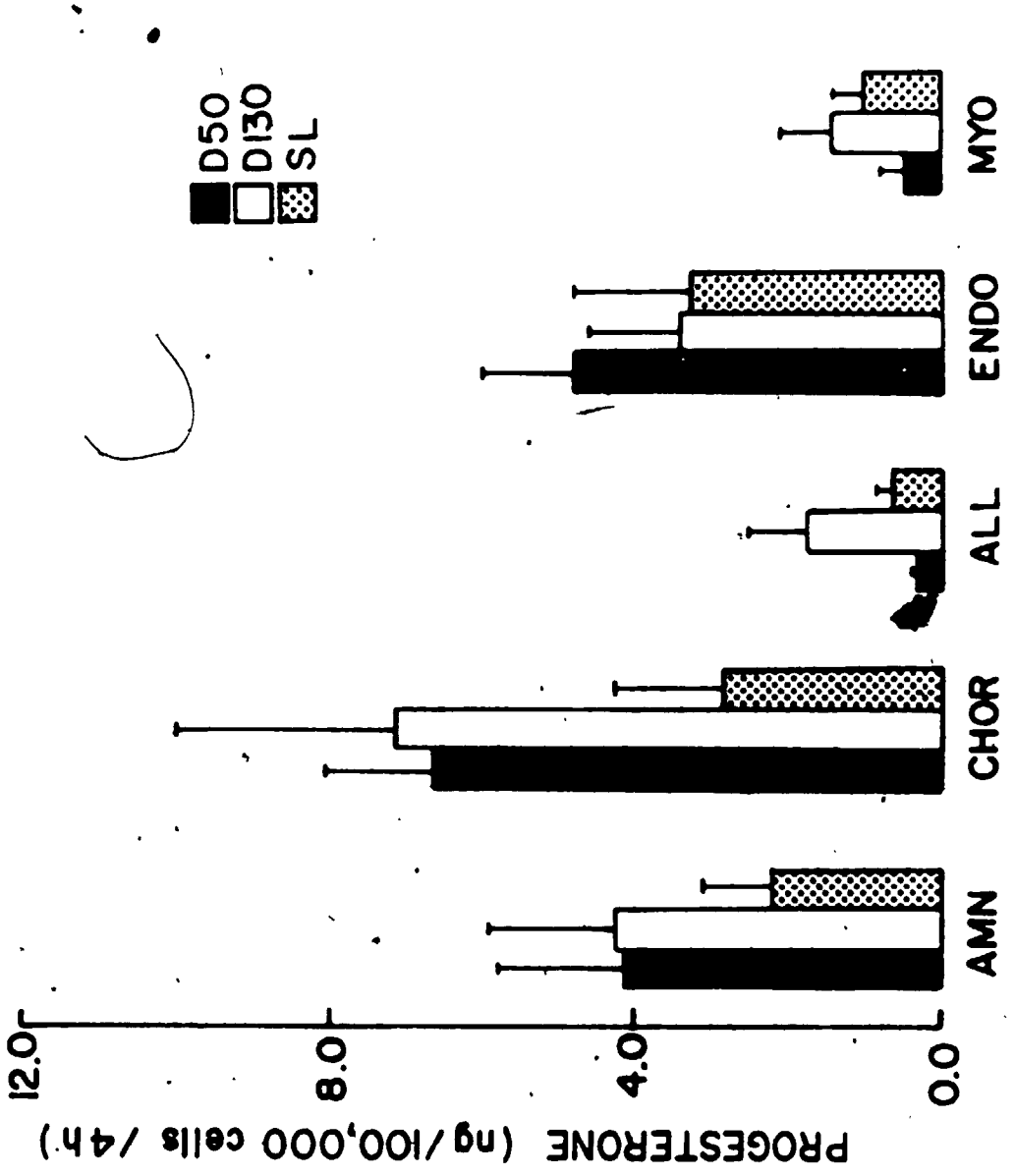

PROGESTERONE (pg/100,000 cells/4 h)
1200
800
400
0
ENDO
CHORION
P5
P5+T
D130
SL

FIGURE 29. Conversion of 20α-dihydroprogesterone (20α-DHP) to progesterone by dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO), and myometrium (MYO) from sheep at d50 and d130 in pregnancy and in spontaneous labour at term (SL). Incubations were performed with 100ng/ml 20α-DHP and the results expressed as pg progesterone formed/100,000 cells/4h (mean ± SEM; n=5-11).

155


D50
D130
SL
AMN
CHOR
ALL
ENDO
MYO
12.0
8.0
4.0
0.0
PROGESTERONE (ng/100,000 cells /4h)

156

TABLE 8. Specific activities of immunoreactive progesterone produced from 20α-dihydroprogesterone by dispersed cells of amnion (AMN), chorion (CHOR) and endometrium (ENDO).

Tissue	Gestational age	Specific activity (CPM/pg) Post Extraction	Post extraction and chromatography
AMN	d 50	1.06	0.99
	d130	1.19	1.16
	Term	1.44	2.04
CHOR	d 50	1.03	0.94
	d130	1.26	1.09
	Term	0.45	0.41
ENDO	d 50	0.33	0.31
	d130	1.07	1.10
	Term	1.89	1.52

suggesting that IR-P4 measured directly after extraction was authentic P4. Insufficient amounts of IR-P4 were produced from 20α-DHP by ALL and MYO to substantiate authenticity.

8.3.2 Metabolism of ^{3}H-Pregnenolone

Levels of radioactivity in one centimeter bands from the origin to the solvent front are shown in Figure 30. Each profile is representative of radioactive material recovered from incubations without cells and with cells from each tissue expressed as a percentage of the total radioactivity recovered. Shown in Table 9 are the RF's of potential products of P5 metabolism in the system used to develop the chromatograms ($CHCl_3$:DEE, 9:3, 1 ascent). Most of the radioactivity recovered cochromatographed with P5 (RF 6.5/16). A peak of radioactivity chromatographed with RF of 4/16. Monohydroxylated derivatives of P5 and 5α-reduced P5 had similar RF's. CHOR cells, free of RBC's, also converted ^{3}H-P5 to the same products (3.3 vs. 2.9 and 5.8 vs. 9.7% total activity, RBC free mixtured vs RBC contaminated mixture). All tissues at all times generated radioactivity with the same RF (Fig. 31). In the AMN, ALL, and MYO mean conversions were not as marked as those in CHOR and ENDO and values at different gestational ages were not significantly different ($P>0.05$). In both CHOR and ENDO mean conversions at term were less than half the values on day 50-60 and day 130-135; however, these differences were not statistically significant ($P>0.05$).

8.3.3 Metabolism of ^{3}H-Progesterone

Levels of radioactivity in one cm bands from the origin to the solvent front of thin layer chromatograms are shown in Fig. 32. Each profile is as described for those showing metabolism of ^{3}H-P5. Most

TABLE 9. Rf values of C21, C19 and C18 steroids on thin layer chromatography developed in chloroform:diethylether (9:3 or 10:3; v/v) Rf (x/16)

	9:3	10.3
5α-pregnane-3,20-dione	10.5	14
5β-pregnane-3,20-dione	10.0	13.5
Pregn-4en-3,20-dione	8.5	12.5
Estra-1,3,5(10)-trien-3-ol-17-one	8.5	12.5
Androst-4-en-3,17-dione	7.5	11.0
5β-pregnane-3β-ol-20-one	7.5	
5α-pregnane-3α-ol-20-one	7.0	10.5
5α-pregnane-20β-ol-3-one	7.0	10.5
Pregn-5en-3β-ol-20-one	6.5	9.0
5β-pregnane-3α-ol-20-one	5.5	9.0
Androst-5-en-3β-ol-17-one	5.5	8.5
Estra-1,3,5(10)-trien-3,17β-diol	5.5	8.0
Pregn-4en-20α-ol-3-one	5.0	7.5
Androst-4-en-17β-ol-3-one	4.5	6.5
Pregn-4en-17α-ol-3-one	4.5	7.0
5α-pregnane-3β,20α-diol	4.0	6.0
Pregn-5-en-3β,17α-diol-20-one	4.0	
Pregn-5-en-3β,20α-diol	4.0	
5α-pregnane-3α,17α-diol-20-one	4.0	4.0
5β-pregnane-3α,20α-diol	2.0	3.5
Pregn-4-en-17α,20α-diol	1.5	2.5
Pregn-5-en-3β,17α,20α-triol	1.0	1.0
5β-pregnane-3α,17α,20α-triol	1.0	1.0

FIGURE 30. Thin layer chromatogram of radioactivity after incubations of amnion (AMN, chorion (CHOR), allantois (ALL), endometrium (ENDO), and myometrium (MYO) with 0.5 μCi [^{3}H]-pregnenolone. Results are expressed as a percentage of the total radioactivity recovered. Retention factor (Rf) is defined as distance travelled by radioactivity/distance to solvent front.

160

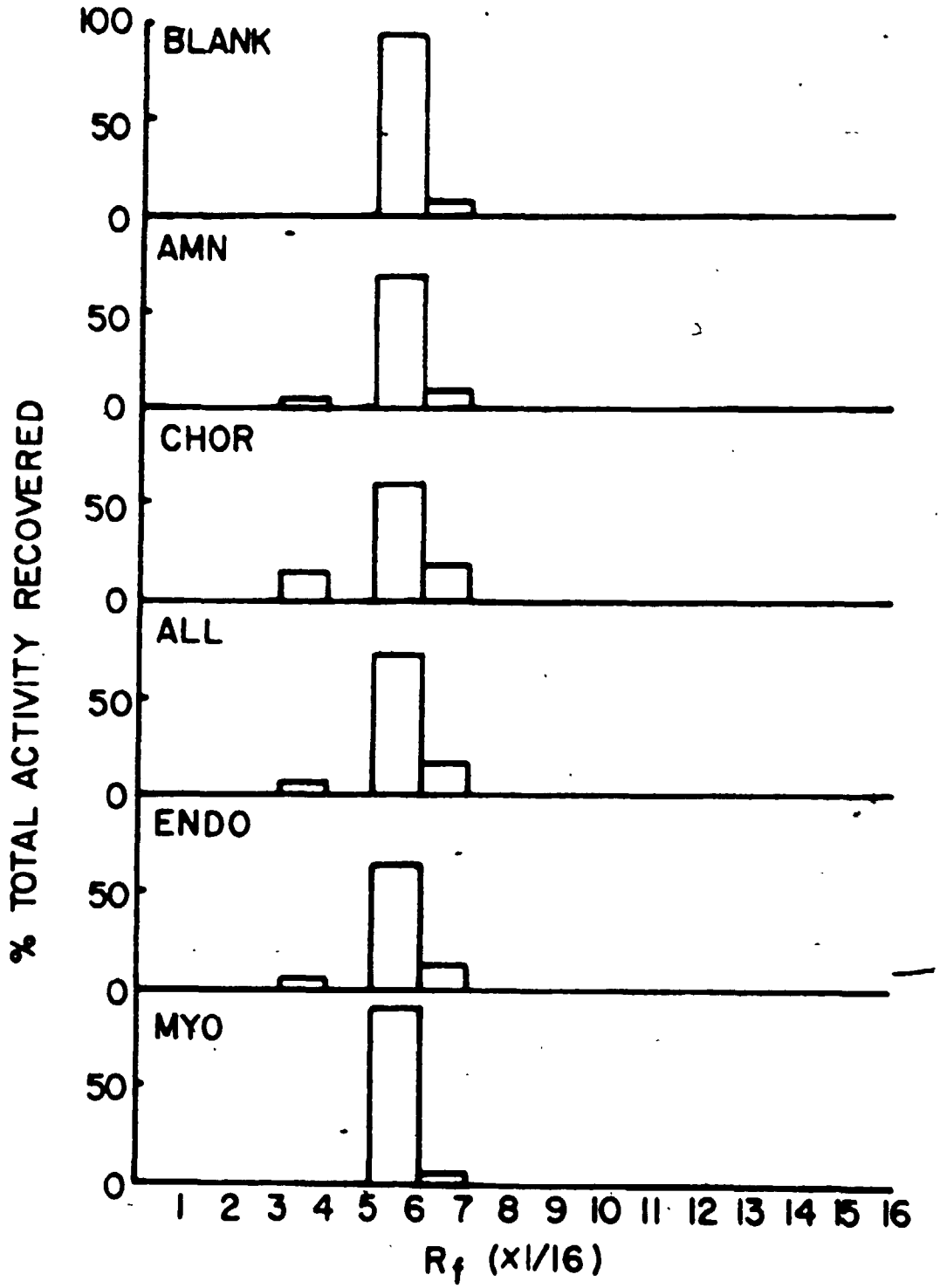

100
50
0
BLANK
AMN
CHOR
ALL
ENDO
MYO
% TOTAL ACTIVITY RECOVERED
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Rf (x1/16)

FIGURE 31. Conversion of [^{3}H]-pregnenolone to the major polar metabolite(s) shown in Figure 30. Amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO), and myometrium (MYO) from sheep at D50 and D130 in pregnancy and in spontaneous labour at term (SL) were incubated with 0.5 μCi [^{3}H]-pregnenolone. Results are expressed as a percentage of the total activity recovered/100,000 cells/4h (mean ± SEM; n=5-11).

