SYSTEMATIC STUDY OF PREIMPLANTATION STAGES OF PREGNANCY IN THE SHEEP

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Summary

Pregnant and non-pregnant ewes were killed at various times during the period up to 15 days after service. The purpose of the study was to examine a number of parameters related to ovarian and uterine activity with a view to establishing the point at which the embryo first has measurable effects on the physiology of the ewe.

The sheep zygote increased slowly in size until days 10–11 when the zona pellucida was shed. After this time the embryo increased rapidly in size, reaching a length of about 10 cm by day 15. In non-pregnant ewes uncleaved ova were recovered, with zona still present, up to day 15. The number of uterine glands and the heights of the uterine and glandular epithelia were similar in pregnant and non-pregnant ewes at all stages examined. The embryo thus causes no morphological alteration of the secretory areas of the uterus before day 15. Evidence is presented to show some alteration of the endometrial caruncular epithelium in pregnant ewes on day 15.

The total number of ovarian follicles was greater in the non-pregnant than pregnant ewes on days 13–15. Corpus luteum weight, even after elimination of variation due to number of ovulations, was a highly variable parameter and did not differ significantly between pregnant and non-pregnant ewes. Two separate measures of pituitary activity (follicle-stimulating hormone and "total gonadotrophin" content) failed to reveal any differences that might be associated with the mechanism that ensures the persistence of the corpus luteum in the pregnant animal.

I. INTRODUCTION

The term implantation is used rather loosely in relation to the physiology of both laboratory and domestic animals. It has been used to describe the "embedding" of the mammalian egg in the uterus and also as a synonym for placentation. Both Boyd and Hamilton (1952) and Amoroso (1952) in their reviews of the subject give the impression that implantation marks the commencement of the broad process of placentation leading to the formation of the definitive placenta. This seems a sensible interpretation and allows localization of the time of implantation to that point in time when there is evidence of morphological alteration of embryonic and maternal tissue due to contact between the two. An early investigation (Green and Winters 1945) concluded that such alteration occurred about day 10. There are now a number of reports to show that this event does not take place until days 16–18 (Boyd and Hamilton 1952; Chang and Rowson 1965; Boshier 1969).

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Of more significance to the present study is not implantation in the physical or anatomical sense but rather the time at which the embryo first signals its presence to the pituitary–ovarian system of the ewe. Somehow, the embryo prevents the regression of the corpus luteum that normally commences on days 14–15 of the oestrous cycle. In this regard, the demonstration by Moor and Rowson (1966) that transfers of blastocysts to the uteri of sheep in the oestrous cycle are only successful when performed before day 13, is of paramount importance. The inference is that day 13 signifies the first difference between the physiology of the pregnant and the non-pregnant ewe.

The purpose of the present study was to compare pregnant and non-pregnant ewes on the basis of a number of parameters related to the activity of the ovary, the uterus, and the pituitary gland in the period up to day 15.

II. Materials and Methods

(*a*) *Sheep and Management*

The ewes were Merino and unidentified Merino crossbred animals, aged 3–5 years, weighed 40–45 kg, and included both parous and non-parous animals. They were maintained indoors at Sydney University where they experienced the natural fluctuations of light and temperature but were protected from rainfall and, to some extent, wind. The ewes were group-fed on a diet of equal parts *M. sativa* and *Triticum* spp. chaff. They were fed once daily at 0900 hr on sufficient of this material to allow slow growth.

(*b*) *Detection of the Day of Oestrus*

Vasectomized rams equipped with marking crayons were placed with the ewes for a preliminary period to record the regularity of oestrous cycles. When the experiment commenced the ewes were placed at random with either entire or vasectomized rams and inspected daily at 0900–1000 hr for evidence of oestrus. This schedule thus involves an error of up to 24 hr in the estimation of the time of onset of oestrus. The day of oestrus was designated day 1.

After service all ewes were placed with vasectomized rams until slaughter, to check returns to service.

(*c*) *Experimental Design*

Groups of pregnant and non-pregnant ewes were killed on days 5, 7, 9, 11, 13, and 15 of pregnancy or the oestrous cycle. Additional ewes, from another experiment, were killed on days 1, 3, 6, 9, and 12 of the oestrous cycle. For each ewe the following were collected or recorded:

(1) blood sample for plasma progesterone determination;
(2) pituitary gland, weighed and stored at −20°C, for gonadotrophin assays;
(3) ovaries, removed and scored for follicular development;
(4) corpus luteum number and weight recorded, and section fixed for histology;
(5) uterus flushed for the presence of an embryo or unfertilized ovum;
(6) section of uterine horn fixed for histology;
(7) appearance of cotyledons noted.

(*d*) *Collection of Tissue*

Prior to slaughter a blood sample (10–15 ml) was drawn from the jugular vein into a heparinized syringe. The sample was centrifuged at 4°C and 2000 r.p.m. for 20 min. Plasma was drawn off and stored at −20°C in glass vials. Sheep were killed by exsanguination. The reproductive tract was removed by severing the suspensory ligaments and the cervix as far caudally as possible. The reproductive tract was wrapped in gauze soaked in 0·9% NaCl until examination, generally within 1 hr of slaughter.

A standardized procedure was followed when examining the reproductive organs. The ovaries were removed and classified with regard to the following criteria:

(1) position—left or right side;

(2) corpora lutea—number and trimmed wet weight;

(3) follicles—total follicle score estimated by attributing a score of 0·1 for each 1 mm of follicular diameter for all follicles greater than 2 mm diameter.

The uterus was then prepared for flushing by placing artery forceps at the cranial edge of the cervix and at a point just caudal to each uterotubal junction. Each uterine horn was flushed with 5·0 ml of 0·9% NaCl using a hypodermic syringe and 23-gauge needle. The flushing medium was collected via a glass funnel into a 20-ml vial. When large embryos were present in the flushing medium this was transferred to a 7 cm diameter Petri dish for further examination under a binocular microscope with transmitted light. Small zygotes or unfertilized eggs were located by examining 2-ml aliquots of the flushing medium in egg dishes at magnifications of ×45. All measurements were made using an ocular micrometer.

