STUDIES ON THE *IN VIVO* CLEAVAGE AND THE *IN VITRO* CULTURE OF NEW ZEALAND ROMNEY SHEEP OVA

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A C K N O W L E D G E M E N T S

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Chapter I

I N T R O D U C T I O N

The field of embryology has been described as the study of the processes and principles of development (Barth, 1953), and can be divided into developmental anatomy and developmental physiology.

Since their discovery, the development of mammalian ova has been studied by examining ova recovered from the female tract at various times after the onset of oestrus or fertile mating, or alternatively by culturing ova in a medium which allows a comparable rate of development to that recorded *in vivo*.

The normal times relative to oestrus when fertilised eggs at various stages of cleavage can be found in ewes of several breeds have been reported by Clark (1934), Green and Winters (1945), Averill (1958), Hancock and Hovell (1961), Chang and Rowson (1965) and Cumming and McDonald (1967).

Brinster (1963) has developed a reliable and successful method for the *in vitro* culture of mouse ova from the 2- cell to the blastocyst stage and has reported that such development proceeds at a rate comparable with that *in vivo*. He has then proceeded to make full use of this method by investigating the effect of osmolarity, hydrogen ion concentration, fixed nitrogen source and energy source on the rate of embryo development (Brinster, 1965). Such a technique has and will continue to give valuable information on developmental anatomy and physiology.

Sheep ova have been successfully cultured within the reproductive tract of the rabbit and the development of these ova has been reported to be similar to that within the sheep (Averill *et al*. 1955). Successful

short term storage of sheep ova at 37°C (Averill and Rowson, 1958) and long term storage at lower temperatures has been reported (Averill, 1956), but sheep ova have proved very refractile to culture at 38°C for long periods of time (Wintenberger *et al*. 1953).

The ultimate proof of the viability of an ovum after culture or storage *in vitro* or in the ligated genital tract of an animal of the same or different species (*in vivo* treatment), is its subsequent development *in vivo*; to gain this proof the treated ovum is transferred to a suitable recipient in which its development can be followed. Successful egg transplantation has been reported in the sheep by many workers (Appendix 1 - Austin, 1961).

In the study of sheep embryology then, the examination of the *in vivo* cleavage of fertilised ova has given much data on developmental anatomy, but the development of better culture techniques should, when used in conjunction with the technique of egg transplantation, give valuable information on developmental physiology as well as developmental anatomy.

The purpose of this present study was to observe the cleavage of fertilised Romney ewe ova *in vivo* and to compare this with cleavage of ova cultured *in vitro*. A method of long term *in vitro* culture of ova was developed and the technique of ovum transplantation used to test ovum viability.

CHAPTER II

REVIEW OF LITERATURE

Chapter II

REVIEW OF LITERATURE

PRODUCTION OF OVA

For egg culture and transfer studies a large number of ova are often required over a limited time period. Because sheep do not naturally produce a large number of ova at each oestrus, gonadotrophic hormones are often used to increase the production of ova for these studies. Pregnant mares' serum gonadotrophin (P.M.S.) has been used to increase the fecundity during the breeding season (Robinson, 1951; Wallace, 1954; Gordon, 1958), and during the anoestrous season (Robinson, 1954; Gordon, 1958). Robinson (1951) and Wallace (1954) reported that the ovarian response to P.M.S. was very variable, and Warwick and Casida (1943) and Wallace (1954), that the administration of gonadotrophin during the follicular phase of the cycle hastened the onset of the following oestrus.

Moore and Shelton (1964) have used Horse Anterior Pituitary extracts for superovulation of sheep, but reported no advantage over P.M.S. except for the apparently more reliable ovarian response to higher doses.

Normal viability of superovulated sheep ova has been reported (Averill, 1958; Moore, Rowson and Short, 1960).

Workers have discussed fully the use of progesterone and its analogues in the suppression and synchronisation of ovarian cycles, both in the anoestrous and breeding seasons (Lamond, 1964; Robinson and Lamond, 1966). They have also discussed the interaction between progesterone and P.M.S. on ovarian function in the ewe, and the poor

fertility often reported after progesterone synchronisation and suppression.

OVA TRANSFER

This technique can be defined as the transfer of follicular oocytes or ova from the normal maternal environment either directly into a recipient (which may be the same individual as the donor), or into a recipient after a period of culture or storage *in vitro* or *in vivo*.

The ova are recovered from the donors for transfer by *in vitro* or *in vivo* methods. The *in vitro* methods involve the flushing and manipulation, or embedding in paraffin and sectioning, of the genital tract which has been removed from the slaughtered animal (Austin, 1961). The *in vivo* laparotomy method of recovery has been described in the sheep (Hunter, Adams and Rowson, 1955) and in the pig (Hancock and Hovell, 1962; Smidt *et al*. 1965). Non-surgical *in vivo* ova recovery has been reported in the cow by Rowson and Dowling (1949), Dracy and Petersen (1951) and Donker (1955). Hafez (1960) reported that the *in vivo* method was approximately 40-60% as efficient as the *in vitro* method, but was invaluable where the donor was to remain alive.

Hunter, Adams and Rowson (1955) have outlined the surgical insertion of ova in the sheep while other workers have outlined and reported successful non-surgical transfer of cow ova (Mutter *et al*. 1964; Rowson and Moor, 1965; Sugie, 1965).

The technique of egg transfer has been reported in sheep by a large number of workers from Warwick *et al*., (1934) to Shelton and Moore (1966).

The following two factors have been found to affect the success of ovum transplantation in sheep.

(a) Synchronisation of onset of oestrus in donor and recipient ewes

A number of workers (Moore and Shelton, 1964; Shelton and Moore, 1966), have reported the need for close synchronisation (± 12 hours) between donor and recipient ewes, while Averill (1956) and Averill and Rowson (1958) have suggested that precise synchronisation is not necessary for successful ovum transplantation. Hancock and Hovell (1961) suggested that the transfer of older ova into younger uteri was generally more favourable to success than the transfer of younger ova into older uteri and suggested that it seemed wise to minimise the chance of oestrus occurring in the recipient before it occurred in the donor.

(b) Site of transfer of ova into the recipient genital tract

Averill and Rowson (1958) reported that no 2- cell, 16% 4- cell and 80% 8- cell ova would develop on transfer into the uterus of the sheep. Usually the site of transfer into the recipient genital tract and the age of the transferred ova are confounded (Moore et al. 1960; Moore and Shelton, 1962b; Shelton and Moore, 1966), and 2- to 4- cell ova placed into the Fallopian tube and $\geq$ 6- cell ova into the uterus (Hancock and Hovell, 1961; Cumming, 1965). Using this practice, most success has been reported with uterine transfers. However Moore and Shelton (1964) did not confound age of ova and site of transfer and reported tubal transfers as being the most successful.

Averill and Rowson (1958) reported that the success of individual transfers was not significantly related to the breed of recipient or donor, season, or the number and results of previous transfers to the same recipient.

Moore, Rowson and Short (1960) and Cumming (1965) found that as the number of ova transferred was increased, the chance of a pregnancy resulting did not increase and the ovum survival rate was lowered. However Moore and Shelton (1962b) reported that the transfer of greater than one egg in tubal transfers did not significantly affect egg survival, but significantly increased the chance of a successful pregnancy. Moore et al. (1960) found that the ewes receiving 5 ova and subsequently becoming pregnant had significantly more lambs at term and embryos at autopsy than those only receiving 2 ova.

The following are some workers suggestions for the applications of the technique of egg transfer (Willet, 1953; McLaren and Michie, 1956; Moore and Shelton, 1962b).

1. To shorten the Generation Interval: The generation interval in domestic animals is long, and if it is to be reduced it would entail a precocious puberty induced by hormones and the transplantation of the resultant eggs into mature synchronised hosts. In this respect workers have studied the calf (Casida et al. 1943; Marden, 1951, 1953; Black et al. (1953), Lamb (Casida, 1934, 1935; Mansour, 1959) and piglet (Casida, 1934, 1935). Ova produced by immature animals have developed normally to produce young (Marden, 1953; Adams, 1953).

2. To make maximum use of a genetically superior female over her breeding life (McLaren and Michie, 1956). The superior female could be frequently superovulated over her breeding life and the recovered fertilised ova transferred to synchronised recipients of inferior genetic merit.

3. Transfer of ova from one country to another (Chang and Marden, 1954; Hunter et al. 1961).

4. Studies on developmental physiology (Austin, 1961). After treatment *in vitro* the ova can be transferred into recipients to test for viability.

5. Studies on maternal and embryonic factors in development (Fekete and Little, 1942; Fekete, 1947; Venge, 1950; Hunter *et al.* 1954; Michie and McLaren, 1958; Green and Green, 1959).

OVA CULTURE AND STORAGE

Sheep ova have been successfully cultured within the rabbit genital tract (Averill, Adams and Rowson, 1955; Averill, 1956; Hunter *et al.* 1961) and within the ligated sheep oviduct (Wintenberger - Torres, 1956).

Successful short term storage of sheep ova *in vitro* has been reported by Averill (1956) and Averill and Rowson (1958). Averill reported better transfer results when ova were placed in tubes containing serum at 30°C for 45 - 115 minutes before transfer, than when ova were left in covered watch glasses in a warm chamber. Averill and Rowson developed special dialysis chambers which they reported conferred some advantage over watch glasses when the period of ova storage before transfer was greater than 50 minutes.

Averill (1956) and Averill and Rowson (1959) successfully stored sheep ova at 4.5 - 8.0°C, but Harper and Rowson (1963) reported no egg transfer success after storing ova at 7°C. The storage of ova at 0 - 4°C and -79°C has also been unsuccessful (Averill and Rowson, 1959).

Wintenberger, Dauzier and Thibault (1953) cultured sheep ova at 38°C in homologous blood serum containing streptomycin for up to 140 hours and found that unsegmented ova rarely passed the 6- blastomere stage; 8- to 12- blastomere ova seldom cleaved; but 15- to 20- blastomere ova

cleaved readily and the speed of division was reported to be similar to that *in vivo*. (Ova cultured below 8.0°C failed to cleave during *in vitro* storage; Averill and Rowson, 1959.) Sheep ova have also been successfully stored for 24 or 48 hours in stoppered tubes at room temperature in the dark (Buttle and Hancock, 1964).

The success of culture has been found to depend on -

(a) Species of ova cultured: Mouse ova will culture readily *in vitro* but sheep ova will not (Brinster, 1963; Wintenberger *et al*. 1953).

(b) Stage of development of cultured ova (Wintenberger *et al*. 1953).

(c) Culture media: Wintenberger *et al*. (1953), Averill (1956), Averill and Rowson (1958, 1959), Harper and Rowson (1963) and Buttle and Hancock (1964) used sheep serum containing streptomycin for the culture or storage of sheep ova. However Brinster (1963) has pointed out that the critical factor in the culture of all mammalian cells is the composition of the culture media and so he developed an extremely complex media for the culture of his 2- cell mouse ova, and has achieved much greater culture success than the earlier workers (Hammond, 1949; Biggers and McLaren, 1958). Serum with streptomycin or penicillin added is usually used as the medium for the transfer of sheep ova, but some workers have used Physiological saline and Locke's solution (Casida *et al*. 1944) and Tyrodes solution and aqueous humor (Warwick and Berry, 1949, 1951). Glycerol has been added to the media for storage of ova at low temperatures (Averill and Rowson, 1959).

The storage of rabbit ova in media containing antibiotics at low concentrations does not have an effect on subsequent embryonic survival, but the survival of ova during culture can be partially or completely inhibited in media containing high concentrations of antibiotics (Hafez, 1963).

OVA CLEAVAGE

Austin (1961) points out that there is little precise information on the cleavage rates of mammalian ova *in vivo*; this is due to the difficulty of knowing the exact time of ovulation, to the fact that fertilisation may be initiated at any time over a period of 12 hours (or longer) after ovulation, and in polytocous animals to the scatter in time of penetration of the eggs. Also it is reasonable to suppose that the eggs in any one species do not all develop at the same rate. Finally, since the actual process of cleavage occurs relatively rapidly, direct observation is rare, the time of cleavage has generally to be inferred from the condition of eggs on recovery from the animal and a large number of observations are necessary for even approximate estimates.

The maturation of sheep ova has been described (Pitkjanen, 1958; Berry and Savery, 1958; Dziuk and Dickmann, 1965), and the relationship of the stage of maturation and fertilisation to the time of ovulation established (Dziuk, 1965). Also the maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes has been described (Edwards, 1965).

The classical work on cleavage stages of the sheep egg was done by Assheton (1898) who obtained a series of ova and blastocysts from the 2nd to 11th day of development.