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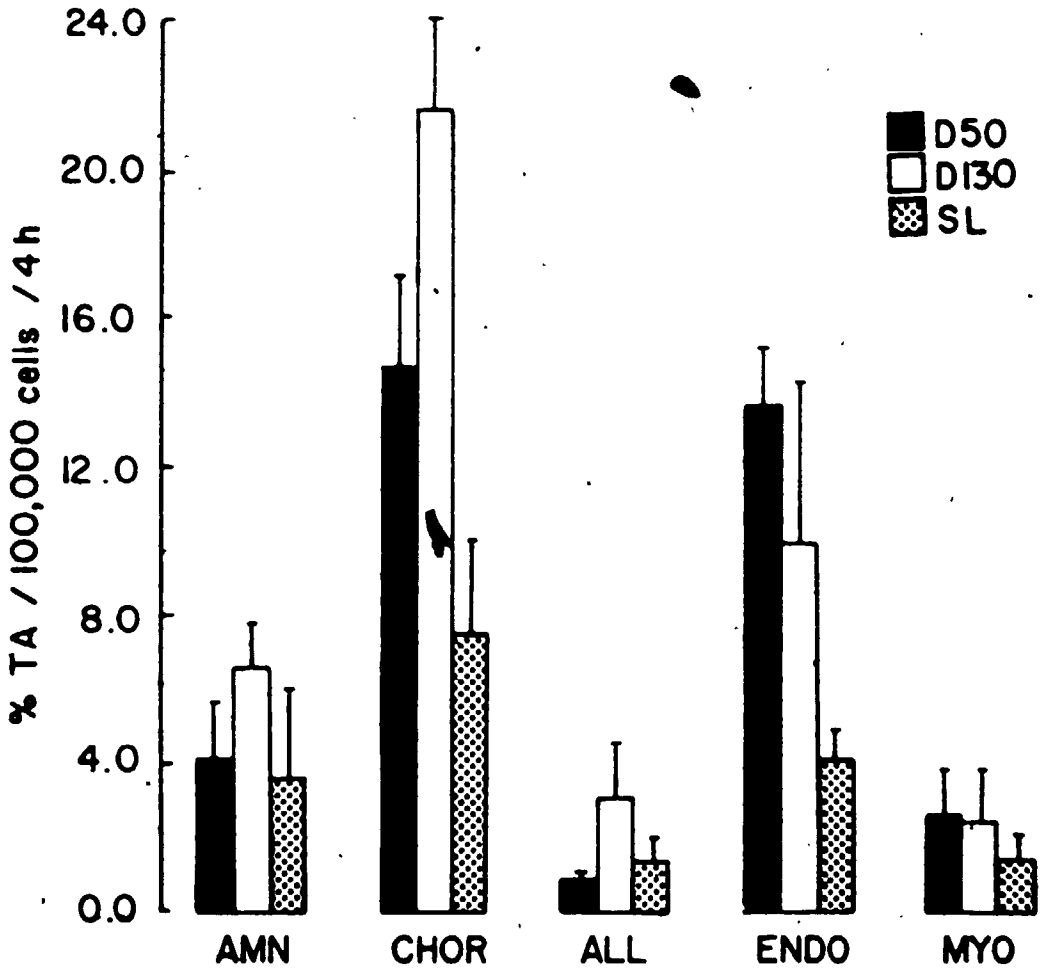
24.0
20.0
16.0
12.0
8.0
4.0
0.0
% TA / 100,000 cells / 4 h
D50
D130
SL
AMN
CHOR
ALL
ENDO
MYO

163

of the radioactivity recovered cochromatographed with P4 (RF=12.5/16, Table 9; $CHCl_3$:DEE, 10:3, 2 ascents). A peak of radioactivity chromatographed with an RF 7-8/16. All tissues at all times generated radioactivity with the same RF (Fig. 33). Mean conversions were most marked in the CHOR and ENDO on day 50-60 and day 130-135. In the CHOR and ENDO the mean conversions on day 130-135 were two-three fold the values at term; however these differences were not statistically significant ($P>0.05$). In the AMN, ALL and MYO the mean conversions at different gestational ages were not significantly different ($P>0.05$).

Radioactivity eluting in bands 6-8 cm (Fig. 32) was purified using HPLC techniques. Shown in Figure 34 are representative profiles of radioactivity (solid histograms) with retention times from 13-30 min for AMN, CHOR, ALL, and ENDO at day 130-135. Eluates with shorter retention times did not contain significant levels of radioactivity. Also shown in Fig. 34 are the retention times of ^{14}C-P4 (dashed histogram) and non-radioactive testosterone (a), 17α-P4(b), P4(c) and 20α-DHP(d) (uv absorbance) chromatographed simultaneously with the tritiated products in bands 6-8. Radioactivity produced by AMN, CHOR, ALL and ENDO eluted with retention times similar to 20α-DHP. Shown in Table 10 are the percentages of radioactivity cochromatographing with 20α-DHP produced by each tissue at each gestational age. Levels of radioactivity for MYO at day 130-135 and term were too low for analysis. The lower percentages for CHOR at each gestational age were associated with significant production of a metabolite or metabolites with similar retention times as testosterone and 17α-P4. The radiochemical purities of the radioactive

FIGURE 32. Thin layer chromatograms of radioactivity after incubations of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO), and myometrium (MYO) with 0.5 μCi [^{3}H]-progesterone. Results are expressed as a percentage of the total activity recovered. Retention factor (Rf) is defined as distance travelled by radioactivity/distance to solvent front.

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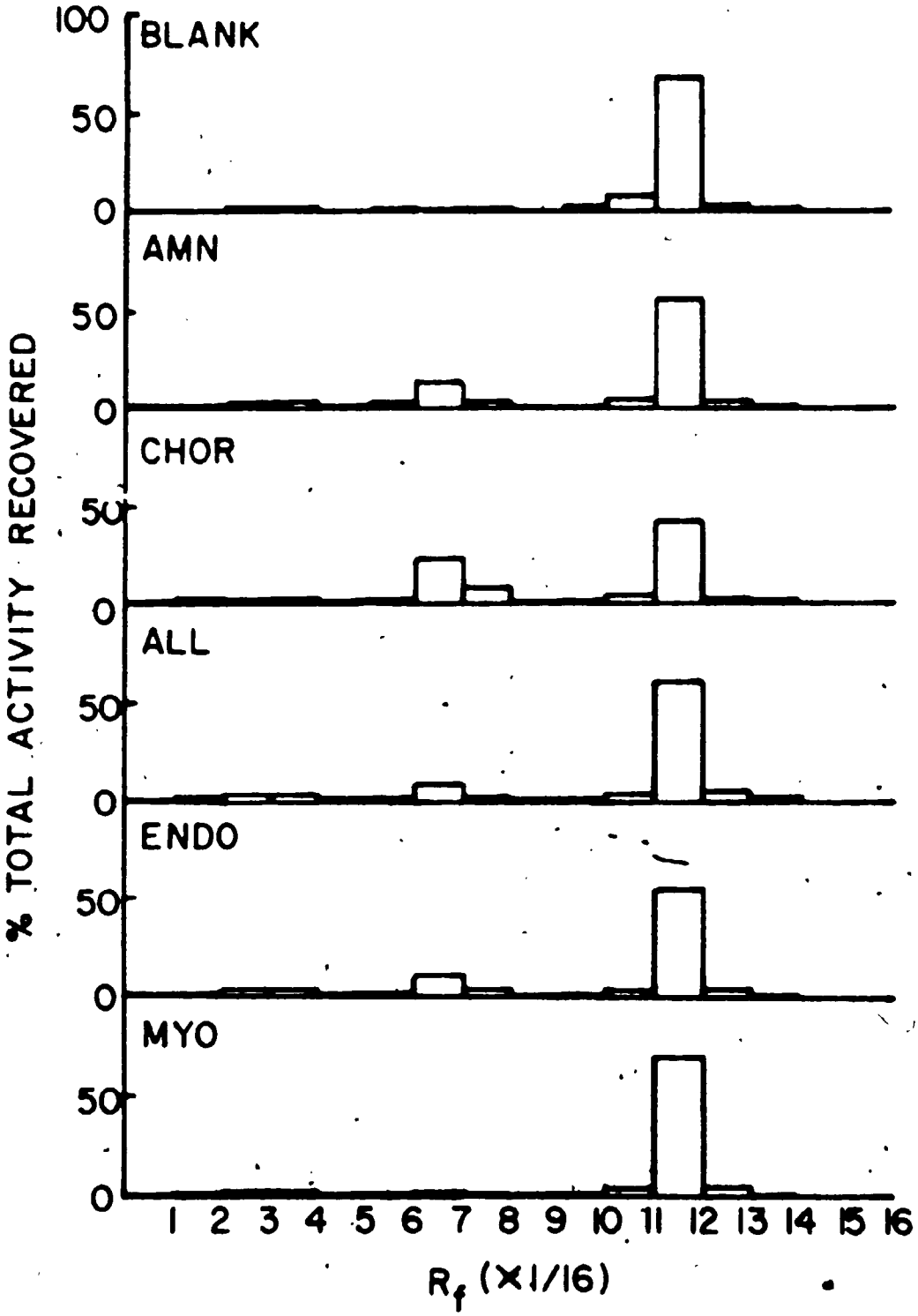
100
BLANK
50
0
AMN
50
0
CHOR
50
0
ALL
50
0
ENDO
50
0
MYO
50
0
% TOTAL ACTIVITY RECOVERED
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
R_f (×1/16)

FIGURE 33. Conversion of [^{3}H]-progesterone to the major metabolite(s) shown in Figure 32. Dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO) and myometrium (MYO) were incubated with 0.5 μCi [^{3}H]-progesterone. Results are expressed as a percentage of total activity (TA) recovered/100,000 cells/4h (mean ± SEM; n=5-11).

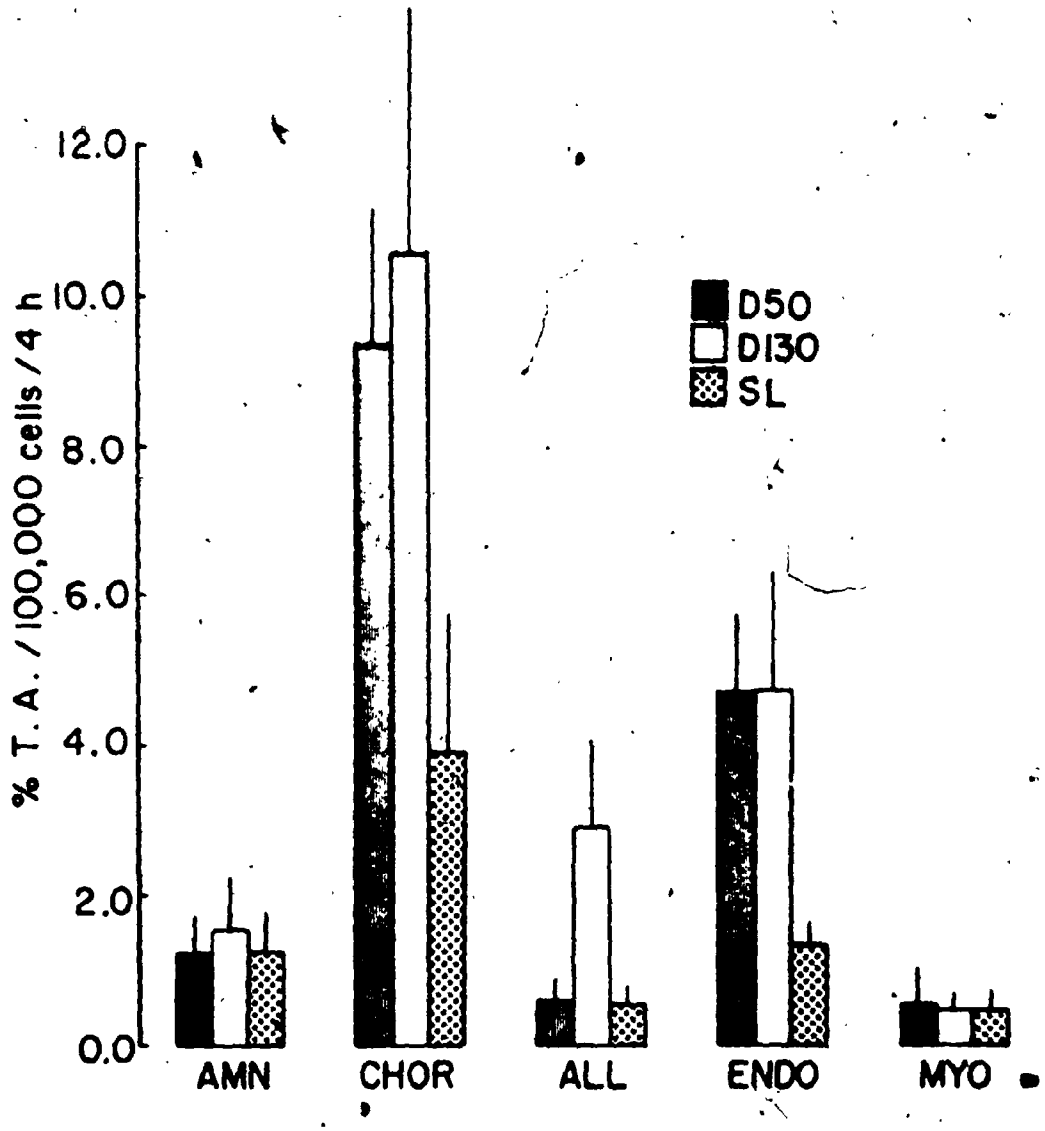
12.0
10.0
8.0
6.0
4.0
2.0
0.0
% T. A. / 100,000 cells / 4 h
D50
D130
SL
AMN
CHOR
ALL
ENDO
MYO