The hypophysis was quickly located and excised and placed in a vial maintained at 4°C. This procedure was generally completed within 5 min of death. Within the next 30–60 min the pituitary gland was trimmed of connective tissue, the posterior lobe removed, and the wet weight of the anterior lobe recorded. The gland was placed in a new vial and frozen in a mixture of CO_2 and ethanol before storage at −20°C.

(*e*) *Tissue Samples for Histology*

After flushing the reproductive tract, a segment of uterine horn approximately 1 cm in length was taken from the same side as the ovary containing the corpus luteum. Where both ovaries contained corpora lutea, one side was sampled at random. The segment was taken at roughly the mid-point of the uterine horn and stored in 15 ml Bouin's fixative for at least 24 hr. The tissue was subsequently maintained in 70% ethanol until processing.

Samples of corpora lutea were prepared for histology in the same manner. All samples were then dehydrated, embedded in Paraplast, and duplicate cross-sections 8 μm thick were cut. The sections were stained with haematoxylin and eosin and a cover-slip positioned with mounting medium.

(*f*) *Bioassay of Pituitary Tissue*

Each anterior pituitary gland was homogenized in 5·0 ml NH_4HCO_3 buffer (0·15M, pH = 7·2). Subsamples of this homogenate (generally 0·1 and 0·3 ml) were then assayed for follicle-stimulating hormone (FSH) by the hypophysectomized mouse uterine weight method of Lamond and Bindon (1966), against a standard preparation (NIH–FSH–S3) at doses of 1·1, 3·3, and 10 μg. There were five test animals per dose level of standard and unknown. The results are expressed as μg-equivalents of NIH–FSH–S3 per pituitary gland.

The bioassay for "total gonadotrophin" was a modification of the intact mouse uterine weight test described by Brown and Billewicz (1962). Immature mice (five per group) were injected at 1600 hr on the first day and at 0900 and 1600 hr on the second day with dose levels (usually 0·1, 0·033, and 0·011 ml) of the homogenate described above. The mice were killed at 1000 hr on the third day and body weights and uterine weights recorded. The standard preparations were extracts of human post-menopausal urine known to contain both FSH and luteinizing hormone activities. One of the two standard preparations (Pergonal, Cutter Laboratories; Humegon, Organon Laboratories) was used each week (doses of 15 and 30 μg) when approximately three pituitary glands were assayed. The results are expressed as μg-equivalent of Pergonal per pituitary gland.

(*g*) *Determination of Plasma Progesterone*

The method used is that described by Bassett and Hinks (1969) and Thorburn, Bassett, and Smith (1969). This test, based on the competitive protein-binding principle, responds to compounds other than progesterone, including 17α-hydroxy-4-pregnen-3,20-dione and 20α-hydroxy-4-pregnen-3-one. The results are more correctly "total progestagen" levels although progesterone has been

TABLE 1

RECOVERY, FORM, AND DIMENSIONS OF SHEEP ZYGOTES DURING EARLY PREGNANCY

Day of Pregnancy	No. of Ewes Examined	Total No. of Corpora Lutea	Total Zygotes Recovered	Zygotes with Zona	Description of Zygotes	Diameter of Early Zygotes		Trophoblast*		Embryonic Disc*	
						Zona (μm)	Vitellus (μm)	Length (mm)	Width (mm)	Length (μm)	Width (μm)
5	5	9	6	6	8 cell to morula	167·5± 6·8	136·2± 9·2	—	—	—	—
7	5	5	5	4	Morula to spherical blastocyst	183·5± 8·9	167·2±20·7	—	—	—	—
9	5	10	6	3	Morula to spherical blastocyst	206·7±47·1	183·3±58·5	0·25±0·03	0·18±0·04	45·0±5·0	45·0±5·0
11	3	4	4	0	Spherical blastocyst	—	—	0·43±0·09	0·34±0·09	67·5±11·8	67·5±11·8
13	4	5	5	0	Expanding blastocysts	—	—	32·9 ±13·1	1·01±0·21	490·0±132	370·2±80
15	5	10	7	0	Elongated blastocysts	—	—	67·9 ±17·2	1·14±0·38	657·0±138	468·6±96

* Dimensions of expanding blastocysts.

indicated as the dominant compound measured in the plasma of non-pregnant sheep (Thorburn, Bassett, and Smith 1969). For convenience the results are referred to as plasma progesterone. Duplicate 1·0-ml samples from each ewe were examined.

(*h*) *Statistical Methods*

Most of the data were insufficiently balanced to allow complete analyses of variance. For this reason use was made of a computer program prepared by Dr. P. J. Claringbold and Mr. N. H. Westwood, Division of Animal Genetics, CSIRO. After appropriate tests of equality of within-group variances an overall comparison of variance within and between groups was made. Further tests of significance of differences between group means were made as described by Snedecor (1956).

The bioassay data were analysed with the aid of a program made available by Mrs. J. Williams, Division of Mathematical Statistics, CSIRO.