Green and Winters (1945) reported in Shropshire sheep that segmentation appeared to be initiated approximately 1½ days after the end of oestrus (ovulation) and that during this interval fertilisation must occur. Chang and Rowson (1965) estimated that ovulation occurred 30-40 hours after the onset of oestrus in the Dorset Horn ewe and assumed that the first cleavage occurred 12-22 hours after ovulation. Clark (1934) recovered 2- cell ova 39½ hours post-coitum and noted that the 2 blastomeres

were of very different size and that this size difference had largely disappeared after the second cleavage, which was at right angles to the first. The normal times relative to oestrus when fertilised eggs at various stages of cleavage can be found in ewes of several breeds has been reported by Clark (1934), Green and Winters (1945), Averill (1958), Hancock and Hovell (1961), Chang and Rowson (1965) and Cumming and McDonald (1967). The results of these workers show that the cleavage rate of sheep ova to the 8- to 12- cell stage is very rapid. Both Clark (1934) and Green and Winters (1945) reported that in 16- cell ova there were marked differences in cell size, and that this could be interpreted as the start of trophoblastic specialisation.

Uncleaved sheep ova must be carefully examined for fertilisation before they are used for egg culture or transfer studies. Fertilised single cell ova have been identified by the presence of numerous surplus spermatozoa found adhering to, or embedded in the zona pellucida (Hart, 1956; Laffey and Hart, 1959), and upon certain changes taking place in the nucleus (Hart, 1956).

Austin (1961) reported that during ovum cleavage the total mass of cytoplasm actually decreases, presumably because yolk materials are used up to provide energy for the maintenance and division of the cells, and that with each successive stage of cleavage, the size of the blastomeres is roughly halved until they reach the size of most of the tissue cells in the organism concerned. In normal sheep ova it has been found that the average diameter of the zonal cavity is 0.147 mm and the average zona pellucida thickness 0.014 mm (Clark, 1934).

OVA ABNORMALITIES

Only normal ova should be utilised in egg culture and transplantation studies. Dutt (1951) showed an estimated loss of 20% of embryos from early-season matings whilst Hart (1956) also showed that a proportion (17%) of all ova shed were abnormal and not able to be fertilised. In the late breeding season Laffey and Hart (1959) found that 40% of ova recovered were abnormal. Braden (1964) classified 12% of his unfertilised sheep ova as being abnormal, but found no significant variation between months. However he observed a significant variation in ova abnormalities between ewes examined 3-4 (29% abnormal); 2-3 (21%); and 0-2 (6%) days after being marked by vasectomised rams. He pointed out that most of the abnormalities developed 1-3 days after ovulation and represented degenerative changes that might well be expected in ageing unfertilised eggs.

It has long been known that both ovarian oocytes and tubal eggs are prone to undergo cytoplasmic division, apparently spontaneously and often in a manner that superficially resembles normal cleavage. This phenomenon has been described in a number of species (e.g. Dziuk, (1960) has reported in the unbred gilt that 80% of the ova recovered from the uterine horn had undergone spontaneous fragmentation), and although several authors were attracted by the idea that parthenogenesis might on occasion be displayed by mammalian eggs, the general conclusion was that most if not all the instances of apparent cleavage were in fact caused by a disorganisation and degenerative fragmentation of the egg (Austin, 1961).

The second-metaphase chromosome group in unpenetrated eggs may not break up but instead give rise to a single nucleus (usually diploid), or alternatively the eggs may show spontaneous resumption of the second

meiotic division and develop a single nucleus (usually haploid). In rabbits and sheep the incidence with which unpenetrated ova would undergo activation or develop nuclei was greatly increased by cold-shock treatment, the second meiotic division being suppressed and single diploid nuclei formed (Thibault, 1949; Chang, 1952a; Thibault and Ortavant, 1949). Pincus (1939c) and Pincus and Shapiro (1940b) claim that parthenogenesis in the rabbit can proceed to the birth of viable young, but Chang (1954) found that activated rabbit ova would develop to give blastocysts, but that these blastocysts would not implant.

Dutt et al. (1959) and Alliston et al. (1961) have reported that some instances of sheep ova abnormalities could be due to high environmental temperatures just prior to ovulation.

CHAPTER III

MATERIALS AND METHODS

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EXPERIMENTAL ANIMALS

Sixty culled for age 5-year-old Romney ewes from the Massey University flocks were used in the 1966 breeding season, 58 in the 1966-67 anoestrous season and 25 in the 1967 breeding season. Two entire Southdown rams of proven fertility and 2 vasectomised Perendale rams ('teasers') were run with the ewes during the 1966 breeding season, and one entire ram and 2 'teasers' during the other two seasons.

MANAGEMENT OF ANIMALS

The animals were grazed on predominantly ryegrass - white clover pasture and managed to maintain constant body weight. All sheep had access to a plentiful supply of water.

Individual ewes were distinguished by ear tags and serially numbered canvas squares attached to the flank wool. The entire and vasectomised rams were fitted with Sire Sine harnesses and the ewes were examined for mating marks every 8 hours (8.o a.m., 4.0 p.m. and 12 midnight) in the presence of these rams (except for the 1966 breeding season where ewes run with the 'teaser' were examined once daily - 8.0 a.m.). A ewe was recorded as showing oestrus if clearly marked over the rump and tail region. The rams were removed whenever the ewes were yarded to prevent rape services.

EXPERIMENTAL PLAN

The experimental plan is outlined in Figures 1, 2 and 3 and is discussed further in the following sections.

FIGURE 1

EXPERIMENTAL PLAN FOR 1966 BREEDING SEASON

Ewes marked
by 'teaser'

↓

Inject with
gonadotrophin

↓

Oestrus
(Entire Ram)

↓ Approximately 60 hours duration

Laparotomy 1

Ova recovered - these ova were either:

(a) Transferred* immediately into a donor ewe (Control transfer)

(b) Cultured in vitro for 24, 48 or 72 hours. Some ova cultured for 24 or 48 hours were transferred* into donor ewes after culture.

(c) Transferred into a donor immediately after recovery and recovered again approximately 24 hours later at laparotomy 2.

The recipients in (a) and (b) were slaughtered if pregnant after ovum transfer.

The donors in (c) could undergo either of two treatments.

Treatment A	Laparotomy 1		Laparotomy 2
Fallopian tube$_1$ (FT_1) →	Ova recovered	Transfer ova into FT_1 and/or FT_2	Ova
Fallopian tube$_2$ (FT_2) →	Ova recovered		recovered

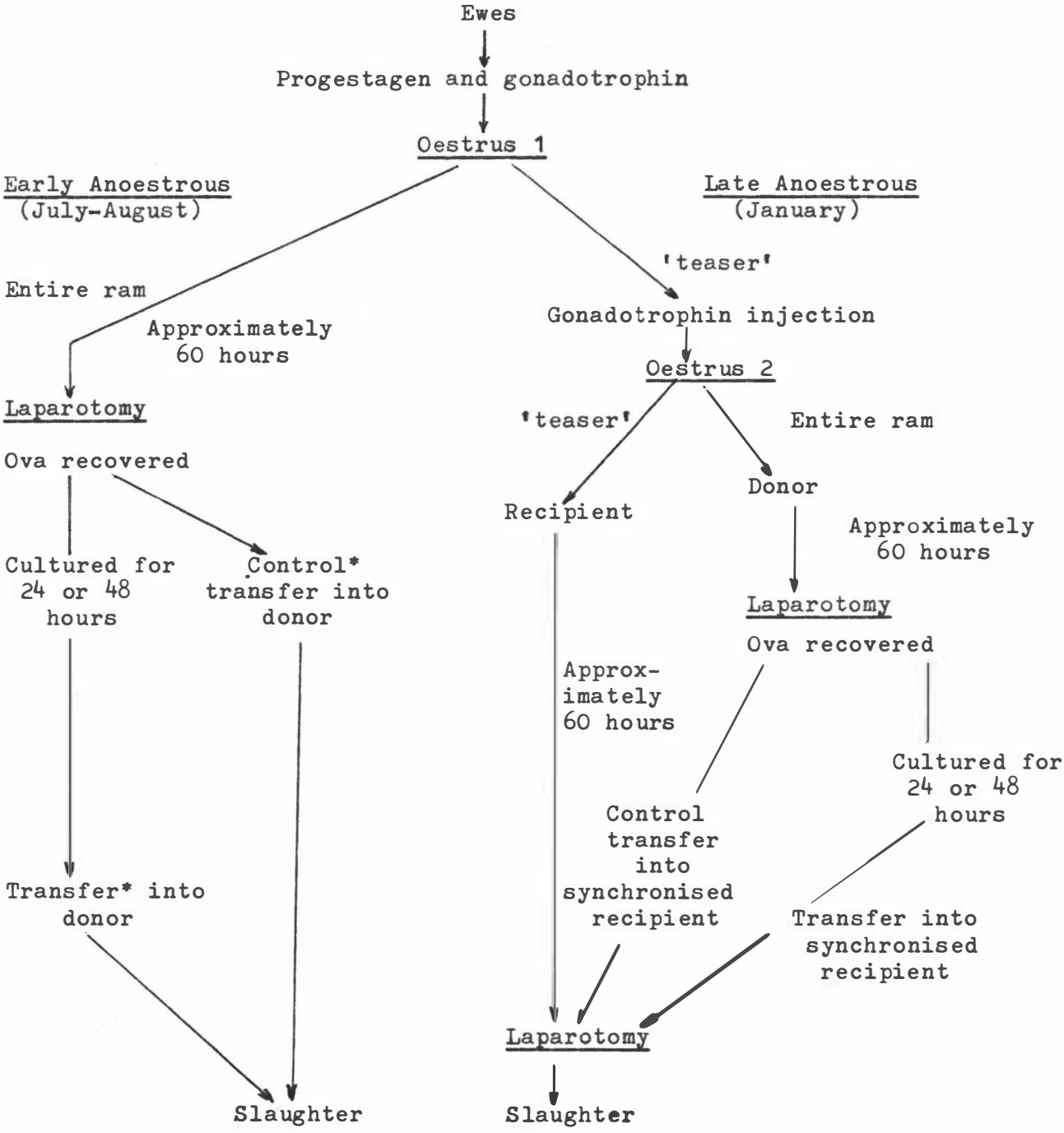
Treatment B	Laparotomy 1		Laparotomy 2
FT_1 →	Ova recovered	Transfer ova into FT_1 →	Ova
FT_2 →			recovered

* Ova only transferred into donors if all ova shed by those donors (based on the number of corpora lutea) had been recovered.

In (a), (b) and (c) the ova were transferred into their own, or a different donor.

FIGURE 2

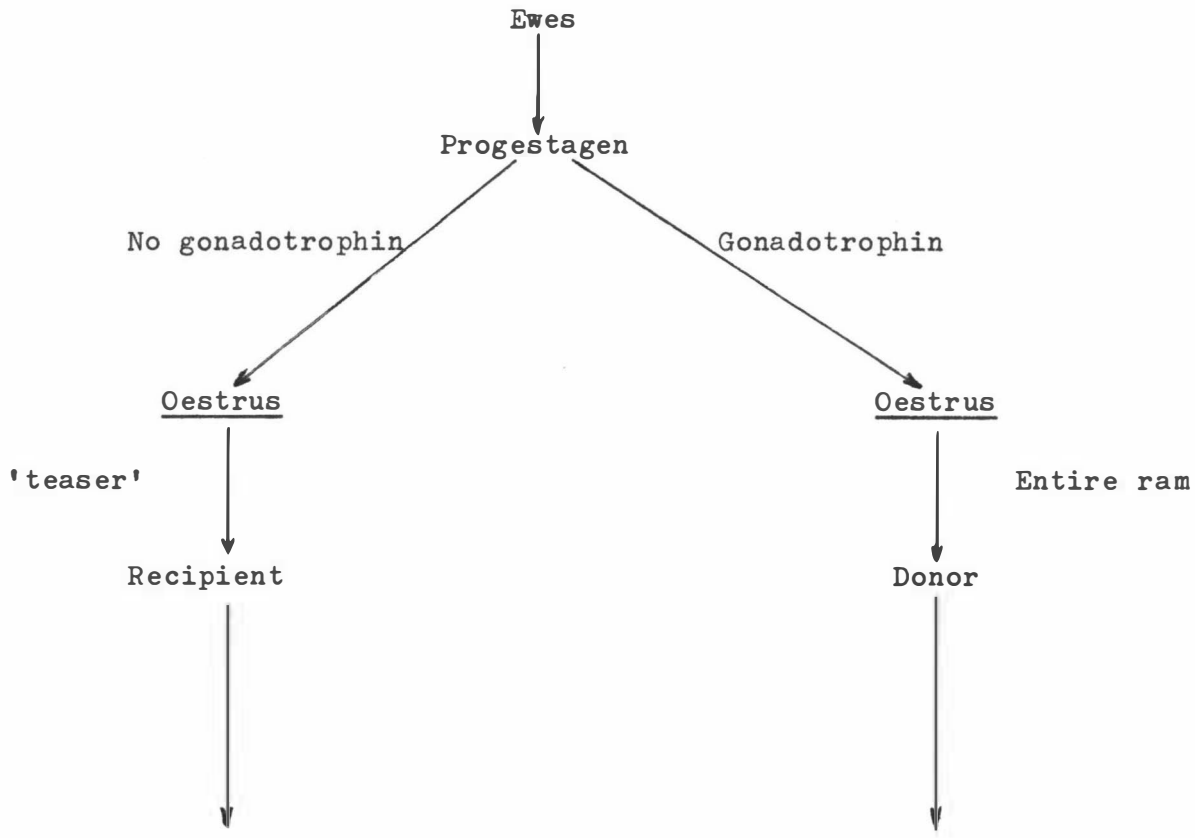
EXPERIMENTAL PLAN FOR 1966-67 ANOESTROUS SEASON



* Ova only transferred into donors if all the ova shed by those donors had been recovered.