FIGURE 6 The concentration of progesterone in amnion and chorion from sheep at different times of pregnancy. Values are mean ± SEM for the number of animals (samples) indicated SL, spontaneous labour

FIGURE 34. High pressure liquid chromatograms of the major radioactive metabolite(s) formed by amnion (AMN), chorion (CHOR) allantois (ALL), and endometrium (ENDO) during incubations with 0.5 μCi [^{3}H]-progesterone. Amount of [^{3}H]-metabolite is expressed as counts per minute (CPM) x 10^{3}. Authentic [^{14}C]-progesterone was added prior to chromatography and its elution is shown in the broken histogram. The elution of non-radioactive testosterone (a), 17α-hydroxyprogesterone (b), progesterone (c) and 20α-dihydroprogesterone (d), as detected by UV absorbance (254nm) is shown in the uppermost panel.

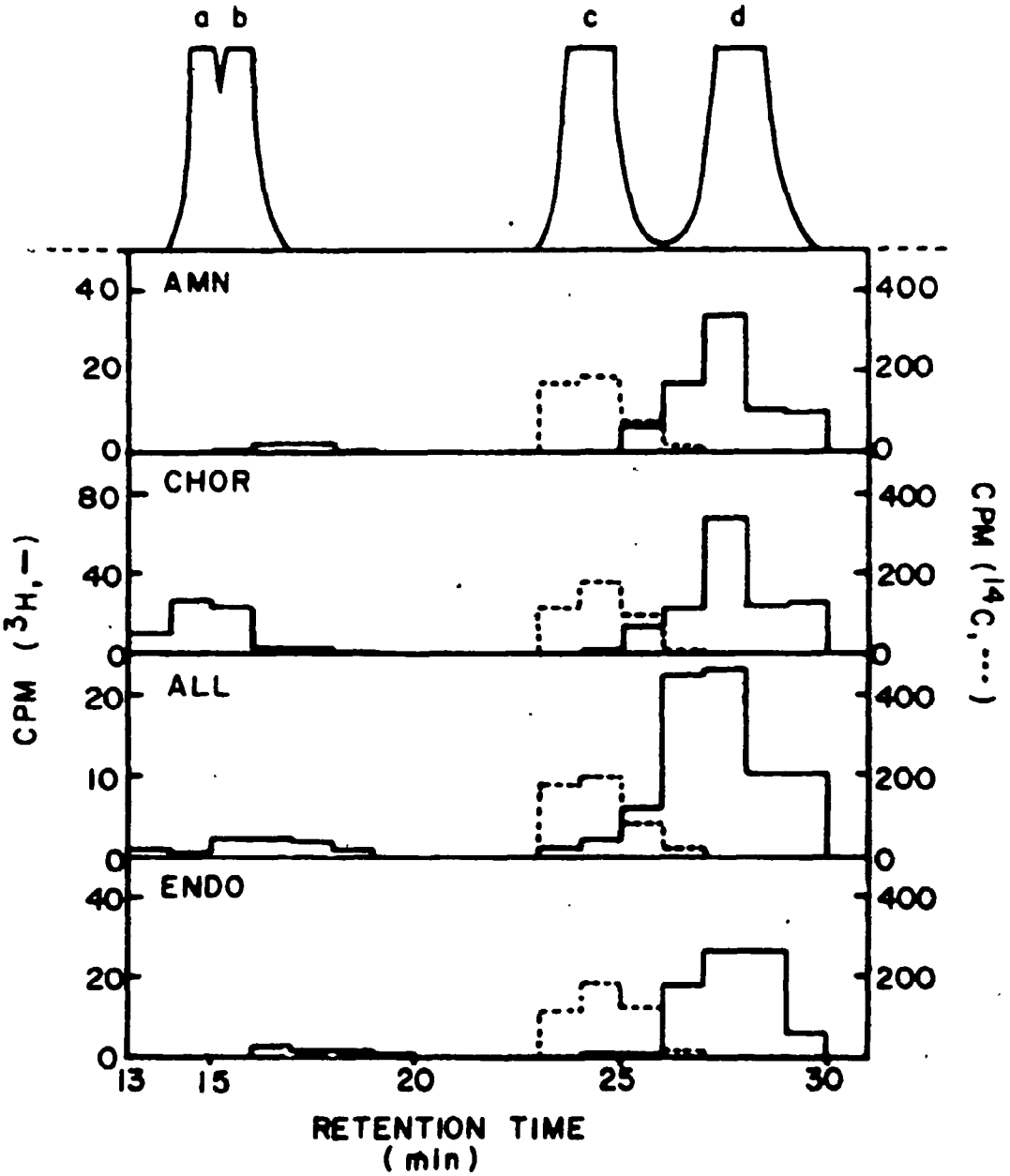
a b
c
d
AMN
CHOR
ALL
ENDO
40
20
0
80
40
0
20
10
0
40
20
0
400
200
0
13
15
20
25
30
RETENTION TIME
(min)
CPM (^{3}H, —)
CPM (^{14}C, ---)

TABLE 10. Precentage of the radioactive metabolite formed from [^{3}H]-progesterone in dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO) and myometrium (MYO) that co-chromatographs with 20α-dihydroprogesterone on HPLC

	Percentage		
Tissue	d50	d130	Term
AMN	75.1	94.1	69.8
CHOR	50.2	64.6	59.0
ALL	70.8	78.6	67.5
ENDO	92.5	92.7	86.2
MYO	77.1	-	-

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TABLE 11. Specific activity of radioactivity* recrystallized with authentic steroid from ethanol with water

Tissue	Steroid	Specific Activity (S.A., c.p.m/mg)			
		X	X_1	X_2	X_3
CHOR	20α-DHP	763.9	489.7	515.8	467.5
ENDO	20α-DHP	1174.1	935.2	1095.3	965.8
AMN	20α-DHP	211.0	80.5	85.3	71.2
ALL	20α-DHP	232.9	134.5	131.5	133.2
CHOR	17α-P_4	877.6	623.1	608.2	569.5

X = S.A...post thin-layer chromatography

X_1, X_2, X_3 = S.A's in successive recrystallizations

20α-DHP = 20α-dihydroprogesterone

17α-P_4 = 17α-hydroxyprogesterone

* Radioactive material had been co-chromatographed previously with authentic steroid.

products were confirmed as 20α-DHP and 17α-P by recrystallization with authentic steroid to constant specific activity (Table 1).

8.3.4 Production of Estrogens

There was no production of E1 or E2 from endogenous substrate by any tissue at any gestational age. All tissues at all times converted E1-S to E1 and E2 (Fig. 35 and 36 respectively). The conversion of E1-S to E1 and E2 was similar for each tissue at day 50 of gestation. However at day 130 and at term, the mean estrone sulphatase activity was greater in AMN and ENDO than in CHOR, ALL and MYO. In the AMN, ALL, and ENDO the mean conversions of E1-S to E1 or E2 did not change significantly ($P>0.05$) between any of the times studied. In the CHOR, the mean conversion of E1-S to E1 at term was significantly lower than the value at day 50 ($P<0.05$) but not significantly lower than that at day 130 ($P>0.05$). The lower mean conversions of E1-S to E1 at term were also associated with lower mean conversions of E1-S to E2; however the differences between mean conversions at different gestational ages were not statistically significant ($P>0.05$). In the MYO, the mean conversion of E1-S to E1 on day 50-60 was about two-fold the value on day 130-135. In contrast the mean conversion of E1-S to E2 on day 50 was about 0.6-fold the value on day 130-135. These differences were not statistically significant ($P>0.05$); however, they suggest the possibility of an increase in the conversion of E1 to E2 in myometrial cells between day 50-60 and day 130-135. There was no significant difference ($P>0.05$) between the mean conversions of E1-S to E1 or E2 on day 130-135 and at term. The formation of ^{3}H-E1 from ^{3}H-E1-S by all tissues (Fig. 37) was con-

FIGURE 35. The conversion of estrone sulphate to estrone by dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO) and myometrium (MYO) of sheep at D50 and D130 in pregnancy and in spontaneous labour at term (S'.). Incubations were performed with 100ng/ml estrone sulphate and the results expressed as pg estrone formed /100,000 cells/4h (mean ± SEM; n=5-11).