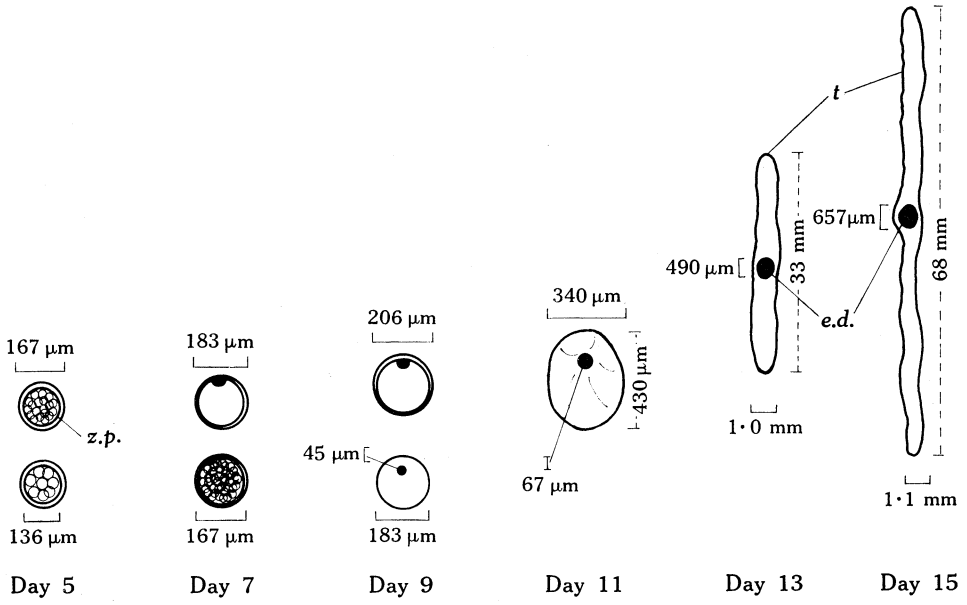


Fig. 1.—Diagrammatic representation of the growth and development of the sheep embryo before implantation. Day 1 is the day of oestrus. Day 5 shows the eight-cell to morula stage, day 7 morula to blastocyst, day 9 spherical blastocyst, and days 11, 13, and 15 stages of the expanding blastocyst. Zona pellucida (*z.p.*) is present up to and sometimes including day 9. *t*, trophoblast; *e.d.*, embryonic disc.

III. Results

(*a*) *Growth and Development of the Early Embryo*

The recovery, form, and dimensions of the sheep zygotes recovered are presented in Table 1. The data have been summarized in diagrammatic form against a time scale in Figure 1. A high proportion of these ewes had more than one ovulation and in spite of randomization all the pregnant ewes killed on day 15 had two ovulations. Only 33 out of 43 (77%) of the potential embryos were recovered. Part of this loss may be attributed to losses associated with the flushing procedure early in the study, although the presence of only 7 out of 10 potential embryos on day 15, when it is impossible to miss the embryo, suggests that substantial embryo mortality was

occurring. Such mortality does not include failure of fertilization, since these cases are represented by degenerating eggs, still present in the uterus on day 15 (Bindon 1969).

All zygotes had lost the zona pellucida by day 11. Prior to this, zygote growth was very slow. After day 11 the growth was very rapid, although this was mainly the result of rapid increase in the length of the trophoblast (chorionic vesicle).

There was a suggestion that twin embryos were smaller than singles on day 15. The best estimate of this comparison is presented in Table 2, which includes extra animals from another experiment. The difference in embryo length is not significant.

TABLE 2

COMPARISON OF ALL SINGLE AND TWIN SHEEP EMBRYOS ON DAY 15 OF PREGNANCY

Type	No. of Embryos	Mean Trophoblast Length (cm) (±S.E.)*	Summary of Overall Analysis of Variance			
			Source of Variation	D.F.	Mean Square	*F* Ratio
Single	6	11·72±2·84	Between groups	1	6,357·1	1·90
Twin	13	7·78±1·44	Between embryos, within groups	17	3,342·1	

* Bartlett's test $\chi^2_{(1)} = 0{\cdot}63$ (n.s.). Variances homogeneous.

(*b*) *Fate of the Unfertilized Sheep Ovum*

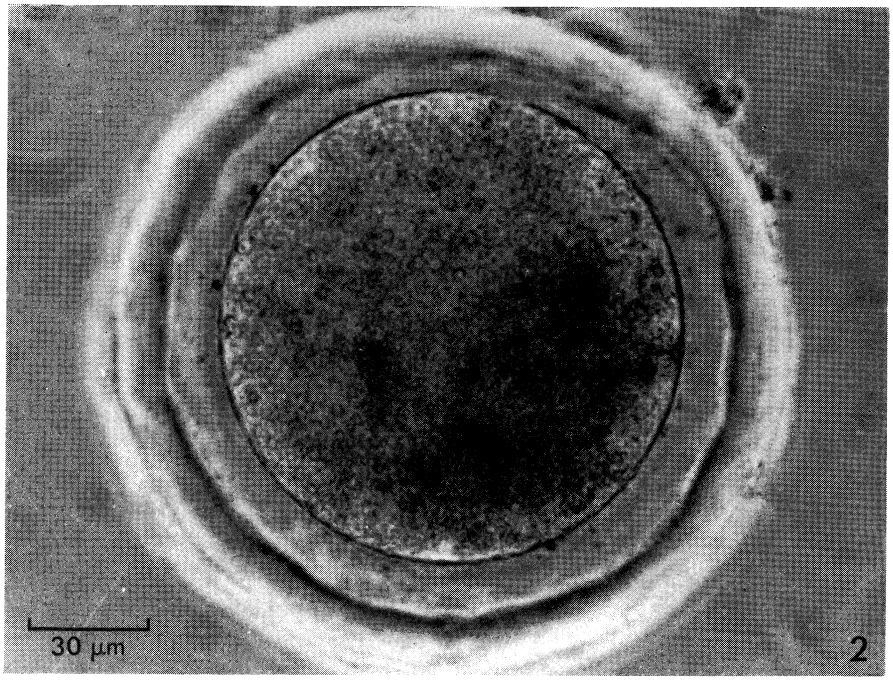
The recovery of ova from the non-pregnant ewes in this experiment has been reported separately (Bindon 1969). Examples of these ova, recovered on day 15, are shown in Figures 2 and 3.