FIGURE 3

EXPERIMENTAL PLAN FOR 1967 BREEDING SEASON



As for 1966-67 Late Anoestrous season (Figure 2)

HORMONES AND TREATMENTS

(a) 1966 Breeding Season (Figure 1)

The ewes were given a subcutaneous superovulating injection of Pregnant Mares' Serum gonadotrophin (P.M.S.) on day 12 or 13 of the oestrous cycle. Records of onset of oestrus (based on mating with the 'teaser') were kept for each ewe, and every second day over the breeding season from 7th April to 1st August (except for periods when operations were not able to be performed), ewes which were at day 12 or 13 of the cycle were injected with either 1,000, 1,200 or 1,500 i.u. P.M.S.

(b) 1966-67 Early Anoestrous Season (Figure 2)

On the 20th July 1966, vaginal sponges containing 60 mg 17α acetoxy - 6α-methylpregn-4-ene3,20 dione (M.A.P.) per sponge were inserted into 18 ewes. These were withdrawn on 4th August and 1,200 i.u. P.M.S. injected subcutaneously on either 3rd August (day -1) or 4th August (day 0).

(c) 1966-67 Late Anoestrous Season (Figure 2)

Forty ewes were treated on 3rd January 1967 with vaginal sponges each containing 60 mg M.A.P. These were withdrawn after 8 or 9 days, or 18 or 19 days and on the day of withdrawal, 1,000 i.u. P.M.S. was injected subcutaneously. On the real or assessed day 12 or 13 of the induced cycle, the ewes were injected with either 1,500 i.u. P.M.S. and placed with the entire ram (donor ewe), or 1,000 i.u. P.M.S. and placed with the 'teaser' (recipient ewe). The P.M.S. used in this season was of 3 brands (See Appendix 1).

(d) 1967 Breeding Season (Figure 3)

Vaginal sponges containing 60 mg M.A.P. per sponge were inserted into the 25 ewes on 30th March and withdrawn after 18 or 19 days. The donors were injected with gonadotrophin (1,500 i.u. P.M.S.) when the sponges were withdrawn, but the recipients were not.

RECOVERY OF OVA

Laparotomies to collect ova from the reproductive tract were carried out 36 - 102 hours after the ewes were first observed in oestrus. The technique used was as described by Hunter, Adams and Rowson (1955) and Cumming (1965).

Sheep serum, the medium used for ovum recovery, was obtained from whole blood collected from recently slaughtered sheep. This blood was allowed to clot within collection vessels (sterilized beakers lined with cheese cloth) which were placed in the refrigerator for 24 hours. The cheese cloth was then gently lifted out of the beakers, taking with it the clot and leaving the serum; this serum was then drawn off, centrifuged, penicillin added (1,000 units per ml.), Seitz-filtered and placed in sterilized bottles before deep-freezing. When required for ovum recovery the serum was thawed and held at 36°C in a water bath.

Immediately after recovery from the genital tract the washings were examined under a binocular microscope for the presence of ova.

CULTURE OF OVA

The ova to be cultured were placed in sterilized culture chambers. These were based on the dialysis chambers described by Averill and Rowson (1958) and a dismantled and assembled chamber is shown in Figure 4.

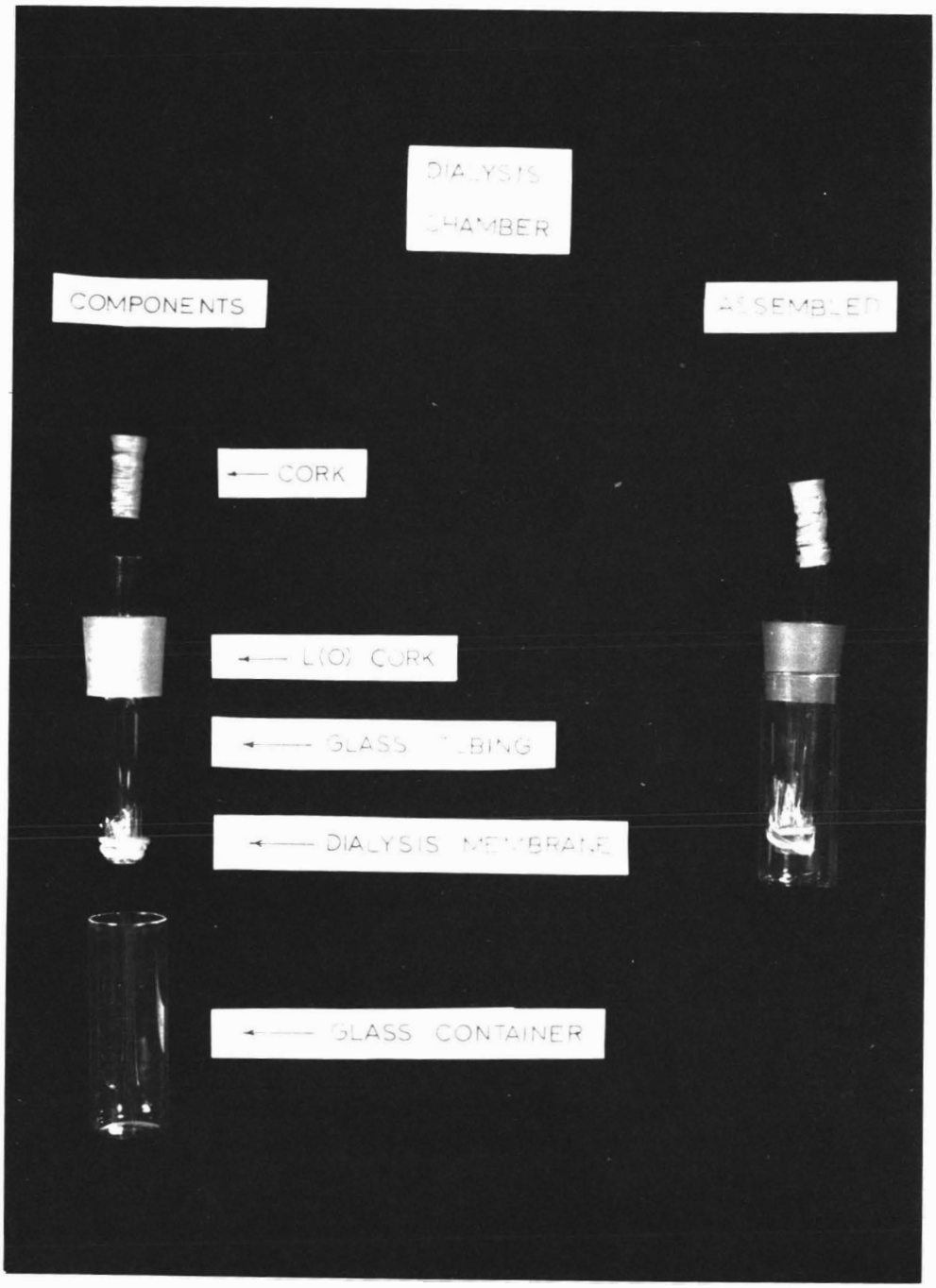


Figure 4 - Dismantled and assembled dialysis chamber

The chambers were constructed from:

1. Dialyzer Tubing Cat. No. 4465-A2 (A.H. Thomas Co. Philadelphia).
2. 10 ml. volume thin-walled glass containers.
3. 6.5 mm. internal diameter glass tubing (8.0 mm. external diameter) cut into sections 7.5 cm. long.
4. B. & T. (L)0 rubber corks.

The dialyzer tubing was placed in saline and then cut into 1" lengths which were split open to give two, 1" squares of membrane. Each membrane square was smoothed over the lower end of the glass tubing and held firmly in place by a rubber band. Care was taken to leave no folds in the secured membrane as these could trap ova and prevent their subsequent isolation and recovery after culture. The chamber components were then autoclaved, after which the dialysis membrane was reimpregnated by being placed in a sterilized 10% glycerin solution for 5-10 minutes and the components then stored in the refrigerator until use.

At the time of ovum transfer into the chambers, approximately 4 ml. of serum at 36°C were placed in the outer container and the chamber assembled. The ova were aspirated into sterilized Pasteur pipettes with a large volume of serum and placed into the inner glass tubing. Additional serum was then added into the glass tubing to make up to a final volume of approximately one ml. Care was taken to flush the sides of the glass tubing so that the ova would be resting on the surface of the dialysis membrane during culture. The glass tubing was then corked and the entire chamber partially suspended in a water bath at 36°C before being transported in a thermos flask to the laboratory. Here the chambers were placed in an incubator at 35 - 36°C for 24, 48 or 72 hours, the serum in the chambers being changed at 24 hourly intervals.

The ova were recovered from the chambers by tipping the contents of the inner glass tubing into a sterilized watch glass, and then flushing

the tubing with serum until all the ova were recovered.

EXAMINATION OF OVA

The ova were examined for the number present, presence of spermatazoa, stage of cleavage and for abnormalities at the following times:

1. At the surgery shed after recovery from the donor and before being transferred into a recipient ewe or a culture chamber. They were located under the microscope using x 17.5 magnification and examined using x 35 and x 70 magnifications.

2. Every 24 hours at the laboratory if they were not to be transferred into recipient ewes. They were recovered from the culture chambers and examined under high-power magnification (x70 - x 250), and then dimensions and photographs of some ova were taken.

3. At the surgery shed after culture for 24 or 48 hours and before being transferred into recipient ewes. These ova had already been examined as in 1, but not as in 2, and here were recovered from the chambers and examined under x35 and x70 magnifications before transfer.

Because fertilised ova at all stages of cleavage were required for egg culture and transplantation studies, it was not possible to identify fertilised one-cell ova by changes taking place in the nucleus. Instead they were examined at recovery for the presence of spermatazoa adhering to, or embedded in the zona pellucida and then transferred back into a donor for 24 hours (culture in vivo), or into a culture chamber (culture in vitro). Any one-cell ova which were recovered with spermatazoa adhering to the zona pellucida and which cleaved normally on culture, were regarded as being fertilised when first recovered and

could be used for egg transplantation and cleavage studies.

TRANSFER OF OVA

The ova were either:

1. Transferred back into the same or a different donor ewe immediately after recovery and examination (Control transfers - Figures 1 and 2).

2. Transferred back into the same or a different donor ewe immediately after recovery at laparotomy 1, and recovered again 24 hours later at a second laparotomy (Figure 1).

3. Transferred back into the same or a different donor after culture for 24 or 48 hours (Figures 1 and 2).

4. Transferred after 24 or 48 hours culture or as control transfers (0 hours culture) into recipients whose tract development was judged to be either synchronised with, or at an earlier stage of development than that of the transferred ova (Figures 2 and 3).

The recipient's tract was exposed and the transfer of ova was accomplished using essentially the same technique as described by Hunter et al. (1955).

The ova were deposited either 3 - 4 cm. along the Fallopian tube via the fimbria (2- to 4- cell ova), or into the lumen of the uterine horns through punctures on the antimesometrial border ($\geqslant$ 6- cell ova). Care was taken in the uterine transfers to ensure that the pipette point was placed inside the uterine lumen and not above the uterine mucosa. After deposition of the ova the pipette was carefully examined to ensure that all ova had in fact been transferred into the recipient tract.

SLAUGHTER OF EWES AND EXAMINATION OF REPRODUCTIVE ORGANS

All ewes to be slaughtered were taken to the local freezing works. Here, within 15 minutes of slaughter, the reproductive tracts were recovered and placed in identified plastic bags for transport to the laboratory where the ovaries, vagina, external genital organs, mesenteries and suspensory ligaments were removed by dissection. The ovaries were examined for corpora lutea and large follicles and were sectioned to check the recorded results.