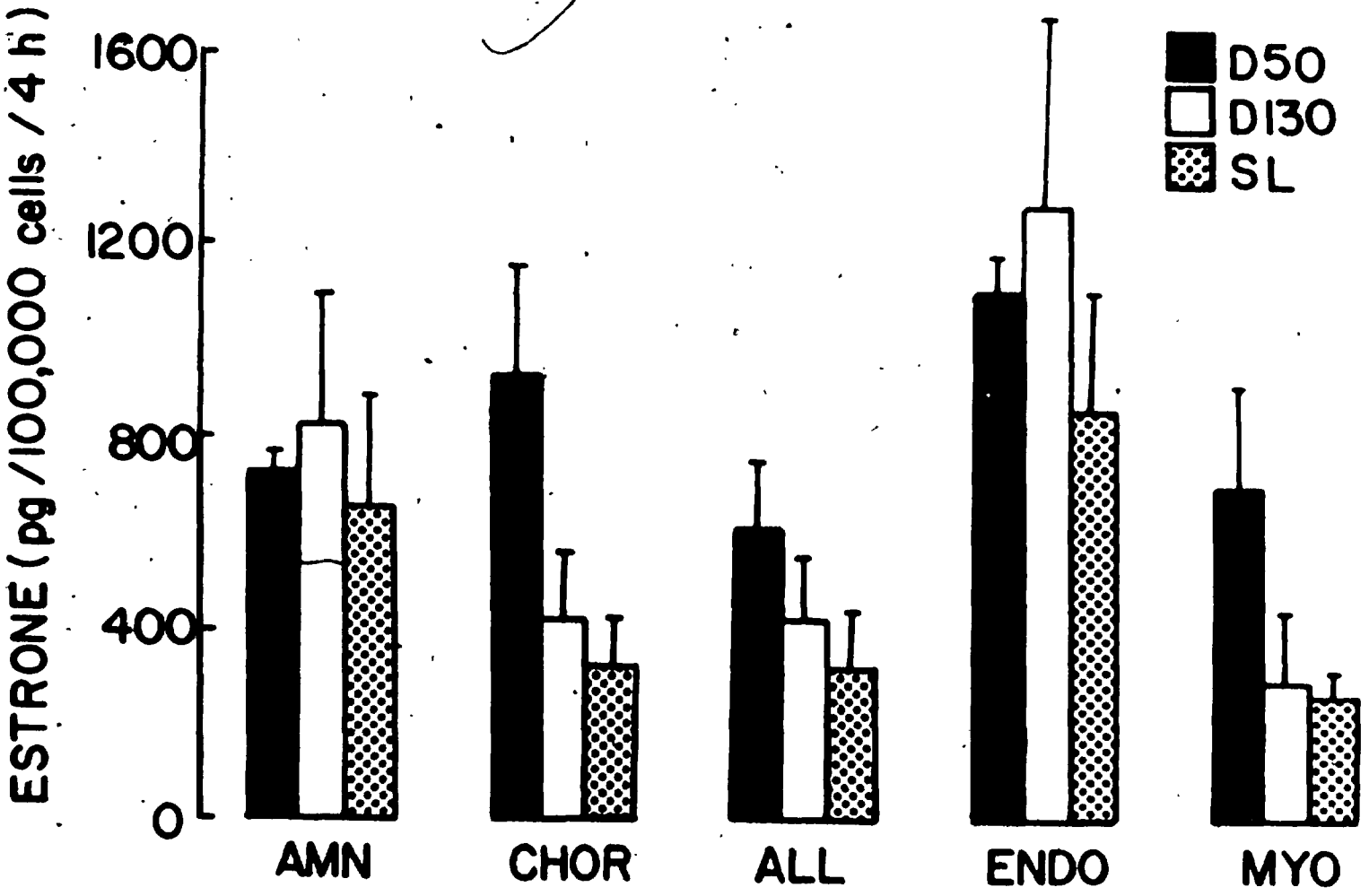
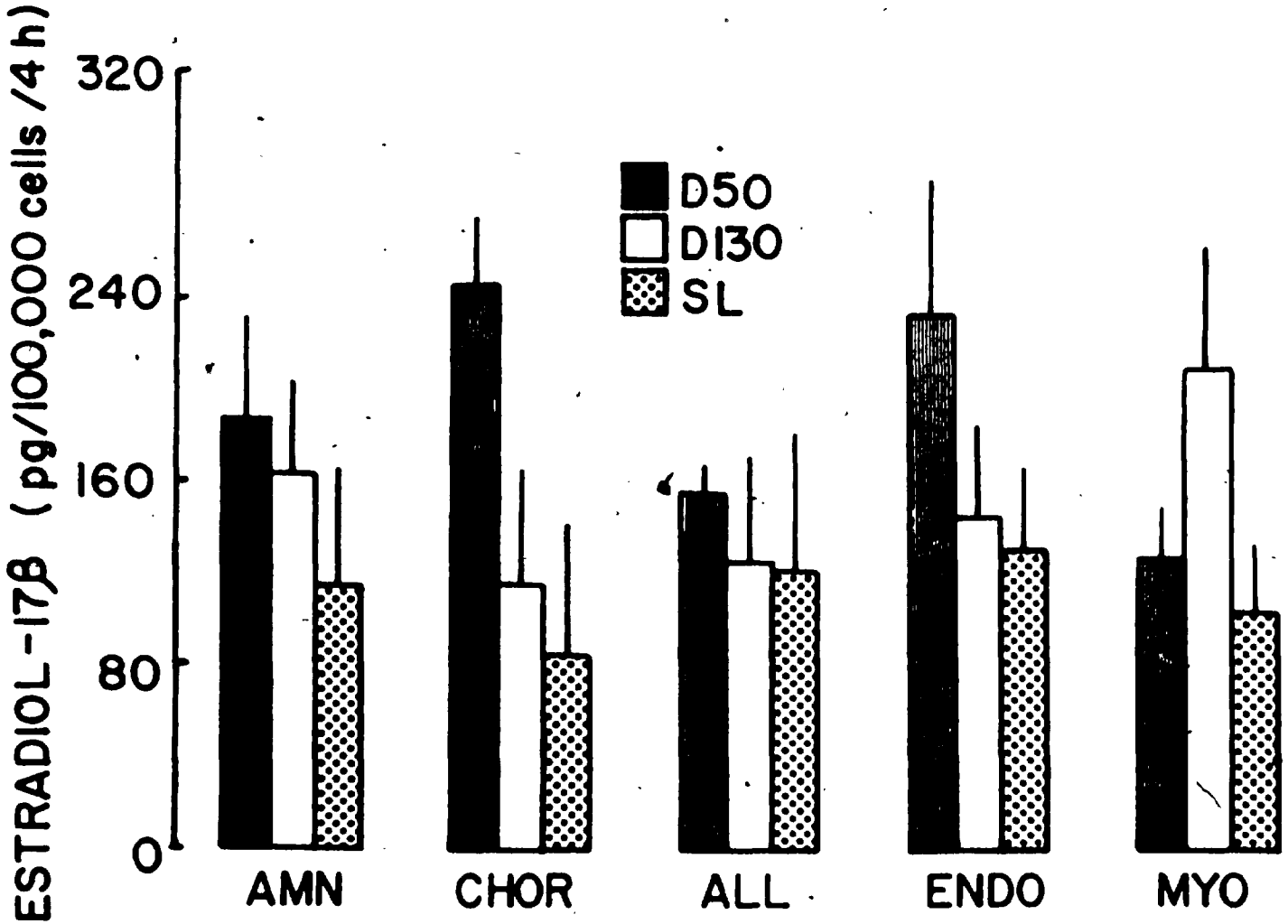

ESTRONE (pg/100,000 cells/4 h)
1600
1200
800
400
0
D50
D130
SL
AMN
CHOR
ALL
ENDO
MYO

FIGURE 36. The conversion of estrone-sulphate to estradiol-17β by dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO) and myometruim (MYO) from sheep at D50 and D130 in pregnancy and in spontaneous labour at term (SL). Incubations were performed with 100ng/m estrone sulfate and results expressed as pg estradiol-17β formed/100,000 cells/4h (mean ± SEM; n=5-11).


ESTRADIOL-17β (pg/100,000 cells /4 h)
320
240
160
80
0
D50
D130
SL
AMN
CHOR
ALL
ENDO
MYO

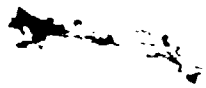


FIGURE 37. Conversion of [^{3}H]-estrone sulphate to [^{3}H]-estrone by dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO), and myometrium (MYO) from sheep at D50 and D130 in pregnancy and in spontaneous labour at term (SL). Incubations were performed with 0.5 μCi [^{3}H]-estrone sulfate. Results are expressed as a percentage of total activity (TA)/100,000 cells/4h (mean ± SEM; n=5-11).

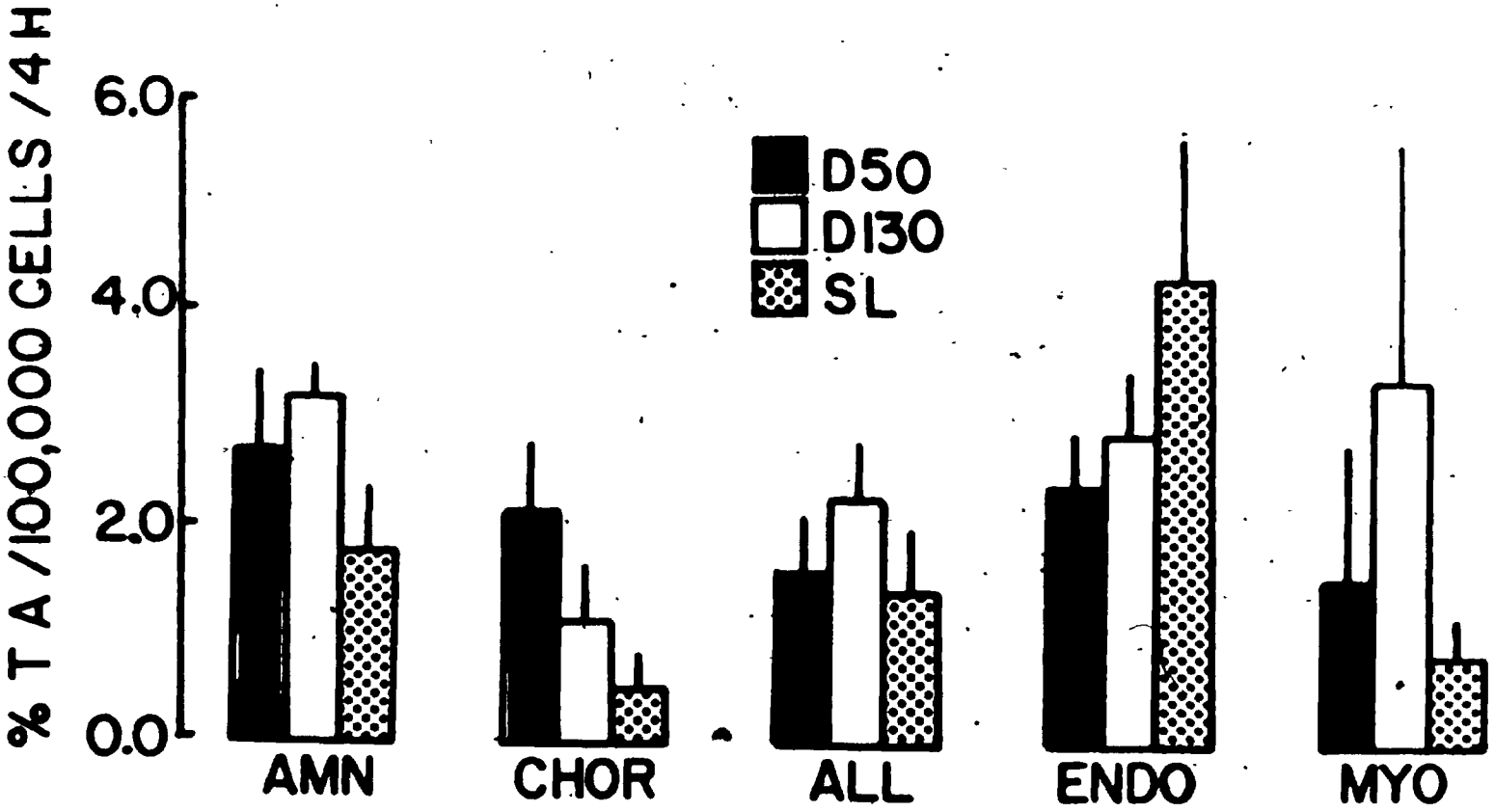
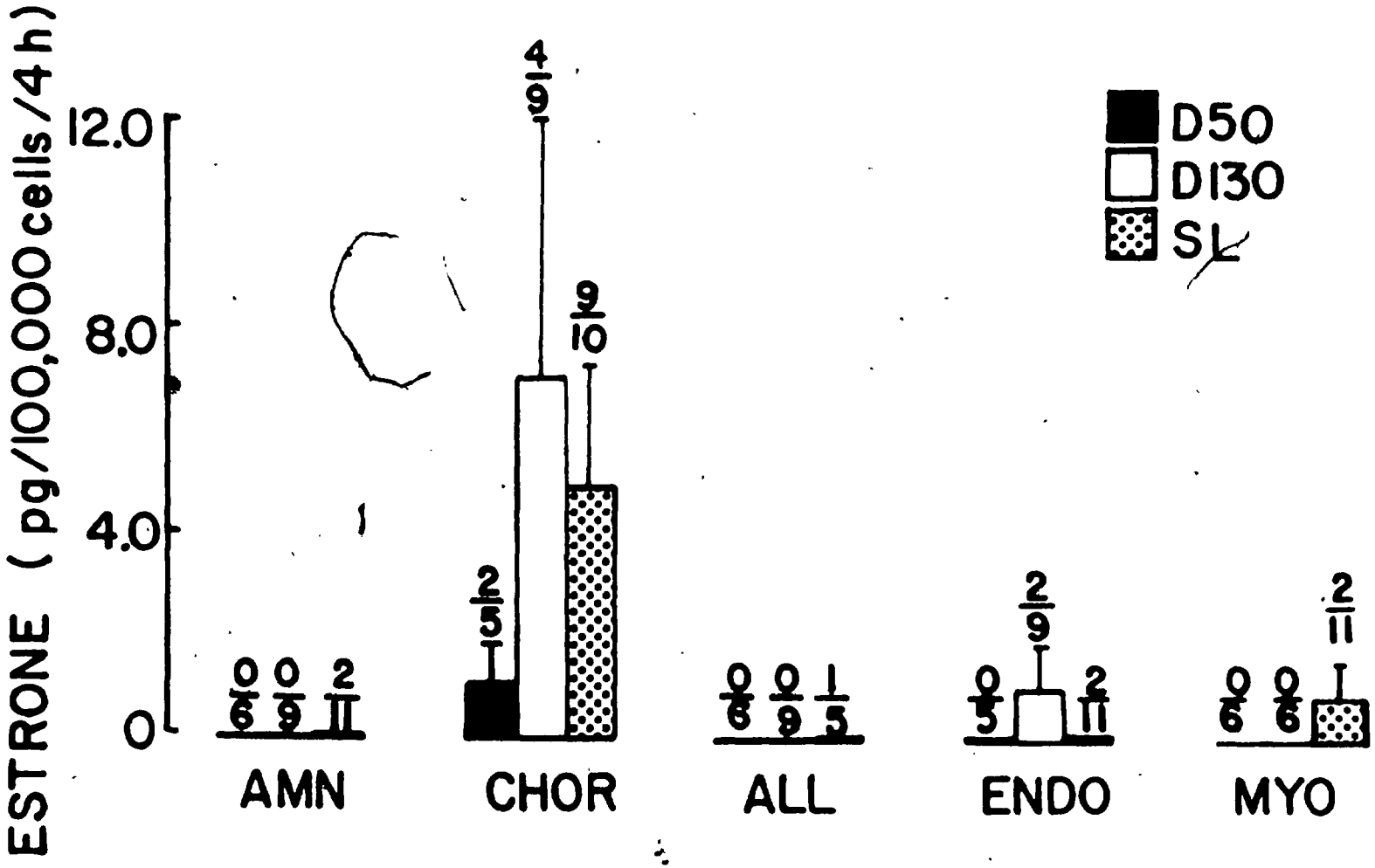

% T A /100,000 CELLS /4 H
6.0
4.0
2.0
0.0
D50
D130
SL
AMN
CHOR
ALL
ENDO
MYO

FIGURE 38. Conversion of androstenedione to estrone by dispersed cells of amnion (AMN), chorion (CHOR), allantois (ALL), endometrium (ENDO), and myometrium (MYO) from sheep at D50 and D130 in pregnancy and in spontaneous labour at term (SL). Incubations were performed with 100ng/ml androstenedione. Results are expressed as pg estrone formed/100,000 cells/4h (mean ± SEM; n=5-11). The number of tissues accumulating measurable amounts of estrone / the total number of tissues studied is shown above the histograms.