(*c*) *Weight and Histology of Corpus Luteum*

Only single ovulations are included here since there is no valid way of comparing luteal weight from animals with more than one ovulation, and in any case, these were represented by variable numbers in the various classifications. Twin ovulations invariably produced a total luteal weight in excess of one single corpus luteum.

The results in Table 3, even after pooling data from several days, are limited by the inequality of subclass sizes. An overall analysis is presented which indicates significant differences among the group means. The only firm conclusion that can be made is that in both pregnancy and the oestrous cycle there is a significant increase in luteal weight between period 1 (days 3–6) and period 2 (days 7–9). The apparent decline in weight in both groups of ewes between days 11–12 and 13–15 is obscured by the small number of observations in some groups.

Representative histological sections from days 5, 7, 9, 11, 13, and 15 of pregnancy and of the oestrous cycle are shown in Figures 4–9 and 10–15 respectively. The only feature that can confidently be discussed is the fluctuation in the relative number of large lutein cells characteristic of peak luteal activity. These cells predominate on days 9 and 11 of the oestrous cycle and days 11 and 13 of pregnancy. It may be significant that both pregnant and non-pregnant ewes showed signs of reduced size and number of lutein cells on day 15. More pronounced signs of luteal regression, degeneration of nuclei of lutein cells, are not expected until day 16 of the oestrous cycle (Deane *et al.* 1966*a*).



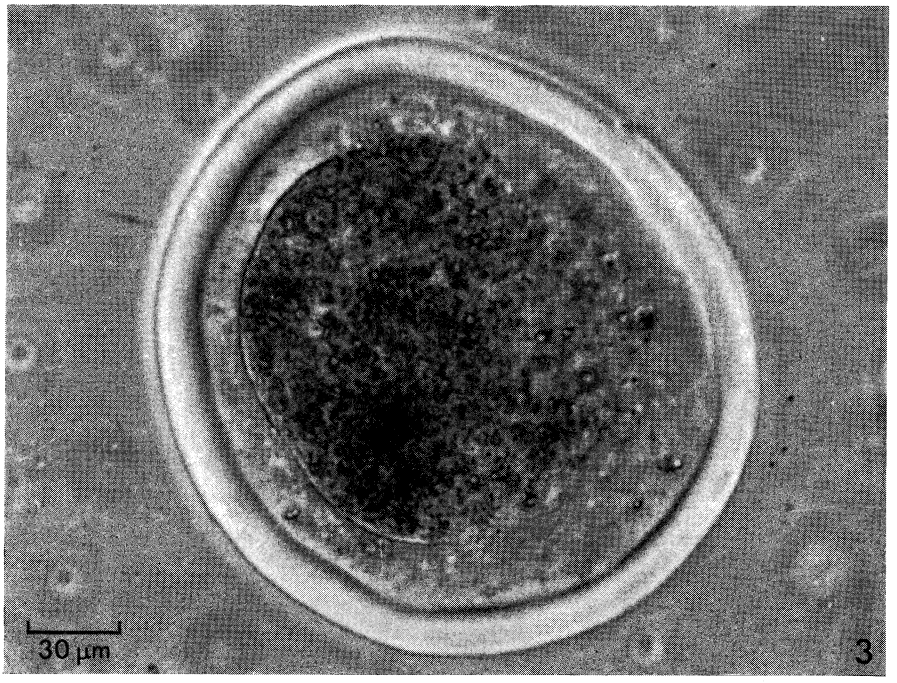
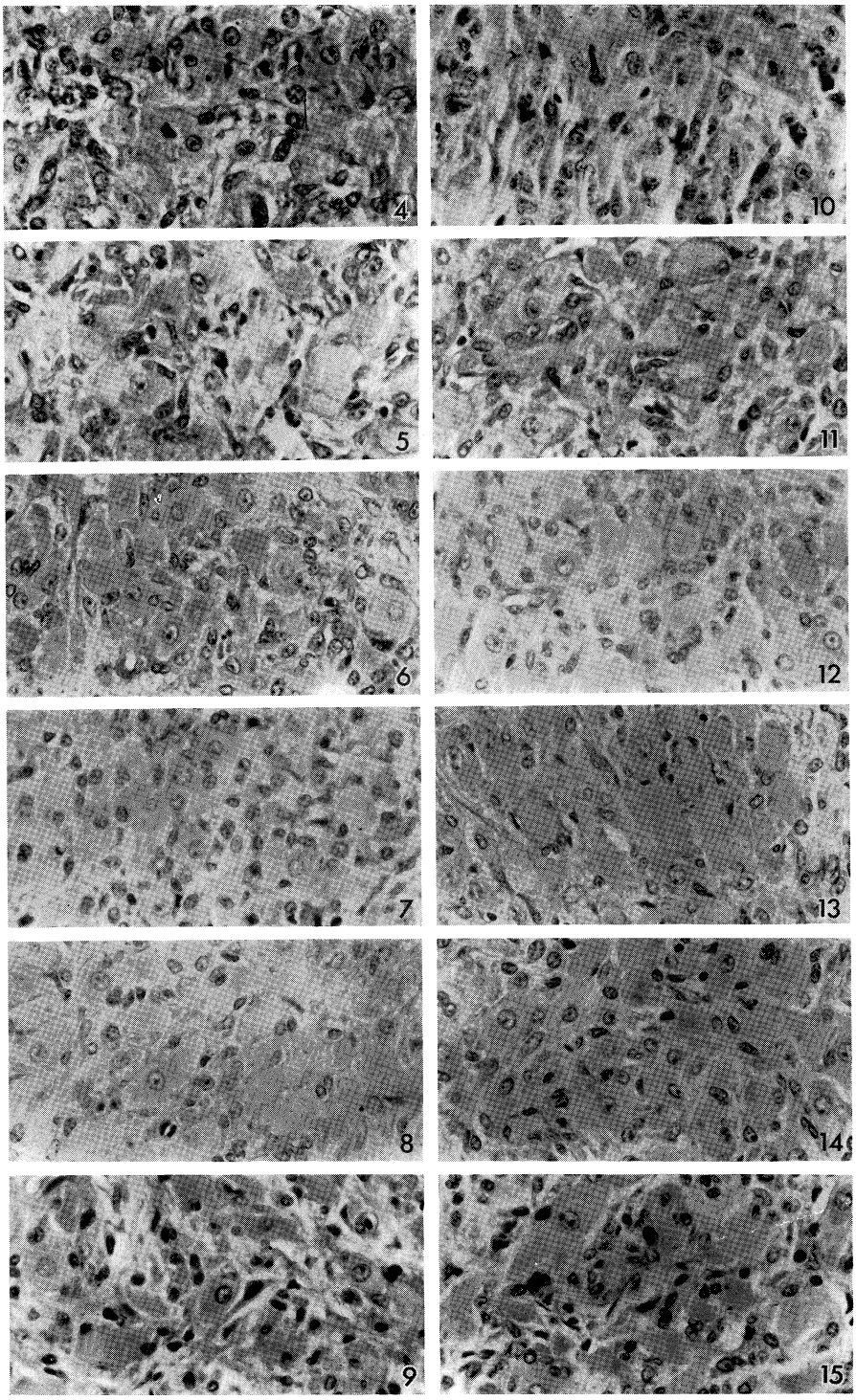