The uterus was placed on a shallow tray and the uterine horns opened with scissors. The recipient ewes from type 1, 3 and 4 transfers were slaughtered approximately 24 days after the onset of the transfer oestrus (provided they had not been marked by the 'teaser' after transfer), and in these ewes the embryos and membranes were carefully removed by flushing the tract with 0.85% sodium chloride. The embryos were observed for heart activity (if heart beat was noted the embryo was considered to be alive at slaughter) and crown-rump length. In ewes 44 - 101 days pregnant from type 2 transfers, poor ova recovery at laparotomy, and in one ewe pregnant from a type 1 transfer, the foetuses were visually examined for the number present, viability and position; the amnion was pierced, the umbilical cord severed 0.5 cm. from the navel and the crown-rump length measured for each foetus.

After examination and measurement some of the embryos were photographed, and then all embryos and foetuses were discarded.

CHAPTER IV

PRODUCTION OF OVA FOLLOWING HORMONAL STIMULATION OF DONOR EWES

Chapter IV

PRODUCTION OF OVA FOLLOWING HORMONAL STIMULATION OF DONOR EWES

The results in this chapter were obtained from the ewes treated with hormones in the 1966 breeding and 1966-67 anoestrous seasons. Only 7 donors were available after the hormonal treatment of 25 ewes in the 1967 breeding season and the results from these ewes were only included in Chapter VII.

INDUCTION OF SUPEROVULATION AND MATING OF DONOR EWES

1. Length of the oestrous cycle following P.M.S. injection during the breeding season

The length of the oestrous cycles following the injection of 1,000, 1,200 or 1,500 i.u. P.M.S. on day 12 or 13 of the cycle are shown in Table 1. An analysis of variance revealed that neither the dose of P.M.S. nor the day of P.M.S. injection had any significant effect on the length of the oestrous cycle.

2. Ovarian Response

The ovarian response of the 131 ewes treated with P.M.S. during the breeding and anoestrous seasons, is presented in Table 2 in terms of the mean number of corpora lutea and corpora lutea and large follicles

TABLE 1

OESTROUS CYCLE LENGTH FOLLOWING INJECTION OF P.M.S. ON DAY 12 OR 13 OF THE CYCLE

Dose of P.M.S. (i.u.)	Day of Oestrous Cycle of Treatment	Number of Ewes	Cycle Length - days (Mean ± S.E.)
1,000	12	3	16.73 ± 0.20
	13	11	16.58 ± 0.18
	Combined days	14	16.61 ± 0.19
1,200	12	15	16.69 ± 0.28
	13	7	16.44 ± 0.29
	Combined days	22	16.61 ± 0.21
1,500	12	24	16.54 ± 0.14
	13	34	16.71 ± 0.20
	Combined days	58	16.64 ± 0.13

ANALYSIS OF VARIANCE

Source of Variation	d.f.	Mean Squares
Between doses	2	0.01 (N.S.)
Between days within doses	3	0.25 (N.S.)
Individuals	88	0.95

N.S. = Not significant

TABLE 2

OVARIAN RESPONSE OF EWES TREATED WITH P.M.S. IN THE BREEDING AND ANOESTROUS SEASONS

Dose of P.M.S. (i.u.)	Number of Ewes	No. Corpora Lutea (Mean ± S.E.)	No. Corpora Lutea and Large Follicles (Mean ± S.E.)
1,000	25	3.08 ± 0.70	4.80 ± 0.91
1,200	31	2.48 ± 0.34	3.42 ± 0.43
1,500	75	4.51 ± 0.51	6.00 ± 0.55

(> 5 mm. diameter) per ewe. Frequency distribution curves were plotted and showed that these data did not follow normal distribution curves. Also Bartlett's test for homogeneity of variance of these data showed that the variance differed between P.M.S. treatments, and that a square root transformation of the observations resulted in similar variances in the different treatments (Appendix 2). The results for ovarian response after transformation are presented in Table 3.

The ovarian response to 1,200 i.u. P.M.S. was lower than that to both 1,000 i.u. and 1,500 i.u. P.M.S., but the analysis of variance in Table 3 showed that the dose of P.M.S. had no significant effect on the ovarian response. There was a significant difference between seasons within doses ($P < 0.01$) for both the number of corpora lutea and the number of corpora lutea and large follicles. A Duncan's Multiple Range Test revealed that for both these measures of ovarian response, there was a significant difference ($P < 0.01$) between seasons within doses for 1,000 i.u. and 1,500 i.u. P.M.S., but not for 1,200 i.u. P.M.S. (Appendix 2). The 7 ewes injected in the anoestrous season (with 1,200 i.u. P.M.S.) to give a similar ovarian response to that recorded in the breeding season, were treated during the early anoestrous. The 11 ewes injected with 1,000 i.u., and the 18 injected with 1,500 i.u. P.M.S. to give a significantly lower ovarian response in the anoestrous season, were treated during the late anoestrous.

3. Recovery of ova

The data in this section were from ewes treated with P.M.S. during the breeding season and unless otherwise indicated were presented on a per ewe basis. Also, the data were presented as percentages and were transformed using an arcsin transformation. This transformation

TABLE 3

OVARIAN RESPONSE AFTER TRANSFORMATION

Dose of P.M.S. (i.u.)	Season	No. of Ewes	No. Corpora Lutea (Mean ± S.E.)	No. Corpora Lutea and Large Follicles (Mean ± S.E.)
1,000	Breeding	14	3.65 ± 0.05	5.02 ± 0.07
	Anoestrous	11	0.92 ± 0.06	3.20 ± 0.03
	---	--	---	---
	Combined	25	2.22 ± 0.04	4.16 ± 0.03
1,200	Breeding	24	2.19 ± 0.02	3.10 ± 0.01
	Anoestrous	7	1.90 ± 0.01	3.10 ± 0.05
	---	--	---	---
	Combined	31	2.12 ± 0.01	3.10 ± 0.01
1,500	Breeding	57	4.54 ± 0.02	6.00 ± 0.02
	Anoestrous	18	0.53 ± 0.05	3.06 ± 0.02
	---	--	---	---
	Combined	75	3.22 ± 0.02	5.20 ± 0.01

ANALYSIS OF VARIANCE

Source	d.f.	No. Corpora Lutea (Mean Squares)	No. Corpora Lutea and Large Follicles (Mean Squares)
Between doses	2	1.68 (N.S.)	3.05 (N.S.)
Between seasons within doses	3	10.81 **	2.68 **
Error	125	0.77	0.62

N.S. = Not Significant

** = $P < 0.01$

is to the angle whose sine is the square root of the percentage and is conducted because the distribution tends to be binomial in form if the variable consists of the proportion of individuals affected.

(a) Effect of P.M.S. dose on the percentage of ova recovered per ewe

Bartlett's Test for homogeneity of variance of these transformed data showed that the variance was homogeneous (Chi^2 = 3.22 - not significant). Table 4 presents the percentage recovery of ova per ewe for the three dose levels of P.M.S. and from the analysis of variance it can be seen that the dose of P.M.S. had no significant effect on the percentage recovery of ova.

If however, for each dose of P.M.S. the percentage of ova recovered was calculated from the proportion of ova recovered of the total number of ova shed (based on a count of the number of corpora lutea) by all the ewes receiving that dose, the recovery percentages would be 63% (1,000 i.u.), 84% (1,200 i.u.) and 80% (1,500 i.u. P.M.S.). The difference in percentage recovery between 1,000 i.u. and 1,200 i.u. P.M.S. was significant (Chi^2 = 6.93 -$P < 0.01$).

(b) Effect of Ovarian Response on the percentage of ova recovered

From Figures 5 and 6 it can be seen that the percentage of ova recovered per ewe was not markedly affected by either the number of corpora lutea or the number of corpora lutea and large follicles present in the ovaries of that ewe.

(c) Effect of the interval from onset of oestrus to laparotomy on the percentage of ova recovered

Any ewe which had two laparotomies performed after oestrus, had a recovery rate recorded for each laparotomy. The results for this section

TABLE 4

EFFECT OF DOSE OF P.M.S. ON THE PERCENTAGE OF OVA RECOVERED AND ON THE PERCENTAGE OF RECOVERED OVA FERTILISED PER EWE (TRANSFORMED DATA)

Dose of P.M.S. (i.u.)	Percentage Ova Recovered (Mean ± S.E.)	Percentage Recovered Ova Fertilised (Mean ± S.E.)
1,000	84.0 ± 1.1	91.1 ± 3.1
1,200	97.6 ± 0.4	97.1 ± 0.7
1,500	91.3 ± 0.3	84.4 ± 0.7

ANALYSIS OF VARIANCE

Source	d.f.	Percentage Ova Recovered (Mean Squares)	Percentage Recovered Ova Fertilised (Mean Squares)
Between doses	2	980.02 (N.S.)	1412.67 (N.S.)
Error	85	519.05	992.78

N.S. = Not Significant

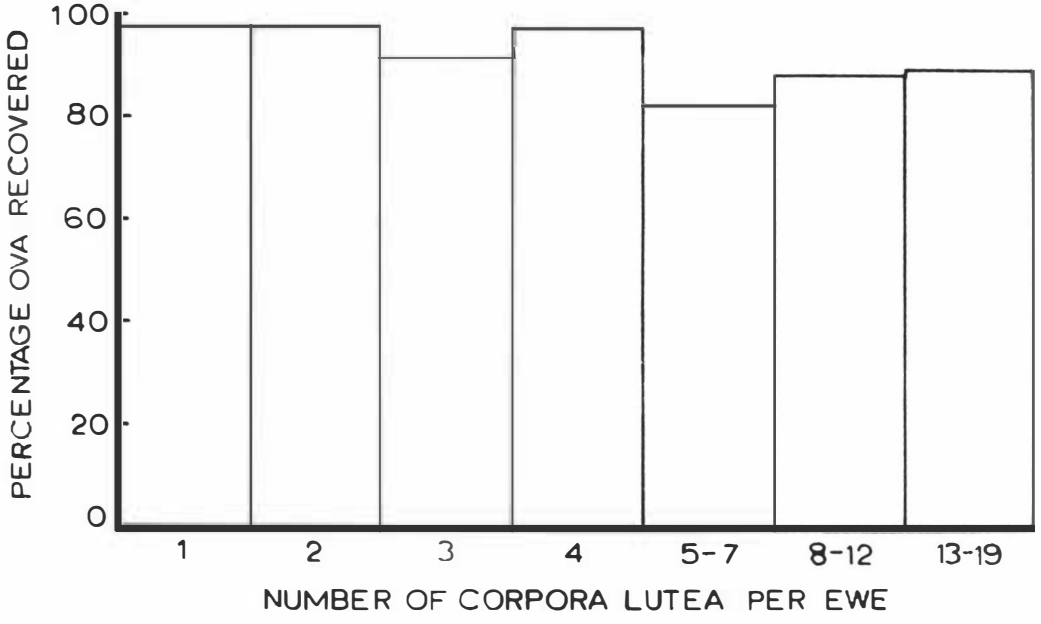


Figure 5 - Mean percentage of ova recovered per ewe relative to the number of corpora lutea in the ovaries of that ewe.

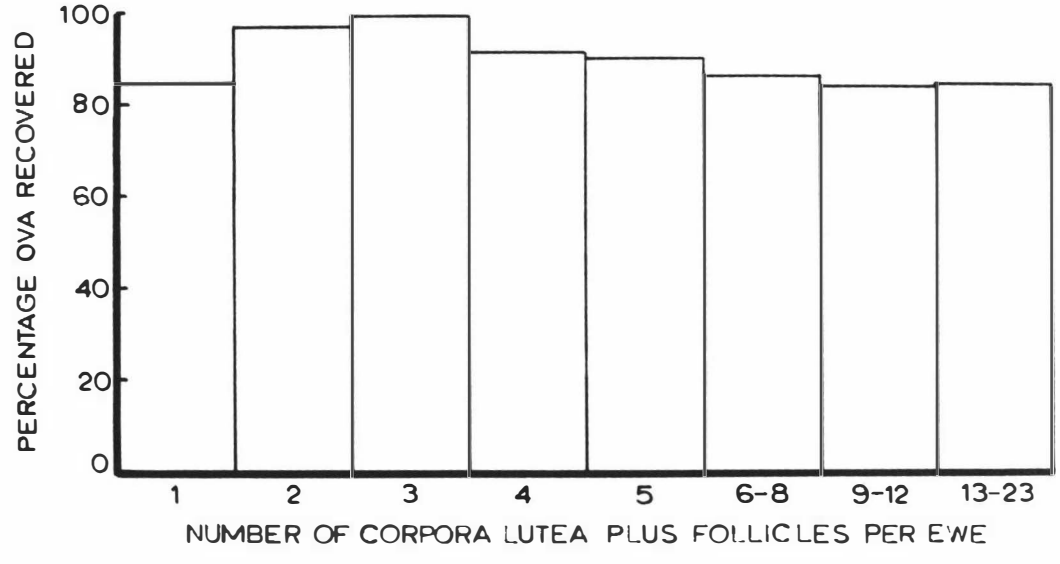


Figure 6 - Mean percentage of ova recovered per ewe relative to the number of corpora lutea plus large follicles in the ovaries of that ewe.

are presented in Figure 7 and it can be seen that increasing the interval from oestrus to laparotomy had no marked effect on the recovery of ova until this interval reached 88 hours. After this time there was a decline in ova recovery.