ESTRONE (pg/100,000 cells/4 h)
12.0
8.0
4.0
0
D50
D130
SL
0/6
0/9
2/11
2/5
4/9
9/10
0/6
0/9
1/5
0/5
2/9
2/11
0/6
0/6
2/11
AMN
CHOR
ALL
ENDO
MYO

firmed by cochromatographing radioactive product with nonradioactive E1 on TLC. The radiochemical purity was substantiated by the recrystallization of radioactivity with authentic E1 to constant specific activity (Table 12). Only the CHOR converted Δ4 to E1 (Fig. 38) and only at term was there conversion of Δ4 to E1 by most animals (2/5, 4/9, and 9/10 samples on days 50-60, day 130-135, and spontaneous labour at term, respectively.) The conversion of Δ4 to E1 was about 1/100 the conversion of E1-S to E1. The specific activity of E1 produced from Δ4 by CHOR at term was unchanged by purification using micro-celite columns (2.50 after extraction vs 2.27 after extraction and purification). Thin layer chromatograms of radioactive material from incubations of all cell types with ^{3}H-Δ4 did not show significant accumulation of any metabolites.

8.3.5 Time Course and Dose Response: Net Production of Progesterone and Estrone

Cells, dispersed from tissues collected from two animals in late pregnancy were incubated for 0-8h with 100ng/ml P5, 20α-DHP, and E1-S or for 4h with several concentrations of the substrates. The net production of P4 from P5 in the CHOR, ENDO and cotyledons increased up to 8h incubation; however, the greatest net production occurred in the first 1h (Figure 39). In these two animals the ENDO and cotyledons produced P4 in similar amounts and both produced more than the CHOR. The net production of P4 increased as the concentration of P5 increased from 10 to 500ng/ml (Figure 39). The net production of P4 from 20α-DHP had similar time course and dose response relationships (Figure 40) with the one exception that the fetal cotyledons of one animal did not produce any P4 irrespective of

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TABLE 12. $^{3}H:^{14}C$ ratios * in successive recrystallizations of estrone

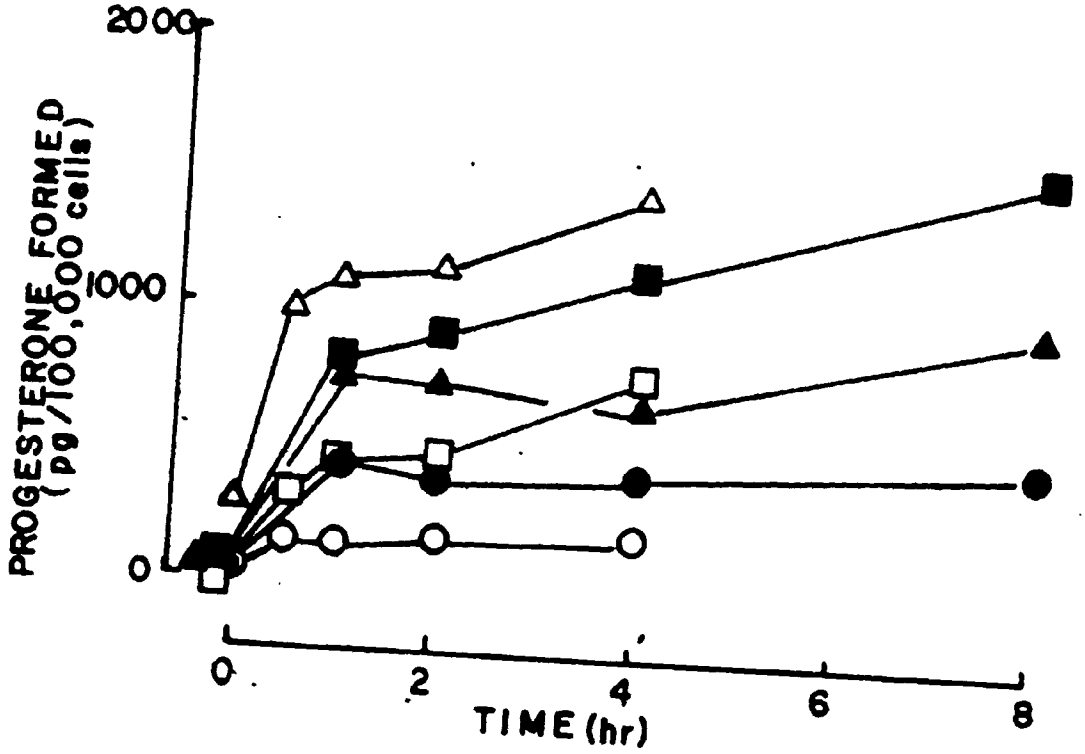
Tissue	$^{3}H : ^{14}C$			
	X	X_1	X_2	X_3
CHOR	0.404	0.281	0.275	.280
ENDO	0.489	0.373	0.361	.331
AMN	0.996	0.690	0.621	.613
ALL	1.014	0.690	0.601	.609
MYO	0.426	0.213	0.177	.205

X = $^{3}H : ^{14}C$ ratio post thin-layer chromatography

X_1, X_2, X_3 = $^{3}H:^{14}C$ ratios in successive recrystallizations of estrone from ethanol with water

* Tritiated material was co-chromatographed with authentic estrone and added to purified ^{14}C-estrone prior to recrystallization.

FIGURE 39. Time course (upper panel) and dose response relationships (lower panel) of the conversion of pregnenolone to progesterone in endometrium (squares), chorion (circles) and fetal cotyledons (triangles). Open and closed symbols represent data from two different animals. Cells were incubated with 100ng/ml pregnenolone for 0-8h (time course) or with several concentrations of substrate for 4h (dose response). Each symbol is the mean of quadruplicates. Error bars were smaller than the symbols and, therefore, not shown.


2000
1000
0
PROGESTERONE FORMED
(pg/100,000 cells)
0
2
4
6
8
TIME (hr)

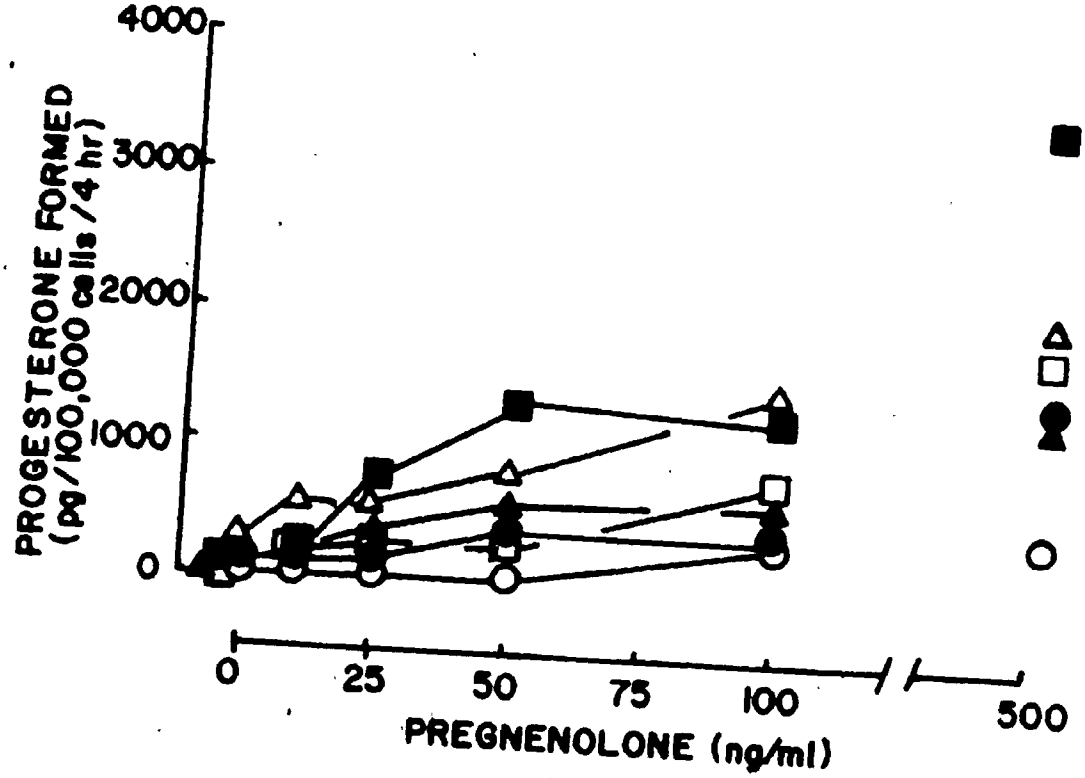
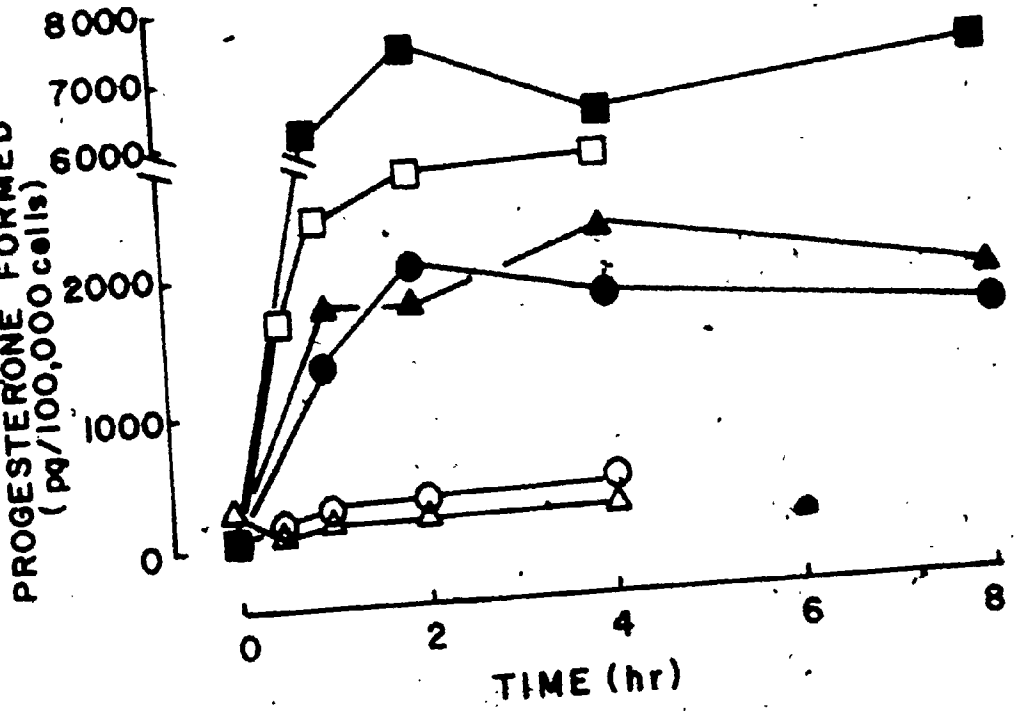

4000
3000
2000
1000
0
PROGESTERONE FORMED
(pg/100,000 cells/4 hr)
0
25
50
75
100
500
PREGNENOLONE (ng/ml)

FIGURE 40. Time course (upper panel) and dose response relationships (lower panel) of the conversion of 20α-dihydro-progesterone to progesterone in endometrium (squares), chorion (circles) and fetal cotyledons (triangles). Open and closed symbols represent data from two different animals. Cells were incubated with 100ng/ml 20α-dihydroprogesterone for 0-8h (time course) or with several concentrations of substrate for 4h (dose response). Each symbol is the mean of quadruplicates. Error bars were smaller than the symbols and, therefore, not shown.