Fig. 2.—An unfertilized sheep ovum recovered from the uterus on day 15 of the oestrous cycle. Note intact vitelline membrane and zona pellucida.

Fig. 3.—An unfertilized sheep ovum recovered from the uterus on day 15 of the oestrous cycle. Note fragmentation of the vitelline membrane.



Figs. 4–15.—Sections of sheep corpora lutea, stained with haematoxylin and eosin. Figures 4–9 represent days 5, 7, 9, 11, 13, and 15 of pregnancy. Figures 10–15 represent days 5, 7, 9, 11, 13, and 15 of the oestrous cycle. ×200.

(*d*) *Uterine Histological Measurements*

Changes observed in the uterine sections were placed on a quantitative basis by the procedure described by Restall (1966). By means of an ocular micrometer the heights of the uterine and glandular epithelia were measured in each section. Care was taken to choose an area that contained cells cut in the one plane. The number of glandular coils in an intercotyledonary area was estimated by counting the number of coils that transected an arbitrary line, 1 mm in length, extending from the myometrium to the edge of the lumen.

TABLE 3

MEAN CORPUS LUTEUM WEIGHT AT FOUR STAGES OF EARLY PREGNANCY OR THE OESTROUS CYCLE OF THE EWE

Single ovulations only

Period	Pregnancy		Oestrous Cycle	
	No. of Ewes	Mean Corpus Luteum Weight (mg) (±S.E.)	No. of Ewes	Mean Corpus Luteum Weight (mg) (±S.E.)
I(days 3–6)	18	265·7±51·1	8	283·1±38·8
II(days 7–9)	18	548·2±26·2	8	587·9±50·8
III(days 11–12)	13	671·9±35·5	4	788·3±40·0
IV(days 13–15)	4	561·0±97·3	10	594·6±60·5

Bartlett's test for homogeneity of variances: $\chi^2_{(7)} = 12{\cdot}9$ (n.s.)

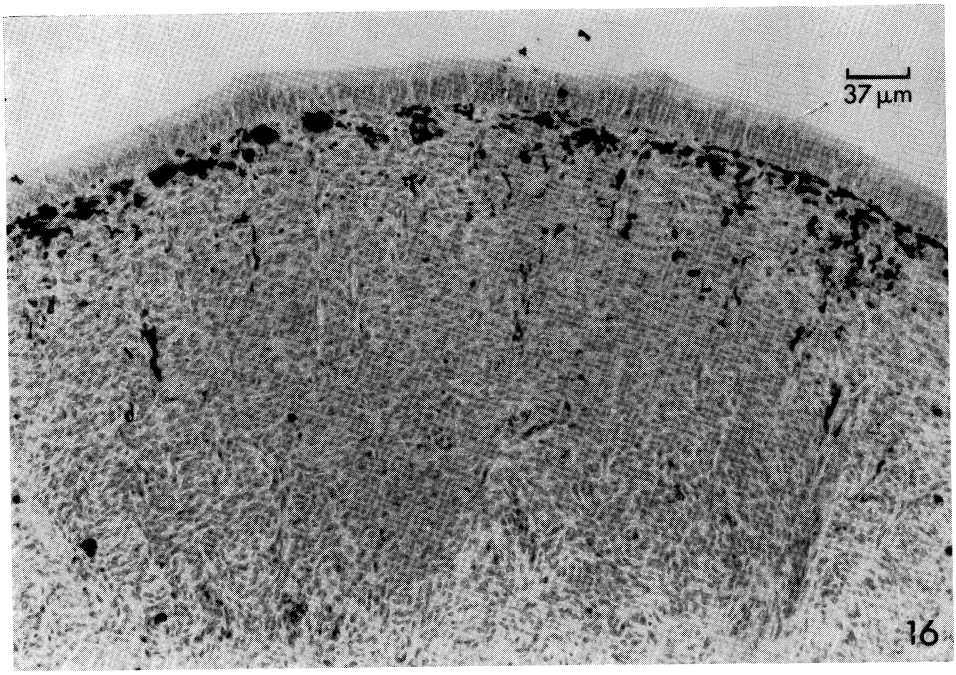
Summary of overall analysis of variance

Source of Variation	D.F.	Mean Square	*F* Ratio
Between groups	7	326,915·9	12·94***
Within groups	75	25,256·3	

*** $P < 0{\cdot}001$.

The mean results are shown in Table 4. The amount of glandular development did not differ significantly in any of the groups. Similarly the height of the uterine epithelium remained fairly constant. The height of the glandular epithelium declined significantly between periods I and II in both pregnant and non-pregnant ewes. The uterine glands are apparently more active during the early period following ovulation than later in the preimplantation period.