4. Fertilisation of recovered ova

Ova which were normally cleaved at recovery, and uncleaved ova which had spermatazoa adhered to, or embedded in, the zona pellucida at recovery and which cleaved normally when cultured *in vivo* or *in vitro* for 24 hours (Chapter III), were classified as being fertilised at recovery.

The data in this section were from the ewes treated with gonadotrophin during the breeding season, and were transformed using an arcsin transformation and unless otherwise indicated were presented on a per ewe basis.

(a) Effect of dose of P.M.S. on the percentage of recovered ova fertilised per ewe

After arcsin transformation, Bartlett's Test for homogeneity of variance of these data showed that the variance was homogeneous (Chi^2 = 2.71 - not significant). The percentage of recovered ova fertilised per ewe is presented in Table 4 for the three dose levels of P.M.S., and it can be seen from the analysis of variance that the dose of P.M.S. had no significant effect on the percentage of recovered ova fertilised.

If the percentage of recovered ova fertilised was not calculated on a per ewe basis (i.e. was calculated from the proportion of ova recovered fertilised of the total number of ova recovered from all the ewes receiving each dose level of P.M.S.), the fertilisation rates would

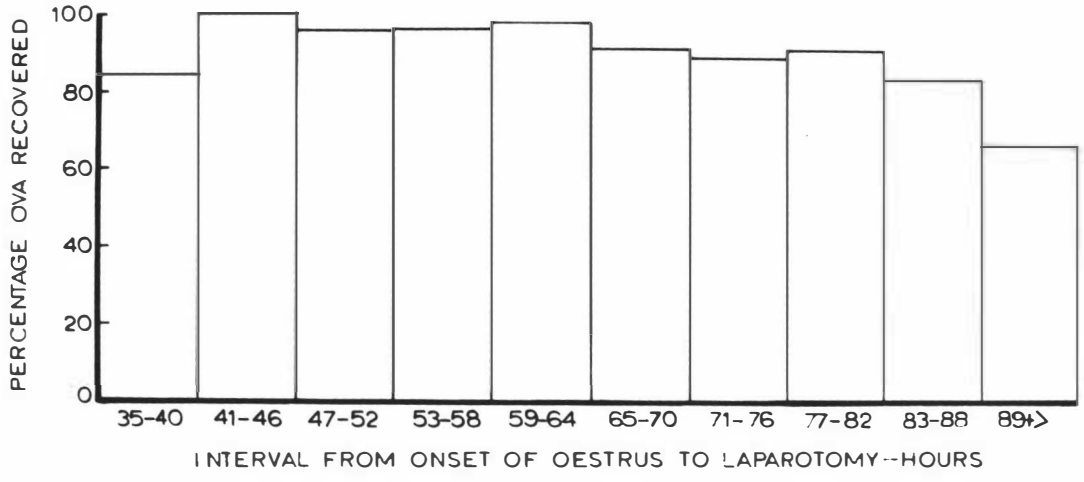


Figure 7 - Mean percentage of ova recovered per ewe relative to the interval from onset of oestrus to laparotomy.

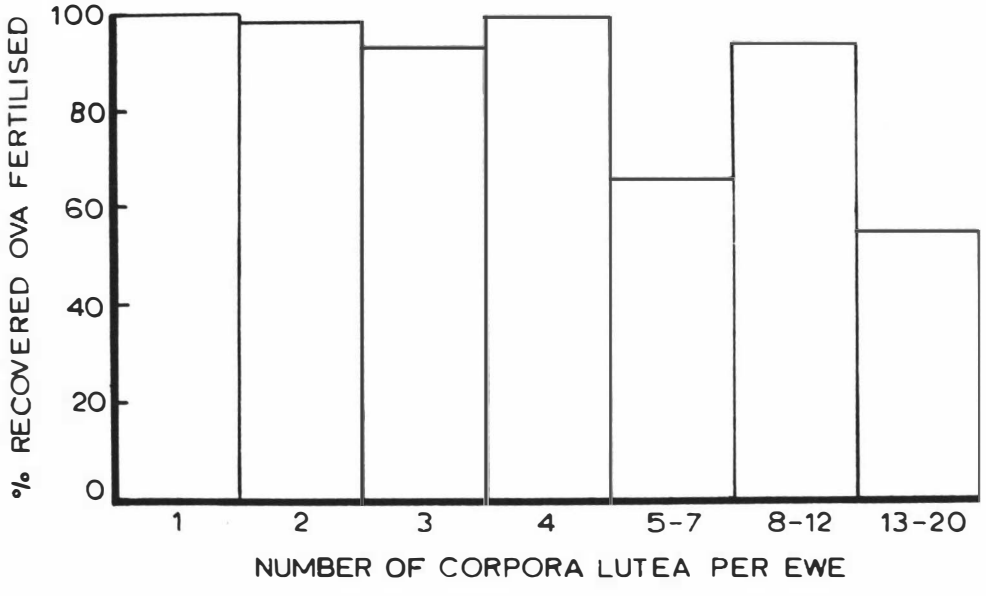


Figure 8 - Mean percentage of recovered ova fertilised per ewe relative to the number of corpora lutea in the ovaries of that ewe.

be 71% (1,000 i.u.), 91% (1,200 i.u.) and 62% (1,500 i.u. P.M.S.). The difference in fertilisation rate between animals injected with 1,500 i.u. P.M.S. and animals injected with 1,000 i.u. and 1,200 i.u. P.M.S. was significant ($Chi^2 = 13.3$; $P < 0.01$).

(b) Effect of number of corpora lutea on the percentage of recovered ova fertilised per ewe.

The results presented in Figure 8 show that the percentage of recovered ova fertilised was not markedly affected by the number of corpora lutea up to 8 to 12, but above this number there was a decline in fertilisation. The result for 5 to 7 corpora lutea was low, but only 5 ewes contributed to this result.

(c) Effect of number of corpora lutea plus large follicles on the percentage of recovered ova fertilised per ewe.

From Figure 9 it can be seen that the percentage fertilisation declined markedly after an ovarian response of 6 to 12 corpora lutea plus large follicles.

(d) Percentage of recovered ova fertilised per ewe as influenced by the interval from onset of oestrus to laparotomy.

Figure 10 shows that after and including 53 hours from the onset of oestrus, increasing intervals to laparotomy had no marked effect on the percentage of recovered ova which were fertilised. Prior to this time however, the fertilisation rate was greatly reduced.

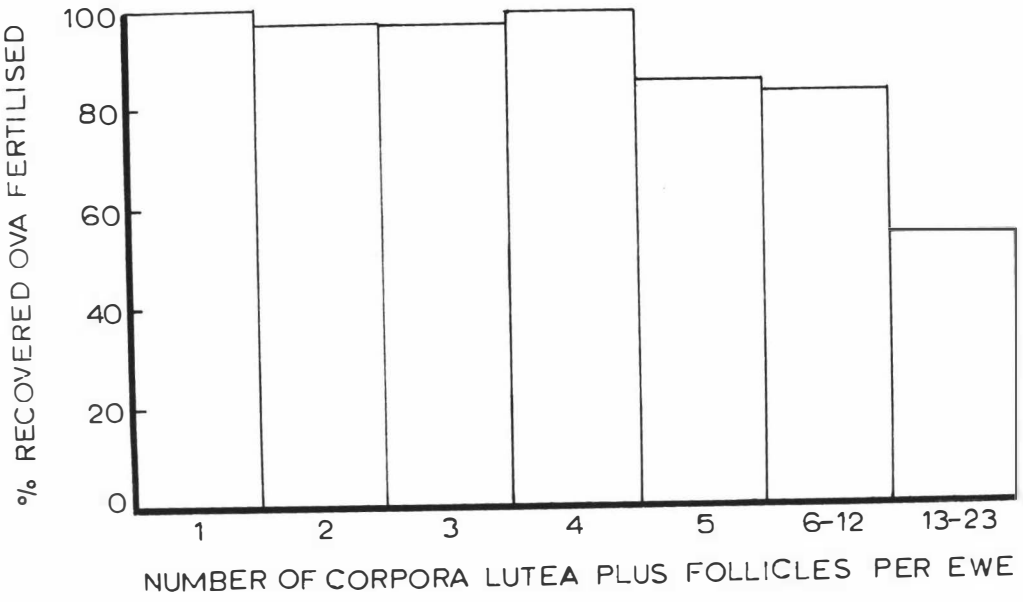


Figure 9 - Mean percentage of recovered ova fertilised per ewe relative to the number of corpora lutea plus large follicles in the ovaries of that ewe.

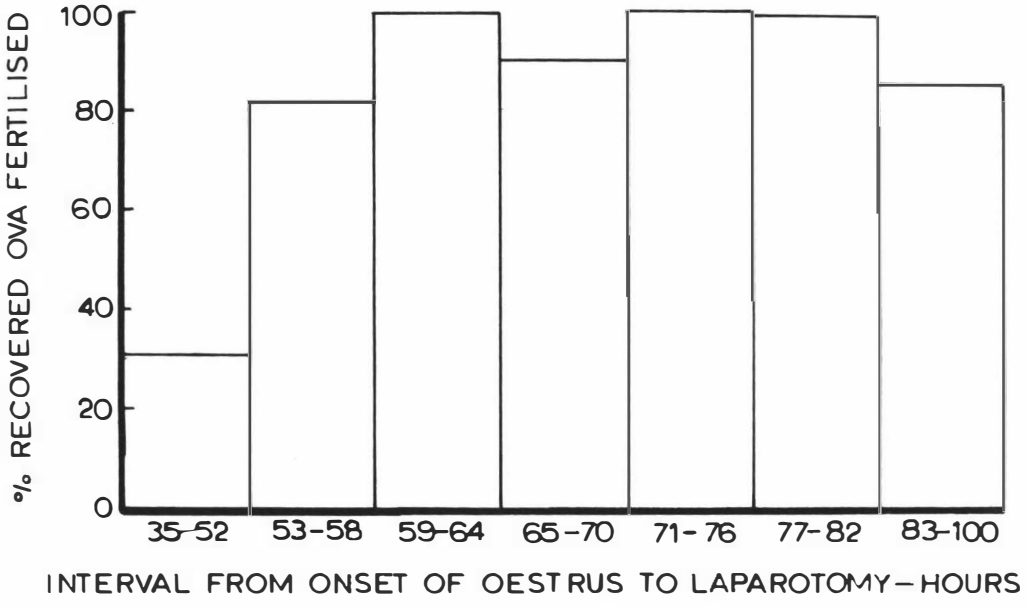


Figure 10 - Mean percentage of recovered ova fertilised per ewe relative to the interval from onset of oestrus to laparotomy.

PRODUCTION OF OVA FROM HORMONALLY STIMULATED EWES IN THE EARLY AND LATE ANOESTROUS SEASONS

1. Early anoestrous

Eleven of 18 ewes treated with intra-vaginal sponges were not pregnant as a result of breeding season experimentation at the time of sponge insertion. Of these 11 ewes, 5 were injected subcutaneously with 1,200 i.u. P.M.S. on the day before sponge withdrawal (day -1), and 6 injected on the day of withdrawal (day 0). Two ewes injected with P.M.S. on day 0 were not stimulated by the hormonal treatment (i.e. did not exhibit oestrus or ovulate), one ewe injected on day -1 and one on day 0 experienced a 'silent oestrus', while the remaining 7 ewes all experienced normal oestrus and ovulation.

(a) Effect of time of P.M.S. injection relative to Progestagen withdrawal on the interval to onset of oestrus and on the ovarian response.

Table 5 shows that the time of P.M.S. injection had no marked effect on the interval to onset of oestrus (from progestagen withdrawal), but that the injection of P.M.S. on day -1 gave the greatest ovarian response. Insufficient data were available to reveal the significance of these differences.

(b) Recovery and fertilisation of ova per ewe.

The mean percentage recovery of ova was 98.3 $\pm$ 1.7% and the mean percentage of recovered ova fertilised was 93.3 $\pm$ 6.7%. Comparison of these results with those in Table 4 for breeding season ewes treated with 1,200 i.u. P.M.S., shows that the treatment of anoestrous ewes with progestagen and 1,200 i.u. P.M.S. had no marked effect on ova recovery or fertilisation.