PROGESTERONE FORMED
(pg/100,000 cells)
8000
7000
6000
2000
1000
0
0
2
4
6
8
TIME (hr)

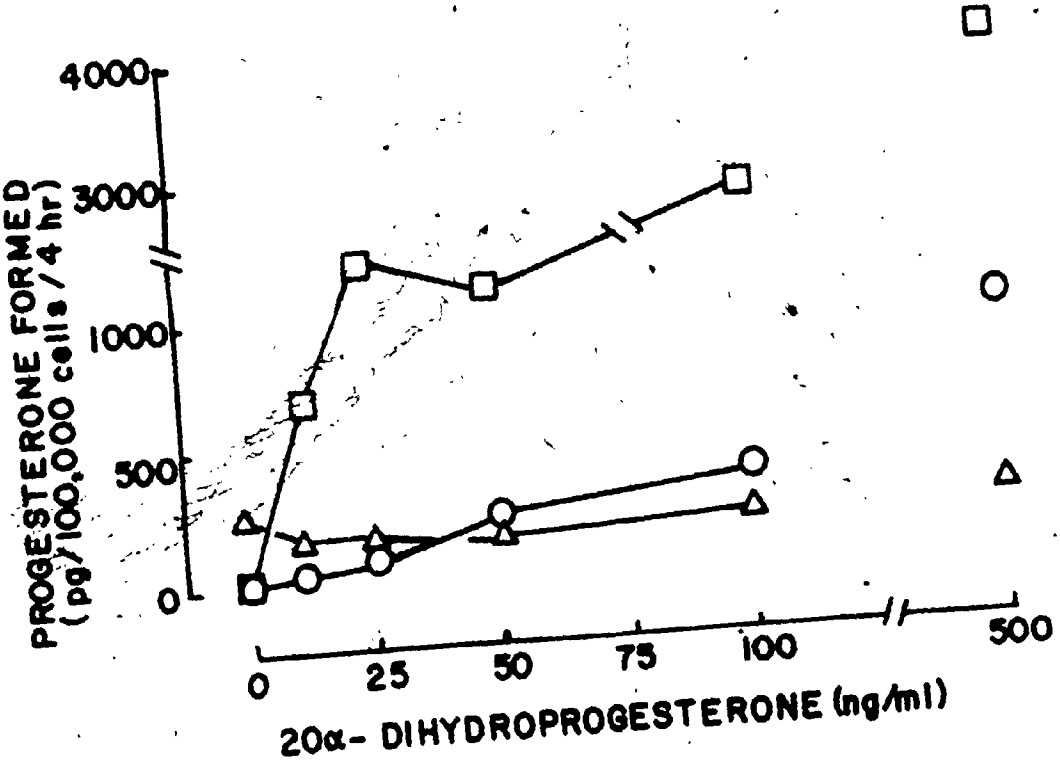
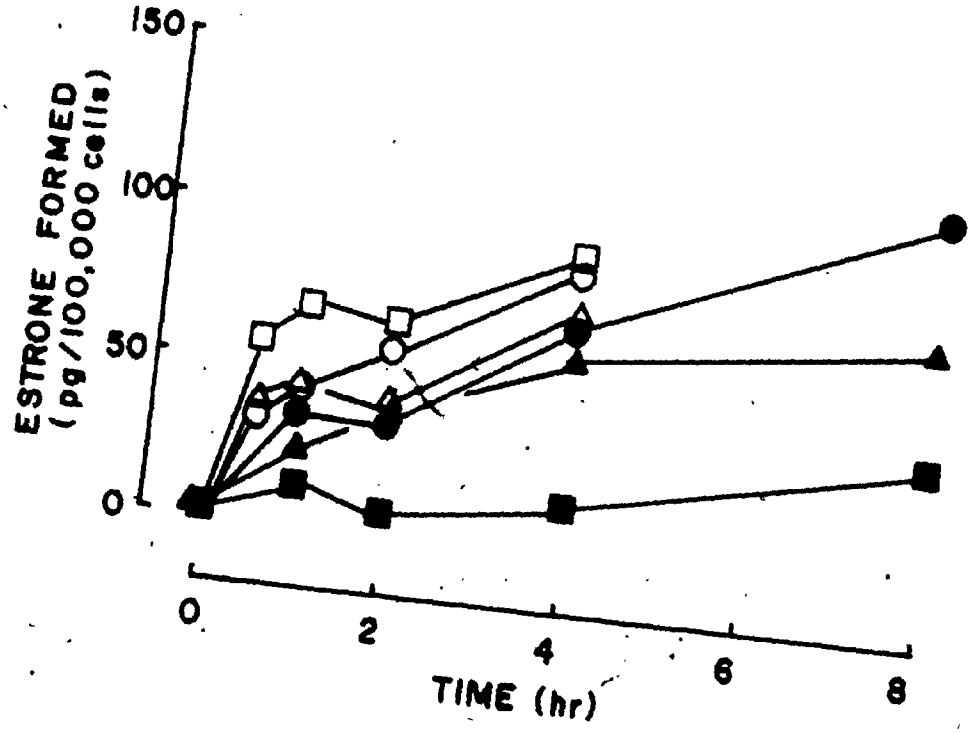
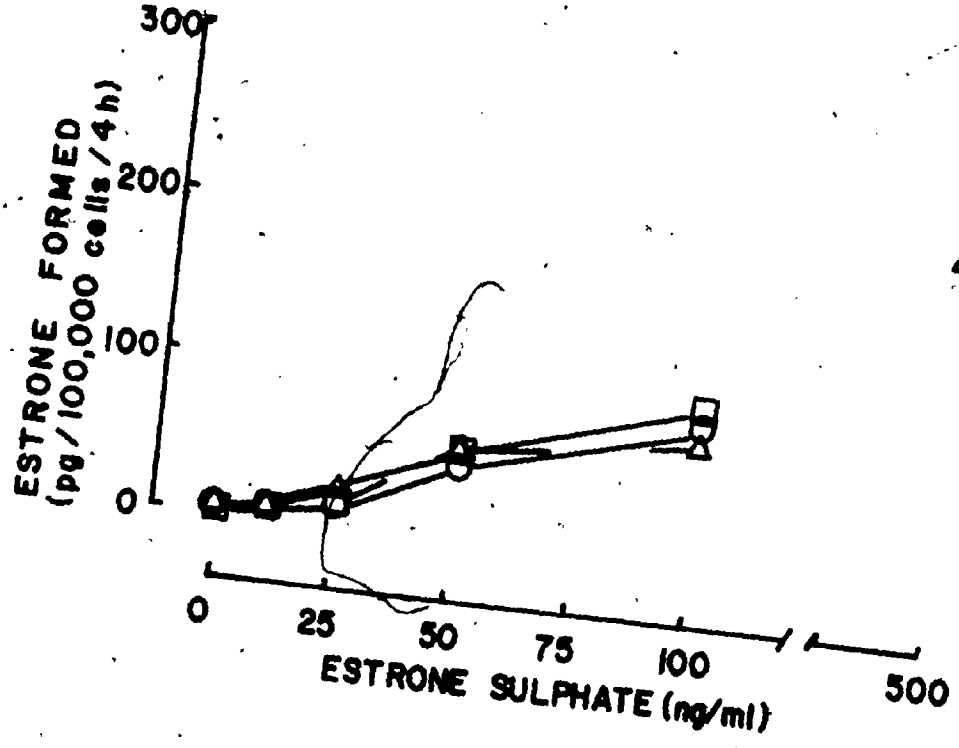

PROGESTERONE FORMED
(pg/100,000 cells/4 hr)
4000
3000
1000
500
0
0
25
50
75
100
500
20α- DIHYDROPROGESTERONE (ng/ml)

FIGURE 41. Time course (upper panel) and dose response (lower panel) of the conversion of estrone sulphate to estrone in endometrium (squares), amnion (circles) and fetal cotyledons (triangles). Open and closed symbols represent data from two different animals. Cells were incubated with 100ng/ml estrone sulphate for 0-8h (time course) or with several concentrations of substrate for 4h (dose response). Each symbol is the mean of quadruplicates. Error bars were smaller than the symbols and, therefore, not shown.


150
100
50
0
ESTRONE FORMED
(pg/100,000 cells)
0
2
4
6
8
TIME (hr)


300
200
100
0
ESTRONE FORMED
(pg/100,000 cells/4h)
0
25
50
75
100
500
ESTRONE SULPHATE (ng/ml)

incubation time and substrate concentration. The fetal cotyledons of the other animal produced P4 from 20α-DHP in similar amounts as the CHOR and both produced less than the ENDO. The conversions of E1-S to E1 in ENDO, AMN and cotyledons were similar with respect to net production and time course and dose response patterns (Figure 41).

8.3.6 Pregnenolone in Intrauterine Tissues

IR-P5 measured after purification on micro-celite columns was 53%, 111%, 84% and 72.6% that measured directly after extraction from AMN (537.5±100.6 pg/mg protein ± SEM; range 31.4-1627.7) CHOR (405.6±72.2; 44.5-853.5); ENDO (282.7±48.5; 29.2-686.4) and MYO (135.8±29.5; 25.3-431.6) respectively.

8.4 Discussion

I have shown that intact cells of intrauterine tissues possess several enzymatic activities capable of synthesizing and metabolizing P4 and Es and that the distribution of these enzymatic activities is tissue specific. P4 was produced from P5 (3β-HSD activity) in cells from CHOR and ENDO and from 20α-DHP (20α-HSD activity) in cells from all tissues, but more so in the CHOR, AMN, and ENDO than in the ALL and MYO. E1 was produced from Δ4 (aromatase activity) in cells from CHOR and from E1-S in cells from all tissues, but more so in the ENDO and AMN. In these experiments production of P4 was about ten-fold greater with 20α-DHP as substrate and production of E1 was about 100-fold greater with E1-S as substrate. E2 was produced from E1-S (estrone sulphatase and 17β-HSD activities) in cells from all tissues. Since the interconversion of E1 and E2 was not examined directly, it is difficult to draw any conclusions as to levels of 17β-HSD activity

in the cells. The 3β-HSD, 20α-HSD, estrone sulphatase and 17β-HSD activities were present throughout gestation but no significant change in the net production of P4 or ES from a constant level of substrate could be associated with the onset of parturition.

Both ^{3}H-P5 and ^{3}H-P4 were metabolized to polar metabolites cochromatographing with their monohydroxylated derivatives on TLC. In both experiments conversions were most prominent in the CHOR and ENDO and relatively low in the ALL and MYO. The similarities between the relative levels of ^{3}H-P5 and ^{3}H-P4 metabolism throughout gestation and between tissues suggests that ^{3}H-P5 and ^{3}H-P4 were substrates of the same enzyme(s). Most of the radioactive products from ^{3}H-P4 metabolism in AMN, ALL, ENDO and MYO cochromatographed on HPLC with 20α-DHP suggesting reduction of P4 by 20α-HSD in these tissues. The radioactive products from ^{3}H-P4 metabolism in CHOR eluted in two peaks on HPLC with similar retention times as 20α-DHP and both testosterone and 17α-hydroxyprogesterone. These results suggest P4 may be metabolized in the CHOR by 20α-HSD and 17α-hydroxylase. The production of 20α-DHP and 17α-P4 was confirmed by the recrystallization data. The relative levels of 20α-HSD activity (20α-DHP to P4) throughout gestation and between tissues bears resemblance to that of the conversion of ^{3}H-P4 to ^{3}H-20α-DHP. In these experiments, the 20α-HSD activity showed, generally, no preference for the reduced or oxidized substrate.

Chorionic cells of the day 21 sheep conceptus produce progesterone from endogenous substrate (Marcus, Ainsworth and Lucis, 1979) and metabolize exogenous radioactive progesterone (Marcus, Lucis, Ainsworth, 1979). In particular progesterone was metabolized by 20α-HSD, 17α-OHase and 5α-reductase. My studies established the

presence of 20α-HSD, 3β-HSD, and 17α-OHase throughout gestation in CHOR but did not identify 5α-reductase activity under the conditions of the experiments. It is interesting that after incubations of subcellular preparations of CHOR and AMN with ^{3}H-P5 (described in Chapter 7) only radioactivity corresponding to ^{3}H-P5 and ^{3}H-P4 was isolated. In those experiments cofactor availability (NAD+) was optimized. The lack of 3β-HSD in the amnion of intact cells and of any other metabolism of ^{3}H-P5 in the subcellular studies may be explained by differences in cofactor availability and the oxido-reductase state of the preparations. In the subcellular studies 3β-HSD activity in the CHOR was higher at term compared to earlier times in gestation. These findings were not verified in the intact cell preparations; however, the substrate P5 and the produced P4 were metabolized extensively by other enzymes in the intact cells. The human fetal membranes also possess several steroid metabolizing enzymes. 3β-HSD has been demonstrated in subcellular preparations of chorion laeve (Gant, Milewich, Calvert and MacDonald, 1977, DHEA-S as substrate); (Gibb et al, 1978, P5 and DHEA as substrate) and amnion (Gibb et al, 1978). 3β-HSD activity is low in amnion and there is significantly greater 3β-HSD activity in chorion and decidua (Gibb et al, 1978; Mitchell et al, 1982; Gibb, Lavoi and Morin-Gonthier, 1985). Both amnion and chorion laeve homogenates metabolize ^{3}H-P4 to tritium labelled 20α-DHP and 5α-pregnan-3,20-dione and, thus, possess 20α-HSD and 5α-reductase activities (Milewich, Gant, Schwarz, Prough, Chen, Athey and MacDonald, 1977). In the same studies 5α-pregnan-3, 20-dione was further metabolized to 5α-pregnan-3β-ol-20-one. The specific activity (per mg protein) of 20α-HSD was higher in chorion and that of 5α-reductase was higher in amnion.