There is nothing in these results to suggest that the embryo is causing significant alteration of the uterus up to day 15. An observation which may reflect this, however, is presented in Figures 16 and 17. There appears on day 15, beneath the caruncular epithelium of pregnant ewes only, some darkly stained unidentified material that may reflect alteration of the caruncular vascular supply or the early stages of shedding of the caruncular epithelium. The phenomenon is apparently not a fixation artefact since it was seen only in the caruncular area and was confined to the day 15 pregnant



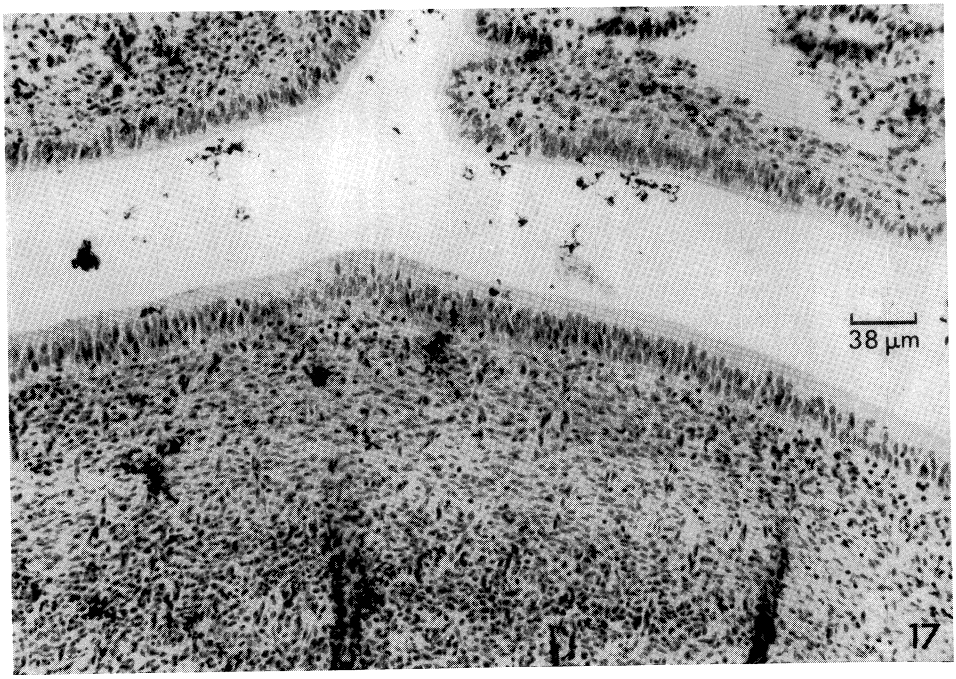


Fig. 16.—Section through endometrial caruncle of ewe on day 15 of pregnancy, stained with haematoxylin and eosin. Dark-staining elements thought to reflect weakening of luminal epithelium prior to invasion by trophoblast.

Fig. 17.—Section through endometrial caruncle on day 15 of the sheep oestrous cycle. Contrast with Figure 16.

TABLE 4

SUMMARY OF HISTOLOGICAL OBSERVATIONS ON SHEEP UTERI DURING EARLY PREGNANCY AND THE OESTROUS CYCLE

Period	Pregnancy				Oestrous Cycle			
	No. of Ewes	(i) No. of Gland Coils	(ii) Uterine Epithelial Cell Height (μm)	(iii) Glandular Epithelial Cell Height (μm)	No. of Ewes	(i) No. of Gland Coils	(ii) Uterine Epithelial Cell Height (μm)	(iii) Glandular Epithelial Cell Height (μm)
I(days 3–6)	5	5·80±0·66	32·4±4·21	20·2±1·34	11	5·91±0·71	31·6±1·43	21·4±1·29
II(days 7–9)	10	6·80±0·70	37·6±2·84	16·7±1·11	11	5·91±0·73	33·8±2·11	16·6±1·07
III(days 11–12)	2	6·0 ±0·71	38·9±3·76	15·4±2·21	7	7·57±0·78	33·8±1·49	15·6±1·39
IV(days 13–15)	8	5·50±0·80	35·3±1·78	14·4±1·65	10	5·90±0·82	35·5±2·27	14·0±1·12

Bartlett's test for homogeneity of variance: $\chi^2_{(7)}$ = (i) 2·35 (n.s.); (ii) 8·62 (n.s.); (iii) 1·73 (n.s.)

Summary of overall analyses of variance

Source of Variation	D.F.	Mean Squares (i)	Mean Squares (ii)	Mean Squares (iii)	*F* Ratios (i)	*F* Ratios (ii)	*F* Ratios (iii)
Between groups	7	3·47	386·2	612·3	0·67	0·85	4·25***
Within groups	56	5·16	455·8	144·1			

*** $P < 0·001$.

ewes. The point is raised here because it has recently been shown (D. P. Boshier, personal communication) that extravasation of Evans' Blue dye can be demonstrated in the caruncular areas of the sheep endometrium on day 15 of pregnancy. Since it is known that the caruncles are the implantation "sites" in the ewe, it is not unreasonable to expect these areas to undergo increased vascular permeability prior to implantation similar to the analogous situation on day 5 of mouse pregnancy.

Pigmentation of caruncles was observed in approximately half the ewes examined. This phenomenon has been described by Amoroso (1952), although its significance is still unknown. In the present study the pigmentation was not associated with either stage of cycle, age of ewe, or parity and occurred in both pregnant and non-pregnant animals.