TABLE 5

EFFECT OF TIME OF P.M.S. INJECTION RELATIVE TO PROGESTAGEN WITHDRAWAL ON INTERVAL TO ONSET OF OESTRUS AND ON OVARIAN RESPONSE

Day of P.M.S. Injection Relative to Progestagen Withdrawal	No. of Ewes	Interval to Onset of Oestrus from Progestagen Withdrawal (Mean - hours)	Ovarian Response	
			No. Corpora Lutea (Mean)	No. Corpora Lutea and Large Follicles (Mean)
0	3	42.0	1.67	3.00
-1	4	40.0	2.25	3.75

2. Late anoestrous

Of 40 ewes treated with intra-vaginal sponges, one was rejected from the experiment because her sponge could not be withdrawn and 39 were injected with 1,000 i.u. P.M.S. on the day of sponge withdrawal. Of these latter ewes, 35 experienced oestrus but all 39 were injected with P.M.S. on the real or estimated day 12 or 13 of the induced cycle. Thirty-one ewes exhibited oestrus after this second P.M.S. injection and 30 of these underwent laparotomy for egg transfer studies (one was not used because no suitable donors were available).

This late anoestrous work is divided into two sections:-

1. Operations performed on 1st and 2nd February 1967 (Anoestrous 1)
2. Operations performed on 10th to 14th February 1967 (Anoestrous 2)

(a) Ovarian Response

The ovarian response data is presented after square root transformation in Table 6. There was no significant difference between doses of P.M.S. for the number of corpora lutea or the number of corpora lutea plus follicles, but there was a significant difference ($P < 0.05$) between anoestrous seasons within doses for the number of corpora lutea.

(b) Recovery and Fertilisation of ova per ewe

The results are presented in Table 7, and although inadequate to analyse they indicate that when compared with the breeding season results for each dose of P.M.S. (Table 4), the hormonal treatment given to these anoestrous ewes did not markedly affect the recovery or fertilisation of ova.

TABLE 6

OVARIAN RESPONSE OF EWES TREATED IN THE LATE ANOESTROUS SEASON

Dose of P.M.S. (i.u.)	No. of Ewes	Stage of Season	No. Corpora Lutea (Mean ± S.E.)	No. Corpora Lutea and Large Follicles (Mean ± S.E.)
1,000	7	Anoestrous 1	1.37 ± 0.10	3.28 ± 0.05
	4	Anoestrous 2	0.36 ± 0.13	3.06 ± 0.06
1,500	7	Anoestrous 1	1.90 ± 0.18	3.46 ± 0.06
	11	Anoestrous 2	0.10 ± 0.03	2.86 ± 0.02

ANALYSIS OF VARIANCE

Source	d.f.	No. Corpora Lutea (Mean Squares)	No. Corpora Lutea and Large Follicles (Mean Squares)
Between doses	1	0.38 (N.S.)	0.00 (N.S.)
Between seasons within doses	2	2.89 *	0.07 (N.S.)
Error	25	0.66	0.33

N.S. = Not Significant

* = $P < 0.05$

TABLE 7

RECOVERY AND FERTILISATION OF OVA IN THE LATE ANOESTROUS SEASON

Dose of P.M.S. (i.u.)	No. of Ewes	Stage of Season	Percentage Ova Recovered (Mean)	Percentage Recovered Ova Fertilised (Mean)
1,000	1	Anoestrous 1	100.00	100.00
	1	Anoestrous 2	100.00	0.00
1,500	4	Anoestrous 1	81.80	75.00
	2	Anoestrous 2	100.00	85.40

CHAPTER V

CLEAVAGE OF OVA IN VIVO

Chapter V

CLEAVAGE OF OVA IN VIVO

One hundred and one ewes were laparotomised to supply data on ovum cleavage rate *in vivo*. Eighty-seven were laparotomised during the 1966 breeding season, 49 of these giving data from laparotomy 1 alone, 15 date from Treatment A (Figure 1) and 23 data from Treatment B (Figure 1). The remaining 14 ewes were laparotomised during the anoestrous season and gave data from laparotomy 1 alone.

In Treatment B, data on cleavage rate *in vivo* could be obtained from one side of a genital tract at laparotomy 1 and after transfer, at laparotomy 2. The advantage of this Treatment over Treatment A was that these data on cleavage rate, could be compared to the cleavage rate of ova in the other side of the genital tract of the same ewe, which was handled at laparotomy 1, but flushed only at laparotomy 2.

NUMBERS OF CLEAVED, UNCLEAVED, FERTILISED OR UNFERTILISED OVA RECOVERED

The numbers of ova recovered cleaved, uncleaved, fertilised or unfertilised is recorded in Table 8 for both the breeding and anoestrous seasons. Season was found to have no significant effect on the proportion of uncleaved ova recovered, but a significantly greater proportion of unfertilised ova were recovered during the breeding season ($P < 0.05$). It can be seen that 13 ova in the breeding season and 10 ova in the anoestrous season were initially recorded as being uncleaved at recovery, but using the criterion of fertilisation outlined in Chapter III, these 23 ova were subsequently found to be fertilised.

TABLE 8

NUMBERS OF CLEAVED, UNCLEAVED, FERTILISED OR UNFERTILISED OVA RECOVERED

Season	Number of ova Recovered	Number of Cleaved Ova Recovered	Number of Uncleaved Ova Recovered	Percentage of Recovered Ova Uncleaved	Number of Fertilised Ova Recovered	Number of Unfertilised Ova Recovered	Percentage of Recovered Ova Unfertilised
Breeding	342	212	130	(38.0) (a)	225	117	(34.2) (c)
Anoestrous	31	17	14	(45.2) (b)	27	4	(12.9) (d)
Total	373	229	144		252	121	

Comparison of differences between seasons

(1) Proportion of uncleaved ova recovered: a v b : $Chi^2 = 0.59$ (N.S.)

(2) Proportion of unfertilised ova recovered: c v d : $Chi^2 = 5.97$ *

N.S. = Not Significant
* = $P < 0.05$

At laparotomy 1, 11 ewes yielded both cleaved and uncleaved ova and 7 ewes (including 6 of the former ewes) both fertilised and unfertilised ova.

CLEAVAGE OF FERTILISED OVA

1. Shortest and longest interval after the onset of oestrus to the recovery of each cleavage stage

The shortest and longest interval to the recovery of each cleavage stage is shown in Table 9. It can be seen that there was a wide possible time-range during which each stage of cleavage could be recovered.

2. Mean interval after the onset of oestrus to the recovery of each cleavage stage

The results presented in Table 10 and Figure 11 show that the fertilised ova cleave at a rapid rate to the 8- to 12- cell stage. From Figure 11 it can again be noted that each cleavage stage is recovered over a very wide time-period.

(a) Effect of P.M.S. dose level

The mean interval after the onset of oestrus to the recovery of most cleavage stages is presented in Table 11 for the three dose levels of P.M.S. The dose of P.M.S. had no marked effect on the interval to recovery of ova at $\geq$6- to 8- cell stages. With 2- cell to 4- to 6- cell ova, the lower the dose of P.M.S., the shorter was the interval to recovery of each stage. However the converse applied to the 6- cell ova.

TABLE 9

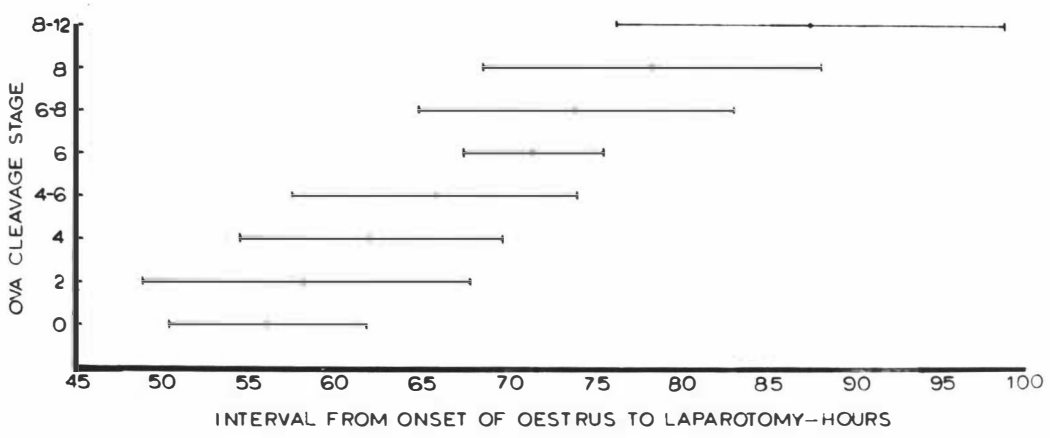
SHORTEST AND LONGEST INTERVAL AFTER THE ONSET OF OESTRUS TO RECOVERY OF EACH CLEAVAGE STAGE

Cleavage Stage (Number of cells per ovum)	Shortest Interval (hours)	Longest Interval (hours)
1	47.00	70.50
2	40.00	95.00
4	40.00	78.00
4 to 6	56.25	80.25
6	69.00	79.25
6 to 8	61.50	94.25
8	62.00	95.50
8 to 12	71.75	102.25

TABLE 10

MEAN INTERVAL AFTER THE ONSET OF OESTRUS TO RECOVERY OF EACH CLEAVAGE STAGE

Cleavage Stage (Number of cells per ovum)	Number of Fertilised Ova	Interval to Recovery (hours)	
		Mean ± S.E.	Variance
1	23	56.10 ± 1.47	32.26
2	64	58.28 ± 1.74	88.20
4	53	62.05 ± 1.46	57.15
4 to 6	14	65.75 ± 3.08	66.38
6	11	71.42 ± 1.62	15.84
6 to 8	35	73.88 ± 2.61	81.57
8	36	78.18 ± 2.60	94.47
8 to 12	16	87.43 ± 4.25	126.29



= Mean ± Standard deviation
Ova cleavage stage = number of cells per ovum
0 ova cleavage stage = uncleaved but fertilised ova

Figure 11 - Times of recovery of fertilised ova at various cleavage stages in relation to the onset of oestrus.

TABLE 11

EFFECT OF DOSE OF P.M.S. ON THE MEAN INTERVAL FROM ONSET OF OESTRUS TO RECOVERY OF EACH CLEAVAGE STAGE

Cleavage Stage (Number of cells per ovum)	DOSE P.M.S.		
	1,000 i.u. Mean ± S.E. (hours)	1,200 i.u. Mean ± S.E. (hours)	1,500 i.u. Mean ± S.E. (hours)
2	50.75 ± 5.5	56.97 ± 2.4	60.31 ± 2.5
4	55.75 ± 4.0	60.38 ± 1.1	65.25 ± 2.0
4 to 6	-	63.92 ± 3.0	67.13 ± 5.2
6	74.13 ± 5.1	70.38 ± 3.8	69.75 ± 0.5
6 to 8	75.63 ± 3.6	74.88 ± 5.1	73.19 ± 3.8
8	79.50 ± 1.0	-	79.18 ± 3.05
8 to 12	-	-	87.43 ± 4.25

TABLE 12

EFFECT OF SEASON ON THE INTERVAL TO RECOVERY OF 2- CELL OVA

Season	No. Ova	Interval from Onset of Oestrus to Recovery of Ova Mean ± S.E. (hours)
Anoestrous	11	54.94 ± 1.59
Breeding	53	59.56 ± 2.29

ANALYSIS OF VARIANCE

Source	d.f.	Mean Squares
Between Seasons	1	123.75 (N.S.)
Error	27	86.88

N.S. = Not Significant

(b) Effect of season on the interval to recovery of 2- cell ova

For ova at the 2- cell stage of cleavage, an analysis of variance was conducted to test the significance of the shorter interval to recovery in the anoestrous season. The results presented in Table 12 show that this difference in interval to recovery was not significant. Data were not available for the other cleavage stages since only a few ewes treated during the anoestrous season produced ova at these other stages.