A number of years ago Tseng, Stolee and Gurpide (1972) reported aryl sulphatase in chorion and aromatase and 17β-HSD activities in both chorion and amnion in experiments designed to study transfer of steroids across human fetal membranes. More recently Mitchell, Cross, Hobkirk and Challis (1984) demonstrated the ability of intact cells of chorion, and, to a lesser extent, amnion, to convert ^{3}H-E1-S to radiochemically pure ^{3}H-E1 and ^{3}H-E2. These authors suggested that the hydrolysis of E1-S was catalysed by a specific estrogen sulphatase since the reaction was not altered by excessive amounts of dehydroepiansdrosterone sulphate. Gibb and Lavoie (1981) confirmed the presense of aromatase in amnion and chorion using subcellular preparations.

The results from my studies extend the initial findings demonstrating 20α-HSD (Rossier and Pierrepoint, 1974b) estrone sulphatase and 17β-HSD (Rossier and Pierrepoint, 1974a) in minced tissue preparations of pregnant sheep myometrium and estrone sulphatase activity in endometrium (Dwyer and Robertson, 1980) Rossier and Pierrepoint (1974b) also demonstrated 5α-reduction of P4 in the myometrium. In the intact myometrial cells of my experiments no 5α-reduction of P4 was apparent. Cell yields of myometrium were relatively low in my experiments and a number of enzymatic activities may not have been detected. The human gestational myometrium (Trolp, Graf, and Breckwoldt, 1982) and decidua (Mitchell et al, 1984) also have the capacity to convert E1-S to E1 and E2β.

Mitchell et al (1984) demonstrated that human chorion cells from tissue obtained at spontaneous labour produced significantly more E1 from E1-S than chorion cells from tissue obtained at cesarean section before the onset of labour. In addition the chorion and decidua

cells from the spontaneous labour group produced significantly more E2 from E1-S than corresponding cells from the cesarean section group. They argued that an increase in E1-S hydrolysis at term could lead to a marked increase in free estrogen levels locally within the intrauterine tissues without significantly altering the maternal peripheral plasma. I have not demonstrated a similar increase in the intrauterine tissues of pregnant sheep.

Milewich et al (1972) reported a decreased rate of P4 metabolism in human chorion and amnion several weeks preceeding normal parturition at term. 20α-HSD and 5α-reductase activities decreased ten- to twenty-fold and two- to six-fold respectively after 33 weeks' gestation. The conversion of exogenous P5 to P4 was higher in decidua obtained at cesarean section than from patients at spontaneous labour (Mitchell, Challis, and Lukash, 1987), which could contribute to a local decrease in P4 production in decidua at the time of labour in women. I could not identify similar changes in the cells of sheep intrauterine tissues.

Despite large differences between mean net conversions, especially characteristic of 3β-HSD activity and 20α-HSD activity in the CHOR and ENDO between d130 and term, effects of gestational age and labour were not statistically significant. The cause of the large variation about the means of these activities is unknown though may be attributed to several factors. The enymatic activities may show fluctuations of activity through time or differ with respect to location in the uterus (i.e. distance from cotyledons). Tissues were taken consistently from the same area of the pregnant uterus (in the inter-cotyledonary space on the ante mesometrial side of the uterus) to decrease any effect different intrauterine locations would have on

the enzymatic activities. The levels of endogenous P4 in CHOR and ENDO also were characterized by large variations about the mean concentrations (Chapter 5). Between day 130 and 135 of gestation the plasma levels of cortisol show marked fluctuations when measured at 15 min intervals over several hours (Challis et al, 1981). These results were interpreted as resulting from intermittent output of cortisol from the fetal adrenal as it regained its responsiveness to ACTH in late pregnancy (Wintour et al, 1975; Glickman and Challis, 1980). The factors regulating steroidogenic enzymes in the intrauterine tissue require elucidation; however, it is interesting to speculate that these tissues are responding to a dynamic environment around day 130 in gestation resulting in gross changes in steroidogenic enzymatic activities and steroid levels over time.

IR-P5 was measured in the fetal membranes and maternal intrauterine tissues at levels comparable to those of P4 (see Chapter 5) in the respective tissues. In the CHOR and ENDO, the two extraplacental intrauterine tissues possessing significant 3β-HSD activity, most of the IR-P5 was authentic P5 on micro-celite columns. Thus P5 is a potential endogenous substrate for conversion to P4 by these tissues. In the AMN, authentic P5 accounted for only ~50% of the IR-P5, but was present at levels similar to those in the CHOR. P5 is present at similar levels in the maternal and fetal plasmas in late pregnancy (Nathanielsz, Elsner, Magyar, Fridshal, Freeman and Buster, 1982). The sulphaconjugated to unconjugated ratio is ~50:1 (Nathanielsz et al, 1982). It is possible that P5-S may be available to the CHOR and ENDO via the circulation for subsequent conversion to P5 or be secreted into the fetal fluids and subsequently made avail-

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able to the fetal membranes. P5-S and P5 have not been measured in the fetal fluids.

The sheep placenta has been well described as having a gestational age dependent ability to produce and metabolize P4 and Es (see literature review). Evidence is now emerging that the extraplacental extraembryonic derived fetal tissues possess similar capabilities. In these experiments the net productions of P4 and E1 from exogenous substrate in several intrauterine tissues were similar to those in the placenta. These enzymatic activities may be important with respect to a tissue's ability to regulate the local hormone milieu. The present studies indicate clearly the potential for steroid production in the fetal membranes endometrium and myometrium of pregnant sheep. However, the lack of change in these activities at term does not suggest that they occupy a major role in the sequence of events leading up to birth.

CHAPTER 9

GENERAL DISCUSSION

The results of the experiments described herein expand our knowledge of the role of progesterone (P4) and estrogens (Es) in the physiological mechanisms leading to parturition. P4 and Es figure prominently in the endocrine control of parturition in many species. The literature abounds with information describing the effects of these steroids on myometrial contractility. Tissues known or presumed to be targets of P4 and Es include the myometrium, endometrium and fetal membranes. Within these tissues P4 and Es have been implicated in the control of a) gap junction formation between myometrial smooth muscle cells b) receptor formation of myometrial stimulants such as oxytocin and adrenergic agents and c) prostaglandin production and release.

Viviparous species have adopted different endocrine controls of pregnancy and parturition thus complicating our understanding of these processes. There exist fundamental differences between sheep and humans. In particular, the falling progesterone and rising estrogen levels in peripheral plasma prior to parturition in sheep do not occur in the pregnant woman. The endocrinology of pregnancy and parturition probably provides only a partial characterization of these processes. We are now learning that there may exist paracrine/autocrine mechanisms that may be similar or different between species.

The objective of the studies herein was to investigate the origin and fate of P4 and Es outside the circulation in their target tissues. The results are summarized below:

1. Progesterone levels in the amniotic and allantoic fluids continue to rise throughout pregnancy until term and do not fall in late pregnancy as do levels in the maternal and fetal peripheral plasma.

2. Progesterone levels in the myometrium (MYO) and endometrium (ENDO) fall at spontaneous labour at term (SL) but not at ACTH induced premature labour. Changes in the MYO were less impressive than those in the ENDO.

3. Progesterone levels in the chorion (CHOR) and amnion (AMN) reach a maximum in late pregnancy but do not change at SL or ACTH-induced premature labour.

4. Estrogen levels in the MYO and ENDO rise at SL but levels in only the MYO rise at ACTH induced premature labour.

5. Estrogen levels in the AMN and CHOR do not change at SL or ACTH induced premature labour.

6. The ratio of estrone (E1) to estradiol-17β (E2) in the MYO and ENDO is 1-2:1 compared to a ratio of 5-10:1 in the maternal peripheral plasma.

7. The ratio of P4:E2 in the MYO decreases at SL and ACTH induced premature labour.

8. There is a tissue specific distribution of steroid metabolizing enzymes in the intrauterine tissues. The enzymes and their relative distribution are as follows:

Estrone sulphatase: ENDO, AMN>MYO, CHOR, allantois (ALL)

17β-hydroxysteroid dehydrogenase: CHOR, ENDO, AMN, ALL, MYO

20α hydroxysteroid dehydrogenase: CHOR, ENDO, AMN>ALL, MYO

3β-hydroxysteroid dehydrogenase: CHOR, ENDO

Aromatase: CHOR

17α-hydroxylase: CHOR

No change in the levels of activity of these enzymes could be identified with the onset of parturition. P4 was produced from 20α-dihydroprogesterone (20α-DHP) and pregnenolone (P5) with a 10:1 preference for 20α-DHP. E1 was produced from androstenedione and estrone sulphate (E1-S) with a 100:1 preference for E1-S.

9. The specific activity of 3β-hydroxysteroid dehydrogenase (3β-HSD; per mg protein) in the CHOR was similar to that in the cotyledons.

10. The Vmax of 3β-HSD in the CHOR was higher at term than at day 50.

11. The activity of 3β-HSD could be inhibited by E1 and E2.

From these results the following conclusions are drawn:

1. A rise in the levels of E1 and E2 in the myometrium was correlated with labour. The onset of labour can occur without changes in the levels of P4 in all tissues studied and without changes in the levels of Es in ENDO, CHOR, and AMN.

2. The levels of steroids in the intrauterine tissues may be influenced by local steroid metabolism; however, net conversions by the steroidogenic enzymes were unchanged at labour.

3. The inhibition of 3β-HSD in the CHOR by Es would appear to be chronic since their levels do not change significantly in this tissue throughout pregnancy and at labour. It supports the possibility of direct effects of steroids on steroidogenic enzymes.

I have not reported changes in endogenous steroid levels with respect to duration of labour. Animals in the studies herein were in labour for various lengths of time. As labour progresses it is

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possible that the levels of P4 and Es may change in tissues in which no change was observed. In addition, it cannot be concluded that the changes in estrogen levels in the MYO preceded the onset of spontaneous labour at term; however, during ACTH infusion the mean levels of E1 and E2 were elevated prior to the onset of labour. Kendall et al (1977) reported that ACTH induced labour occurs in sheep with hypothesectomized fetuses without a rise in the levels of Es in the maternal peripheral plasma. It would be interesting to know whether a rise in the levels of Es in the MYO and ENDO occurs in those animals.

It appears that any alteration of the prostaglandin producing capacity of the fetal membranes at term is not dependent on a change in the levels of P4 and Es in these tissues. P4 and Es may have no role in prostaglandin production by the fetal membranes or may act permissively allowing the action of other stimulants of prostaglandin production. In any case, they have been implicated in the regulation of fetal fluid volumes throughout pregnancy (Wintour et al, 1986).

The significance of steroidogenic enzymes in the intrauterine tissues is unclear. My results describe an overview of several enzymatic activities in five different tissues. The tissue specific distribution suggests specific regulatory processes. I have only analysed the activity of 3β-HSD in the CHOR in detail. Its activity was found to be similar to that in the placenta, a major source of P4 during pregnancy. Kinetic analysis of the other enzymes needs to be performed. In intact cells, the activities of 3β-HSD, 20α-HSD and estrone sulphatase, as expressed as net conversions of exogenous substrate, are similar between several of the major tissues possessing these activities and the placenta. It is interesting to note

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that the endometrium and chorion are the most active tissues. The juxtaposition of these two tissues within the pregnant uterus provides a route of communication between mother and fetal tissues other than at the placenta. Their enzymatic capabilities may allow them to regulate fetal to maternal and maternal to fetal steroid fluxes.