TABLE 5

MEAN OVARIAN FOLLICLE SCORE AT FOUR STAGES OF EARLY PREGNANCY OR THE OESTROUS CYCLE OF THE EWE

Ewes with single and multiple ovulations pooled

Period	Pregnancy		Oestrous Cycle	
	No. of Ewes	Mean Follicle Score (±S.E.)	No. of Ewes	Mean Follicle Score (±S.E.)
I(days 3–6)	20	1·24±0·14	13	1·14±0·14
II(days 7–9)	24	1·60±0·14	11	1·46±0·20
III(days 11–12)	15	2·20±0·28	8	1·74±0·38
IV(days 13–15)	10	1·60±0·19	12	2·50±0·19

Bartlett's test for homogeneity of variances: $\chi^2_{(7)} = 12{\cdot}10$ (n.s.)

Summary of overall analysis of variance

Source of Variation	D.F.	Mean Square	*F* Ratio
Between groups	7	292·97	6·16***
Within groups	105	56·75	

*** $P < 0{\cdot}001$.

(e) *Ovarian Follicular Development*

The paired ovaries of each ewe were scored with regard to all follicles greater than 2 mm diameter, by allotting a score of 0·1 to each 1 mm of follicular diameter. A follicle of 1 cm diameter thus received a score of 1·0. The results for all ewes, regardless of the number of ovulations are shown in Table 5. The significant differences among the means, shown in the overall analysis of variance in Table 5, are due to the peaks of follicular development in the pregnant ewes on days 11–12 and the

non-pregnant ewes on days 13–15 being significantly greater than that found earlier in the cycle or pregnancy.

TABLE 6

WEIGHTS OF ANTERIOR PITUITARY GLANDS OF EWES DURING THE OESTROUS CYCLE AND EARLY PREGNANCY

Period	Pregnancy		Oestrous Cycle	
	No. of Ewes	Weight of Pituitary (mg) (±S.E.)	No. of Ewes	Weight of Pituitary (mg) (±S.E.)
I(days 1–5)	5	630·6±18·3	9	574·2±48·8
II(days 6–9)	10	630·0±25·1	6	564·2±47·0
III(days 10–12)	3	686·0±56·1	5	573·8±42·7
IV(days 13–15)	8	628·3±40·2	7	620·3±45·9

Bartlett's test for homogeneity of variance: $\chi^2_{(7)} = 7{\cdot}98$ (n.s.)

Summary of overall analysis of variance

Source of Variation	D.F.	Mean Square	*F* Ratio
Between groups	7	8,216·5	0·69 (n.s.)
Between ewes, within groups	45	11,908·8	

TABLE 7

FOLLICLE-STIMULATING HORMONE CONTENT OF PITUITARY DURING EARLY PREGNANCY AND THE OESTROUS CYCLE

Values in parentheses are 95% confidence limits

Day	Pregnancy		Oestrous Cycle	
	No. of Estimates	Mean FSH Content (μg-equiv.)*	No. of Estimates	Mean FSH Content (μg-equiv.)*
1	—†		1	61·7 (17·9–134·3)
3	—†		1	291·8 (138–624·4)
5	1	45·7 (8·3–148·2)	1	33·9 (5·9–108·1)
7	3	54·8 (13–153)	2	101·2 (28·1–246)
9	1	17·3 (2·8–43·1)	1	94·9 (23·2–257)
11	2	99·1 (36·8–198)	3	63·3 (23·7–152)
13	1	133·2 (72·2–199·4)	1	88·5 (19·4–238)
15	1	152·5 (67·1–305·6)	1	110·9 (21·7–270·5)

* Values expressed as μg-equivalents of standard NIH–FSH–S3 preparation per pituitary gland.

† Not examined.

The difference between the non-pregnant and pregnant ewes on days 13–15 is just significant ($P<0{\cdot}05$) and probably reflects the commencement of follicular development for the next oestrus and ovulation in the non-pregnant animals.

(*f*) *Pituitary Gland Weights and Gonadotrophin Content*

The mean anterior pituitary gland weights are shown in Table 6. There is no evidence of differences among the means. The mean pituitary FSH levels shown in Table 7 and the "total gonadotrophin" levels in Table 8 do not follow any pattern that

TABLE 8

TOTAL GONADOTROPHIN (PERGONAL) LEVELS IN THE PITUITARY GLANDS OF EWES DURING EARLY PREGNANCY AND THE OESTROUS CYCLE

Values in parentheses are 95% confidence limits

Day	Pregnancy		Oestrous Cycle	
	No. of Estimates	Pergonal Level (μg-equiv.)	No. of Estimates	Pergonal Level (μg-equiv.)
1	—*		3	605 (362–1108)
3	—*		2	642 (542–869)
5	5	626 (269–1183)	4	782 (487–1376)
7	5	1,760 (1136–3684)	3	508 (374–699)
9	5	2,199 (1540–3748)	4	821 (466–1321)
11	3	506 (287–852)	5	1,421 (908–2573)
13	3	1,295 (853–2165)	4	1,157 (774–1886)
15	4	883 (555–1406)	3	970 (529–1777)

* Not examined.

might suggest differences in pituitary function between the pregnant and non-pregnant animals during the period studied, with the possible exception of low FSH on day 9 and low total gonadotrophin on day 11 of pregnancy.