3. Cleavage of ova recovered from the Fallopian tube unflushed at laparotomy 1, but flushed at laparotomy 2 (Treatment B - Figure 1)

The object of this section was to see if the manipulation of the genital tract at laparotomy 1 (when one Fallopian tube was flushed), had any effect on the cleavage rate of ova in the tube not flushed until laparotomy 2 (experimental tube) in the same ewe. The results for cleavage rate of ova in the experimental tube are presented in Table 13. In order to calculate this cleavage rate, the cleavage stage of ova at laparotomy 1 in the experimental tube was assumed to be the same as that of ova recovered from the tube flushed at this time, in the same ewe. Evidence to support this assumption was obtained from 22 ewes where ova were recovered from both Fallopian tubes at laparotomy 1. Nineteen (86.4%) of these gave ova from the two tubes at the same cleavage stage, one (4.5%) gave 6- cell ova from one tube and 8- cell ova from the other, and two (9.1%) yielded uncleaved ova from one tube and cleaved ova from the other. In the latter 2 ewes the uncleaved ova were unfertilised. On the basis of

TABLE 13

CLEAVAGE RATE OF OVA IN THE EXPERIMENTAL FALLOPIAN TUBE

Experimental Fallopian Tube			Interval Between Cleavage Stages (Mean hours)		Result No.
Assumed Cleavage Stage at Laparotomy 1 (No. cells per ovum)	Recovered Cleavage Stage at Laparotomy 2 (No. cells per ovum)	No. Ova Recovered at Laparotomy 2	Exptal Tube	Control (Table 10)	
1	4	1	22.0	6.0	1
1	8	3	24.4	22.1	2
2	6 to 8	3	24.5	15.6	3
2	8	4	24.8	19.9	4
2	8 to 12	3	22.5	29.2	5
4	6 to 8	1	22.5	11.8	6
4	8	3	24.4	16.1	7
4	8 to 12	6	23.5	25.4	8
4 to 6	8 to 12	2	22.3	21.7	9
6 to 8	8 to 12	1	24.0	13.6	10
8	8 to 12	1	21.8	9.3	11

these results it was felt that the assumption made was a valid one, and that the cleavage rate of ova in the experimental Fallopian tube, (i.e. the interval in hours for the ova in the experimental tube to develop from the assumed cleavage stage at laparotomy 1, to the cleavage stage recovered at laparotomy 2), could be calculated and compared with the control cleavage rate between these cleavage stages (from Table 10).

The experimental and control rates of cleavage were similar ($\pm$ 5 hours) in results 2, 4, 8 and 9, but differed by more than 5 hours in the remaining results. In results 5 and 8 the ova cleaved faster in the experimental tract, but in results 1, 6, 10 and 11 the rate of cleavage was much slower in the experimental tube (-10 hours). In result 1, the uncleaved ovum recovered at laparotomy 1 in the flushed tube, had much spermatazoa attached to the zona pellucida and cleaved on subsequent culture in vitro. It was therefore fertilised at recovery and the long interval of time to the recovery of the 4- cell ovum in the experimental tube, could be due to the time interval from initiation of fertilisation to the initiation of the first cleavage division in the uncleaved egg. In result 2, the uncleaved ova recovered at laparotomy 1 were also fertilised and it was assumed that the ova in the experimental tract must have initiated cleavage soon after laparotomy 1, thus resulting in similar experimental and control cleavage rates to the 8- cell stage.

Table 14 shows the interval (after the onset of oestrus) to the recovery of 6- to 8- cell and 8- cell ova from the experimental tubes of 7 ewes (at laparotomy 2), and from 19 ewes at laparotomy 1. The results suggest that the handling of the tract at laparotomy 1, may retard the cleavage rate to both of these cell stages in the experimental tube.

TABLE 14

INTERVAL FROM THE ONSET OF OESTRUS TO RECOVERY OF 6- to 8- CELL AND 8- CELL OVA FROM THE EXPERIMENTAL TUBES AND FROM TUBES AT LAPAROTOMY 1

Cleavage Stage (Number of cells per ovum)	Interval to Recovery (hours after onset of oestrus)			
	Laparotomy 1 (Mean ± S.E.)	No. of Ewes	Laparotomy 2 (Mean ± S.E.)	No. of Ewes
6 to 8	71.45 ± 2.13	10	86.00 ± 8.25	2
8	74.67 ± 2.72	9	84.50 ± 4.39	5

Insufficient results were available from the other cleavage stages to allow similar comparisons to be made.

The effect of the double laparotomy in Treatment B upon the percentage ova recovered per ewe is shown in Table 15. The recovery of ova at laparotomy 1 was significantly greater ($P < 0.01$) than that from the experimental tube at laparotomy 2.

4. Cleavage of ova transferred into one or both Fallopian tubes at laparotomy 1 and recovered at laparotomy 2 (Treatments A and B - Figure 1)

Thirty-three ewes were involved in this section of the experiment, but only 25 supplied information on cleavage rate after transfer. Information was not obtained from the remaining 8 ewes because the transferred ova either were not fertilised (2 ewes), or were not recovered at laparotomy 2 (6 ewes).

Table 16 shows the mean interval taken by the transferred ova to cleave from the transfer to the recovery stage of cleavage. This interval indicates the rate of cleavage after transfer and has been compared to the control cleavage rate between the same ovum cleavage stages. The transfer and control cleavage rates were similar in results 4, 6, 9 and 10 ($\pm$ 5 hours), and in result 9 the ova cleaved faster after transfer. In results 1, 2 and 3 the long interval of time to the recovery of ova at the various cleavage stages after transfer (relative to the control intervals), could again be due to the interval involved from initiation of fertilisation to initiation of first cleavage in the uncleaved egg. However, the remaining results indicate that the rate of ovum cleavage was considerably retarded after transfer.

TABLE 15

EFFECT OF DOUBLE LAPAROTOMY IN TREATMENT B, AND TRANSFER OF OVA AT LAPAROTOMY 1 BEFORE RECOVERY AT LAPAROTOMY 2, ON THE PERCENTAGE OF OVA RECOVERED PER EWE

Treatment(s) (See Figure 1)		Percentage of Ova Recovered (Mean ± S.E.)	No. Ewes
B	- Ova Recovery at Laparotomy 1	99.4 ± 0.2 (a)	23
B	- Ova Recovery at Laparotomy 2 in Experimental Tube	73.5 ± 1.7 (b)	23
A and B	- Ova Recovery at Laparotomy 1	91.3 ± 0.3 (c)	33
A and B	- Ova Recovery at Laparotomy 2 after transfer at Laparotomy 1	58.3 ± 0.8 (d)	33

ANALYSIS OF VARIANCE

Source	d.f.	Mean Squares	
(a) v (b)	1	8091.24	**
(c) v (d)	1	10911.22	**
(b) v (d)	1	1160.78	N.S.

** = P < 0.01

N.S.= Not Significant

TABLE 16

CONTROL CLEAVAGE RATE AND CLEAVAGE RATE OF OVA TRANSFERRED AT LAPAROTOMY 1 AND RECOVERED AT LAPAROTOMY 2

Cleavage Stage (Number of cells per ovum)		No. Ova Recovered after Transfer	Interval Between Cleavage Stages (Mean - hours)		Result No.
Stage Transferred	Stage Recovered After Transfer		After Transfer	Control (Table 10)	
1	2	2	22.8	2.2	1
1	4	2	22.8	6.0	2
1	6 to 8	1	24.5	17.8	3
1	8	2	23.8	22.1	4
2	6 to 8	6	24.3	15.6	5
2	8	4	24.3	19.9	6
4	6 to 8	3	24.0	11.8	7
4	8	3	23.9	16.1	8
4	8 to 12	2	23.5	25.4	9
4 to 6	8 to 12	2	23.3	21.7	10
6 to 8	8	2	22.0	4.3	11
6 to 8	8 to 12	4	24.5	13.6	12
8	8 to 12	2	21.8	9.3	13

Table 17 shows the interval (after the onset of oestrus) to the recovery of 6- to 8- cell and 8- cell ova from 19 ewes at laparotomy 1, and 14 ewes at laparotomy 2 after transfer of ova of various cleavage stages at laparotomy 1. A valid analysis of variance could not be conducted because the 33 ewes involved did not represent a random sample from the population. However, the results suggest that the transfer of ova at laparotomy 1, may retard the cleavage rate of ova to the two cell stages studied.

The recovery of ova at laparotomy 2 after previous transfer (at laparotomy 1), was significantly lower than the recovery of ova at laparotomy 1 ((c) v (d) Table 15 - $P < 0.01$); but was not significantly lower than the recovery of ova at laparotomy 2 from the experimental tube described in the last section ((b) v (d) Table 15 - Not significant).

TABLE 17

INTERVAL FROM THE ONSET OF OESTRUS TO RECOVERY OF 6- to 8- CELL AND 8- CELL OVA AFTER TRANSFER, AND AT LAPAROTOMY 1

Cleavage Stage (Number of cells per ovum)	Interval to Recovery (hours after onset of oestrus)			
	Laparotomy 1 (Mean ± S.E.)	No. of Ewes	Laparotomy 2 (Mean ± S.E.)	No. of Ewes
6 to 8	71.45 ± 2.13	10	82.75 ± 4.69	6
8	74.67 ± 2.72	9	78.34 ± 2.76	8

CHAPTER VI

CULTURE, ABNORMALITIES AND DIMENSIONS OF OVA

Chapter VI

C U L T U R E , A B N O R M A L I T I E S A N D D I M E N S I O N S O F O V A

CULTURE OF OVA

One hundred and seventy-eight ova were cultured for periods of 24, 48 or 72 hours in culture chambers. These ova were recovered from 75 ewes, 63 of which were laparotomised during the 1966 breeding season and 12 during the anoestrous season.

1. Media

One hundred and sixty-two ova were cultured in sterilised sheep serum, and it is from these ova that the results in the following sections were recorded. In addition, 7 ova were cultured in sheep oviduct fluid and 9 in tissue culture medium (Medium 199 - Commonwealth Serum Laboratories, Melbourne, Australia) plus sheep serum (ratios of 75 : 25 and 25 : 75). These two latter media were only used to a limited extent because they gave poor culture results. It was believed that the oviduct fluid gave poor results because it had been collected through alkali impregnated tubing, which caused the medium pH to be too high (8.4) for successful culture, and that the tissue culture plus serum gave poor results because of incorrect osmolarity of the medium.

2. Cleavage of cultured ova

Of 97 ova examined after 24 hours culture and then cultured for a further 24 hours, only 3 (3.1%) continued to cleave over the second

culture period. These 3 consisted of a 12- to 16- cell ovum which continued to cleave to a 32- cell ovum, an 8- cell which cleaved to a 10- cell, and an 8- to 12- cell which cleaved to a 12- to 16- cell ovum. None of 15 ova examined after 24 hours culture and then cultured for a further 48 hours, continued to cleave after the first 24 hours of culture. Because ova did not cleave readily after the first 24 hours of culture, the results presented in Table 18 for normal development, and development of abnormalities relative to the initial cleavage stage of the cultured ova, refer only to the 150 ova recovered from the culture chambers after the first 24 hours of culture.

Uncleaved ova did not cleave readily during culture, a high proportion (40%) of them becoming abnormal. It was believed that the poor development of these cultured uncleaved ova could be explained on the basis of fertilisation. Of the 21 normal uncleaved ova and the 23 abnormal ova recovered after culture, only 4 in each group (19% and 17% respectively) had spermatazoa attached to the zona pellucida. Because the remaining ova did not have spermatazoa attached to the zona pellucida and the 8 ova with spermatazoa present did not cleave normally during culture, all of the normal uncleaved ova and abnormal ova recovered after culture were classified as unfertilised, and would not be expected to cleave normally during culture.

The 2-, 4-, and 6- to 8- cell ova cleaved readily on culture for 24 hours and although most ova cultured at the 8- cell and 8- to 12- cell stages remained normal, they cleaved less readily than most other cleavage stages. The 6- cell ova did not cleave readily, but only 5 ova at this stage were cultured.

The cultured ova were not examined for stage of development at intervals during the 24 hour culture period. Therefore the only

TABLE 18

NORMAL OVA DEVELOPMENT AND DEVELOPMENT OF ABNORMALITIES RELATIVE TO THE INITIAL CLEAVAGE STAGE OF OVA CULTURED FOR 24 HOURS

Initial Cleavage Stage (No. cells per ovum)	No. Ova Recovered After Culture	Ova which Cleaved Normally During Culture		Ova which did not Cleave During Culture but Remained Normal		Ova Becoming Abnormal During Culture	
		No.	(%)	No.	(%)	No.	(%)
1	58	14	(24.1)	21	(36.2)	23	(39.7)
2	16	12	(75.0)	2	(12.5)	2	(12.5)
4	24	19	(79.2)	3	(12.5)	2	(8.3)
6	5	2	(40.0)	3	(60.0)	0	(0)
6 to 8	5	3	(60.0)	2	(40.0)	0	(0)
8	31	12	(38.7)	17	(54.8)	2	(6.5)
8 to 12	11	2	(18.2)	7	(63.6)	2	(18.2)
Total	150	64	(42.7)	55	(36.7)	31	(20.6)

information indicating the cleavage rate of ova *in vitro* was the maximum time (i.e. the culture interval) taken by ova to cleave over most developmental stages *in vitro*. This information does not accurately indicate the rate at which ova cleaved *in vitro* since they may take most, or only a fraction of the culture period to cleave between the recorded stages. From Table 19 where a comparison has been made between the maximum time taken for ova to cleave over most developmental stages *in vitro* and the cleavage interval over the same stages *in vivo*, it can be seen that ova cultured at an early initial cleavage stage would undergo a number of divisions, while ova cultured at later stages would cleave less readily. In result (g), the ovum definitely cleaved faster *in vitro* than would be expected *in vivo*. In results (d), (e) and (f) similar intervals between cleavage stages were recorded *in vitro* and *in vivo*, but in the remaining results, the maximum time for cleavage between two developmental stages *in vitro* was usually considerably greater than the corresponding *in vivo* cleavage time. Because the mean interval to recovery of the fertilised but uncleaved ova in Table 10 does not indicate the time of fertilisation in the uncleaved egg, the longer interval between cleavage stages *in vitro* in results (a), (b) and (c), could be partly due to the interval from fertilisation to initiation of first division in the uncleaved egg.