I have used dispersed cell preparations to investigate the enzymatic capabilities of the intrauterine tissues. I was interested whether potential substrate present in maternal or fetal plasma, fetal fluids or intrauterine tissues could be incorporated into the cells and metabolized. The placenta metabolizes P4 to 20α-DHP (Ainsworth and Ryan, 1967) and releases it into the circulation (Elsner et al, 1980) where it may be distributed to the intrauterine tissues for conversion back to P4. E1-S is the major estrogen in the fetal (Findlay and Cox, 1970; Currie et al, 1973) and maternal (Tsand, 1974; Carnegie and Robertson, 1978) plasmas and fetal fluids (Carnegie and Robertson, 1978; Challis and Patrick, 1980). Hydrolysis of E1-S to unconjugated E1 and subsequent conversion to E2β within intrauterine tissues provides a mechanism to alter local bioactive Es. I have measured P5 in the CHOR and ENDO and found levels similar to those of P4. P5-S is the predominent form of P5 in the maternal and fetal plasma (Nathanielsz et al, 1982). Hydrolysis of P5-S to P5 and subsequent conversion to P4 within the ENDO and CHOR provides a mechanism to alter local P4. Though not investigated in the studies described herein, cholesterol, either provided exogenously in low density lipoprotein (LDL) or produced de novo within the intrauterine tissues, can serve as a substrate for steroid biosynthesis. Trophoblastic tissue from the early sheep conceptus can produce cholesterol from simple precursors (Marcus et al, 1979).

Human trophoblastic cells in primary culture can internalize LDL (Winkel, Gilmore, MacDonald and Simpson, 1980) and increase their output of cholesterol and P4 (Winkel, Snyder, MacDonald and Simpson, 1980). It is worth noting that I used crude cell populations. It is possible that particular cell populations within a tissue may be responsible for a specific enzymatic activity described. A change in its contribution to the total cell population may have masked a change in net conversions per cell. Use of cell separation techniques is required to investigate this possibility. Gibb and Lavoie (1986) found different 3β-HSD:aryl sulphatase ratios in enriched preparations of collagenase dispersed cells from human chorion laeve isolated on Percoll gradients, thus lending support to this contention.

The net production or metabolism of P4 and Es can be regulated at at least two levels. Firstly, there is induction of enzyme synthesis. Cortisol has been shown to increase placental 17α-hydroxylase (Anderson et al, 1978) and aromatase (Ricketts et al, 1980). Progesterone stimulates an increase in human endometrial 17β-and 20α-HSD. (Tseng and Gurpide, 1974, 1975). Catecholestrogens increase the secretion of P4 and E2 from placental explants maintained in organ culture (Barnea and Hasan, 1985). This effect was inhibited by α- and β- adrenergic blockers suggesting an effect mediated through adrenergic receptors. Caritis and Zeleznik (1980) have reported that the β-sympathomimetics, isoproterenol and terbutaline, could stimulate the accummulation of P4 in human placental explant media. β-adrenergic receptors have been found on human placenta (Falkay and Kovacs, 1983). P4 secretion by choriocarcinoma has been shown to be stimulated by epidermal growth factor (EGF; Benvenesti, Speeg,

Carpenter, Cohen, Linder, and Robinowitz, 1978). EGF receptors have been found on human placental syncytiotrophoblast membrane (Richard, Beardmore, Brown, Molloy and Johnson, 1983, Rao, Carman, Chegini and Schultz, 1984; Chegen and Rao, 1985), decidua (Chegini and Rao, 1985) and human fetal membranes (Rao et al, 1984, Chegini and Rao, 1985). Thus steroids, peptide hormones and regulatory transmitters are putative regulators of steroidogenic enzymes in intrauterine tissues. Direct effects of these substances on steroidogenic enzymes in intrauterine tissues needs to be studied in cells grown as monolayer cultures.

Secondly, there are the direct effects of substances on the enzymes altering substrate enzyme affinity and maximum velocity. Powell and Challis (1986) reported an increase in the net conversion of ^{3}H-P5 to ^{3}H-P4 in the presence of 20α-DHP. It was shown that 20α-DHP inhibited metabolism of ^{3}H-P4 to ^{3}H-20α-DHP. Grimshaw, Mitchell and Challis (1983) showed that P4 output by human placental cells could be increased by 5α-dihydrotestosterone and 5α-pregnanedione by preventing P4 metabolism by 5α-reductase. P4 production is dependent not only on the direct effects of steroids such as Es and DHEA on the synthesizing enzymes but also on end product inhibition of the metabolizing enzymes. In addition, it is important to note that these regulatory steroids are not necessarily bioactive. Studies need to be performed to investigate net conversions of substrate to product in the presence of different levels of substrate, product, metabolites and inhibitors.

Sequestration within tissues by specific and non-specific protein receptors is another way the cell can alter endogenous concentrations of P4 and Es within the tissues. Studies on

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progesterone and estrogen receptor levels in intrauterine tissues of pregnant sheep are lacking. The rise in the levels of E1 and E2 at labour may result from an increase in the levels of an estrogen receptor. The relative predominance of E2 in the MYO and ENDO compared to the maternal peripheral plasma may suggest a higher affinity of this receptor for E2. Alternatively 17β-HSD activity may set this predominance of E2. P4 and estrogen receptors have been measured in the human intrauterine tissues during pregnancy (Dawood and Dawood, 1984; Giannopoulos, Goldberg, Shea and Tulchinsky, 1980; Giannopoulos and Tulchinsky, 1979; Tulchinsky and Giannopoulos, 1983). The total estrogen and total P4 receptor levels in the myometrium are lower at term and labour than during the menstrual cycle. Tulchinsky and Giannopoulos (1983) argued that the receptor levels were lowered by the high concentrations of P4 during pregnancy. They also showed a 30% to 50% reduction of both estrogen and P4 receptor levels in the myometrium in labour; however, this result may have been an artifact of sample collection. Cytoplasmic receptors for P4 and Es are low or undetectable at term and the nuclear receptors are almost saturated with endogenous hormone. During the menstrual cycle most of the receptors are cytoplasmic. This distribution of receptor was attributed to the high circulating and tissue levels of both Es and P4 during pregnancy resulting in a greater degree of translocation of cytoplasmic receptor to the nucleus.

In the rat, a species in which there is a withdrawal of P4 and a rise of Es in peripheral blood toward the end of pregnancy, there is a fall of nuclear P4 receptors as well as a rise of nuclear estrogen receptors. (Vu Hai, Logeat, Warembourg and Migrom, 1977). As the

concentration of P4 falls so does the translocation of cytoplasmic P4 receptors to the nucleus. As the peripheral estrogen levels rise there is an increase in the translocation of cytoplasmic estrogen receptors to the nucleus.

Dawood and Dawood (1984) were unable to detect cytoplasmic or nuclear receptors for E2 or P4 in the chorion and amnion; however both tissues had higher levels of E2 and P4 than those in the decidua and myometrium. It is possible that the steroid levels in human fetal membranes are influenced more by local steroidogenesis than by sequestration at receptors.

Intrauterine tissues of both the pregnant sheep and pregnant woman possess the potential to produce P4 and Es. At least in the human, the activity of some of the steroidogenic enzymes change with the onset of labour. These changes might alter the local steroid levels without effecting peripheral plasma levels. These enzyme activities must also be considered when administering steroid either into the peripheral plasma of mother and fetus or into the fetal fluids. They will effect the flux of steroid across fetal membranes and between maternal and fetal tissues.

It is only through an elucidation of the physiological mechanisms that control pregnancy and the onset of parturition will the etiology of preterm labour be determined. In the future it is hoped that the obstetrician will be able to use a more scientific approach to the management of an old obstetrical problem.

SECTION III

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AND

CURRICULUM VITAE

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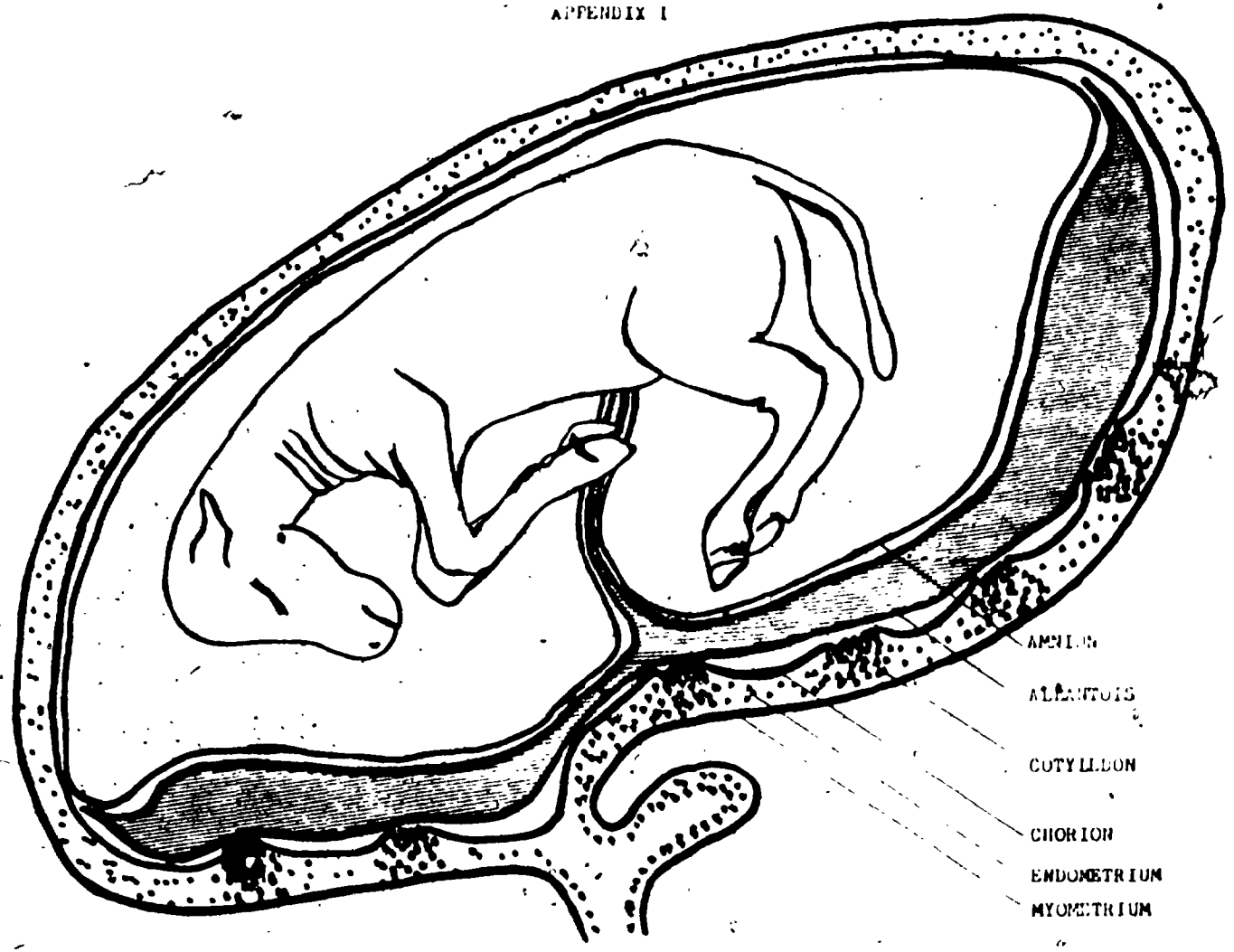
APPENDIX I


AMNION
ALLANTOIS
COTYLEDON
CHORION
ENDOMETRIUM
MYOMETRIUM

APPENDIX 2: Progesterone antibody (Dr. B.J.A. Furr):

cross reactivities

	Percent Cross Reactivity
Pregn-4-en-3,20-dione	100.0
Pregn-4-en-11α-ol-3,20-dione	35.9
Pregn-4-en-21-ol-3,20-dione	23.8
Pregn-4-en-11β-ol-3,20-dione	3.4
Pregn-4-en-11β,21-diol-3,20-dione	0.92
Estra-1,3,5(10)-trien-3-ol-17-one	<0.25
Estra-1,3,5(10)-trien-3,17β-diol	<0.25
Pregn-4-en-17α-ol-3,11,20-trione	<0.18
Pregn-4-en-17α,21-diol-3,20-dione	<0.18
Pregn-5-en-3β,17α-diol-20-one	<0.18
Pregn-5-en-3β-ol-20-one	<0.18
5α-pregnan-3α,20α-diol	<0.18
pregn-4-en-17α,21-diol-3,11,20-trione	<0.18
Cholesterol	<0.18

APPENDIX 3: Estrogen antibodies; cross reactivities

	Percent Cross Reactivity Antibody	
	Estrone	Estradiol-17β
Estrone	100.0	1.14
16β-epiestriol	2.6	3.04
16-oxo-estradiol-17β	1.9	0.35
Estradiol-17α	1.5	0.70
Estradiol-17β	0.1	100.00
Progesterone	0.1	0.1
Pregnenolone	0.1	0.1
Testosterone	0.1	0.1
Cortisol	0.1	0.1