TABLE 9

MEAN PLASMA PROGESTERONE LEVEL DURING EARLY PREGNANCY AND THE OESTROUS CYCLE

Values are from ewes with single ovulations only

Day	Pregnancy		Oestrous Cycle	
	No. of Ewes	Progesterone Level (ng/ml)	No. of Ewes	Progesterone Level (ng/ml)
1	—	—	3	0·03±0·02
4	11	0·84±0·2	—	—
5	3	0·88±0·3	4	0·41±0·2
6	—	—	3	0·21±0·09
7	4	1·26±0·7	1	1·50
8	12	1·81±0·5	—	—
9	3	0·84±0·3	7	1·62±0·4
11	2	1·48±0·5	2	1·34±0·8
12	9	7·25±1·9	—	—
13	3	5·07±1·1	2	0·61±0·5
15	—	—	6	0·48±0·2

(*g*) *Plasma Progesterone Levels*

Ewes with more than one ovulation were excluded from this analysis. The mean progesterone levels for ewes with one ovulation are shown in Table 9. Values for all

days for both groups of ewes are presented since pooling of several days would lead to spurious conclusions; for example, there were nine samples from pregnant ewes on day 12 but none from non-pregnant ewes on this day. This situation precludes any meaningful comparison between pregnancy and the oestrous cycle. Attention will therefore be confined to describing the pattern of progesterone levels in the two groups. In the oestrous cycle there was a gradual increase during the first half to reach a plateau level of 1–2 ng/ml on days 7, 9, and 11. The values show a decline on days 13 and 15. The pattern in pregnancy was different mainly as a result of the values measured on days 8 and 12, the days not examined in the oestrous cycle. That is, there was a plateau period extending from day 5 to day 11 with values of 1–2 ng/ml. On day 12 there was a significant peak of 7·6 ng/ml. The absence of pregnancy values on day 15, due to the ewes examined on this day all having more than one ovulation, is unfortunate. It might be expected that pregnant and non-pregnant values would begin to differ about this time (Deane *et al.* 1966*a*). For this reason plasma progesterone values were examined in detail in a separate experiment.

IV. Discussion

(*a*) *The Zygote*

The results in Table 1 and Figure 1 show that there is some variation in the rate of development and time of zona shedding by the sheep zygote. This may be related in part to the error (up to 24 hr) in the identification of the onset of oestrus from which all other events are timed. On average the blastocyst stage is reached on days 8–9, the zona pellucida is shed on days 9–10, and rapid elongation of the trophoblast (chorionic vesicle) occurs between days 11 and 13. This pattern agrees well with the description of these events provided by the photographic and histological evidence of Boyd and Hamilton (1952). Other reports in agreement are those of Clark (1934), and Wintenberger-Torrès (1967). The latter study provides detailed quantitative analysis of the rate of cell division in the embryo up to day 12 of pregnancy. There is strong evidence, supported by the present results, that cell division up to day 10 is very slow but beyond this point there is a spectacular increase in growth, due mainly to rapid cell division in the trophoblast. It is thought to be significant that the loss of the zona pellucida marks the commencement of this rapid growth phase.

The persistence of the zona pellucida of unfertilized ova until day 15 is surprising since the zona of the cleaved ovum disappears around day 9. These results suggest that the shedding of the zona pellucida is a function of the developing zygote rather than the uterine environment. This contrasts with the suggestion that in laboratory rodents, at least, zona loss is the result of steroid-induced changes in the uterine environment (Psychoyos 1966).

(*b*) *The Uterus*

The histological measurements reported here suggest that there are no marked morphological responses by the uterus to the presence of the embryo prior to day 13 that might be identified with the "signal" that must pass from the zygote to the maternal organism by this time (i.e. Moor and Rowson 1966). There is a significant decline in epithelial height of the uterine glands between days 3–6 and 13–15 in both

pregnancy and the oestrous cycle. Secretory activity is apparently greater at oestrus (Restall 1966) than in the period when the rapidly growing embryo is entirely dependent for its nutrient supply on the uterine secretions.

(c) *The Ovary*

Corpus luteum weight, even after elimination of variation due to multiple ovulation, was a highly variable parameter. Apart from the expected increase in weight between days 3 and 7 there is nothing in the results (Table 3) to suggest differences between the pregnant and non-pregnant ewe during the period to day 15.

Plasma progesterone values and corpus luteum histology do not clarify the situation, except to suggest that both pregnant and non-pregnant ewes experience a decline in luteal activity during days 13–15. This pattern does not agree with the histological and corpus luteum progesterone data reported in abstract form by Deane *et al.* (1966*b*). There is significant evidence in the cow, however, to show that luteal progesterone declines significantly between days 14 and 28 of pregnancy (Zimbleman, Loy, and Casida 1961). The significance of the elevated plasma progesterone levels on day 12 of pregnancy will be discussed in a later paper.

(d) *The Pituitary Gland*

The trend for pituitary weight to be heavier in pregnant than non-pregnant ewes (Table 6) was not significant. The bioassays for FSH and total gonadotrophin (Tables 7 and 8) illustrate the large variation between ewes in the one group with respect to these parameters. Any differences between pregnant and non-pregnant ewes would have to be very large, therefore, to show up as significant. It is stressed that these results are based on valid 4- and 6-point assays. The variable pituitary gonadotrophin levels are not therefore the result of assay error and merely reflect the inadequacy of assessing pituitary activity on the basis of a single pituitary hormone measurement.

Clearly, blood levels of the pituitary gonadotrophins throughout early pregnancy and the oestrous cycle are required. Hopefully, the radio-immunoassay techniques will solve this problem. Plasma levels of luteinizing hormone are now being measured in the ewe (Niswender *et al.* 1968; Pelletier *et al.* 1968; Goding *et al.* 1969; Wheatley and Radford 1969). All these reports show a discrete peak of luteinizing hormone lasting a few hours just prior to ovulation, and stable low values throughout the remainder of the oestrous cycle. Only Niswender *et al.* (1968) and Goding *et al.* (1969) have studied early pregnancy (up to day 20) and plasma luteinizing hormone values were low and stable as in the cycle. It appears that fluctuations in luteinizing hormone are not responsible for the preimplantation changes that lead to the persistence of the corpus luteum in the pregnant ewe.

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VI. References

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