3. Recovery of ova from the culture chambers

The per chamber recovery of ova after 24 hours culture was 98.6%, after 48 hours culture was 99.6% and after 72 hours culture was 100%. These figures show that the recovery of ova from the chambers was not affected by the length of culture, and also that the chambers used in this study allowed a very high ova recovery rate.

TABLE 19

COMPARISON OF THE MAXIMUM TIMES FOR CLEAVAGE OF OVA IN VITRO WITH THE CLEAVAGE INTERVAL IN VIVO

Cleavage Stage at Commencement of Culture (No. cells per ovum)	Cleavage Stage After Culture (No. cells per ovum)	No. Ova Cultured	Interval Between Cleavage Stages (hours)	
			In Vitro	In Vivo (Table 10)
1	2	7	25.0	2.2 (a)
1	4	3	25.6	6.0 (b)
1	6	3	22.9	15.3 (c)
1	8	1	23.3	22.1 (d)
2	4	5	25.5	3.8
2	6	3	22.4	13.1
2	6 to 8	1	29.8	15.6
2	8	2	21.5	19.9 (e)
2	8 to 12	1	29.3	29.2 (f)
4	6 to 8	2	27.1	11.8
4	8	11	26.1	16.1
4	8 to 12	5	29.5	25.4
4	12	1	22.3	> 25.4 (g)
6	8 to 12	1	23.3	16.0
6	12	1	22.3	> 16.0
6 to 8	8 to 12	2	28.0	13.6
6 to 8	12 to 16	1	26.0	> 13.6
8	8 to 12	3	27.2	9.3
8	12	4	26.0	> 9.3
8	12 to 16	4	27.6	> 9.3
8	16	1	30.0	-
8 to 12	12 to 16	1	26.1	-
8 to 12	16	1	28.0	-

ABNORMALITIES OF OVA

Ova were examined at laparotomy and after culture for the abnormalities described by Hart (1956) [Involution and vacuolation], and Laffey and Hart (1959) [Broken zona pellucida and degeneration or fragmentation of cytoplasm], and for any additional abnormalities.

1. Abnormalities of ova recovered at laparotomy

Of the 373 ova recovered at laparotomy 1 or 2, 43 (11.5%) were found to be abnormal. Table 20 records the types of abnormal ova recovered and their incidence. It can be seen that the predominant abnormality was involution of the vitellus and that a high proportion of the involuted ova were uncleaved (81%) and unfertilised (78%). The incidence of ova with fragmented cytoplasm and abnormally thick zonae pellucidae was low; all ova exhibiting these abnormalities were unfertilised.

The incidence of abnormalities in 121 unfertilised ova as affected by the interval from onset of oestrus to the recovery of these ova, is recorded in Table 21. This incidence increased with increasing intervals from onset of oestrus to laparotomy.

In the 1966 breeding season, 24.6% of the ova recovered in the early season (April to May inclusive) and 6.5% of the ova recovered in the late season (June to July inclusive) were abnormal. Eighty-one per cent of the abnormal ova in the early breeding season were involuted and if the involuted ova were deleted from both the early and late breeding seasons, the incidence of abnormal ova would have been 5.8% and 2.9% respectively.

TABLE 20

THE TYPES AND INCIDENCE OF ABNORMAL OVA RECOVERED AT LAPAROTOMY

Type of Abnormality	No. Ova	For each Type of Abnormality		
		% of Total Abnormal Ova	% Abnormal Ova Uncleaved	% Abnormal Ova Unfertilised
Involution	31	72.09	80.65	77.50
Fragmentation	5	11.63	100.00	100.00
Vacuolation	0	-	-	-
Abnormally thick Zona Pellucida	7	16.28	100.00	100.00
Total Abnormal Ova	43			

TABLE 21

EFFECT OF INTERVAL FROM ONSET OF OESTRUS TO LAPAROTOMY ON THE INCIDENCE OF ABNORMAL UNFERTILISED OVA

Interval from Onset of Oestrus to Laparotomy	No. Unfertilised Ova Recovered	No. Abnormal Unfertilised Ova Recovered	% Unfertilised Ova Abnormal at Recovery
0 - 2 days	12	2	16.7
2 - 3 days	92	28	30.4
3 - 4 days	17	6	35.3
	121	36	29.8

2. Abnormalities of ova after culture

A description of the ova abnormalities at the commencement of culture and after 24, 48 and 72 hours culture is shown in Table 22 and Figure 12.

The abnormal ova after 24, 48 and 72 hours culture only include ova which were either normal at insertion into the chambers and which became abnormal during culture, or ova which were abnormal at insertion and changed abnormality type during culture. It can be seen that there was a marked increase in the incidence of abnormal ova as the culture period lengthened. The incidence of ova with involuted vitellus decreased markedly over the first 24 hours of culture, but was opposed by a marked increase in the incidence of ova with fragmented cytoplasm. The incidence of vacuolated ova increased over the first 24 hours of culture and then remained relatively constant. The ovum with the thick zona pellucida abnormality fragmented during the first 24 hours of culture.

Of the 14 involuted ova placed into the chambers, 10 were classified as unfertilised, and 9 (90%) of these fragmented over the first 24 hours culture.

The marked increase in the incidence of fragmented ova during the first 24 hours of culture, was only partly explained by the fragmentation of ova abnormal at insertion into the chambers. In addition 8 normal uncleaved and 7 normal cleaved ova fragmented during the first 24 hours of culture.

The effect of the initial cleavage stage of the ova on the incidence of abnormalities over culture is noted in Table 23. The uncleaved ova had a high level of abnormalities after 24 hours culture and this level increased as the culture length increased. The other

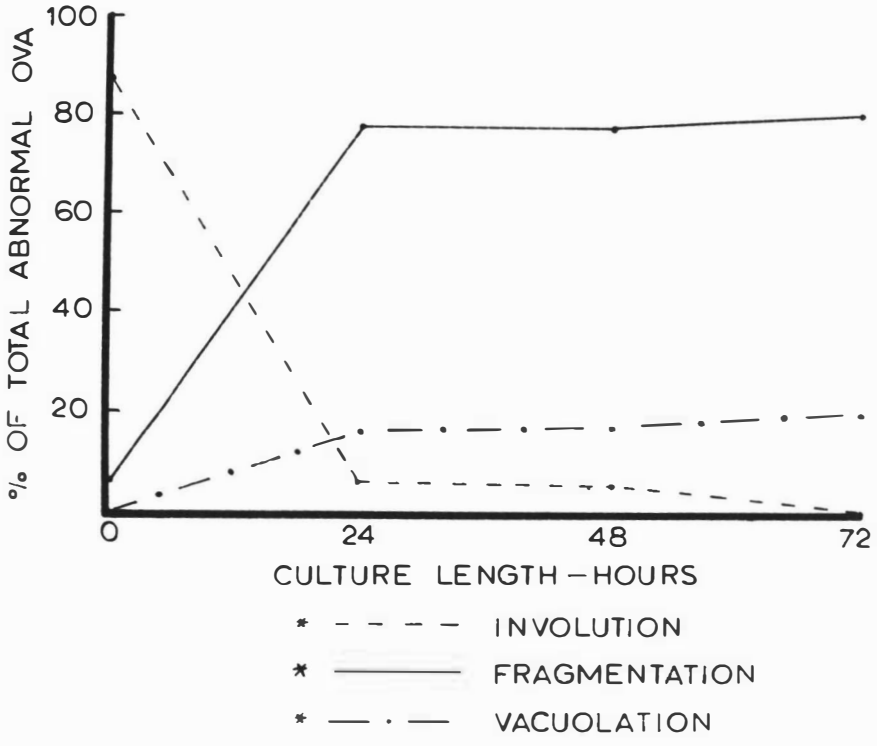
TABLE 22

EFFECT OF CULTURE LENGTH ON THE INCIDENCE OF OVA ABNORMALITIES

Culture Length (hours)	No. Ova Cultured	No. Ova Abnormal (3)	% Ova Abnormal	Type of Abnormality							
				Involution		Fragmentation		Vacuolation		Thick Zona pellucida	
				(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
0 (Insertion of Ova into Chambers)	162	16	9.9	14	(87.5)	1	(6.3)	0	(0)	1	(6.3)
24	150	31	20.7	2	(6.5)	24	(77.4)	5	(16.1)	0	(0)
48	98	35	35.7	2	(5.7)	27	(77.1)	6	(17.1)	0	(0)
72	15	10	66.7	0	(0)	8	(80.0)	2	(20.0)	0	(0)

(1) = No. of ova in each type of abnormality.

(2) = (1) expressed as percentage of (3).



* = Type of abnormality

Figure 12 - Incidence of the types of abnormal ova at the commencement of and during culture.

TABLE 23

EFFECT OF INITIAL CLEAVAGE STAGE OF OVA ON THE INCIDENCE OF ABNORMALITIES AFTER CULTURE

Initial Cleavage Stage (No.cells per ovum)	Culture Length								
	24 hours			48 hours			72 hours		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
1	58	23	(39.7%)	35	16	(45.7%)	9	7	(77.8%)
2	16	2	(12.5%)	6	1	(16.7%)	-		
4	24	2	(8.3%)	12	2	(16.7%)	3	0	(0%)
6	5	0	(0%)	5	0	(0%)	-		
6 to 8	5	0	(0%)	10	6	(60.0%)	-		
8	31	2	(6.5%)	22	5	(22.7%)	1	1	(100%)
8 to 12	11	2	(18.2%)	8	5	(62.5%)	2	2	(100%)

(1) = No. ova recovered after culture

(2) = No. abnormal ova recovered after culture

(3) = % recovered ova abnormal after culture

cleavage stages had a lower level of abnormalities after 24 hours culture, but the level of abnormalities increased markedly over the second 24 hours of culture for both 6- to 8- and 8- to 12- cell ova.

Figures 13 - 16 are examples of normal and abnormal ova recovered after culture.

DIMENSIONS OF OVA

The following dimensions were recorded from 99 normal ova recovered after culture:

1. Total diameter (T).
2. Zonal cavity diameter (Z), i.e. the diameter within the zona pellucida.
3. Average blastomere diameter (b).

Dimensions 1 and 2 were measured once on each ovum, but dimension 3 was obtained from two measurements on each blastomere in 2- cell ova and one measurement on most blastomeres in more fully developed ova. The average dimensions for each ovum cleavage stage are recorded in Table 24. The analysis of variance conducted on the data showed that as the ova continued to cleave, there was no significant increase in T or Z, but there was a highly significant ($P < 0.01$) reduction in average blastomere diameter.

For ova at all cleavage stages, the average T was 175.04 microns, the average Z was 145.95 microns and the average zona pellucida thickness was 14.55 microns. The average zona pellucida thickness of the 7 ova with abnormally thick zonae pellucidae was 28.31 microns.

For each ovum cleavage stage, the mean blastomere volume was calculated using the formulae $V = \frac{4}{3}\pi r^3$ (V= mean blastomere volume, r= mean blastomere radius). Multiplying this volume by the number of

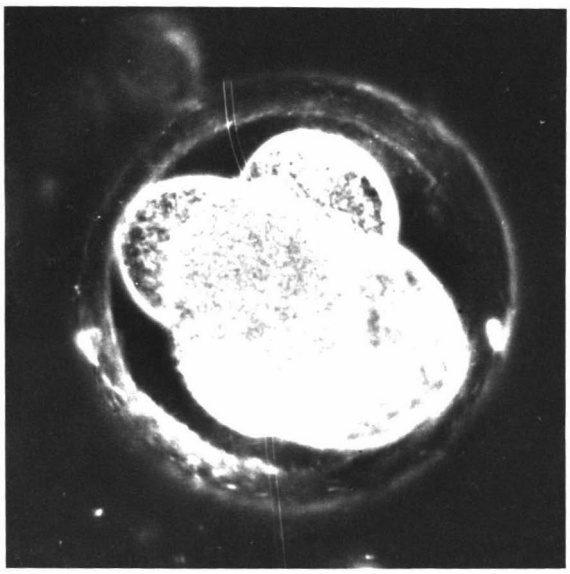


Figure 13 - Normal 4- cell ovum recovered after 24 hours culture. This ovum was fertilised but uncleaved when inserted into the culture chamber.
(Approx. x 300)

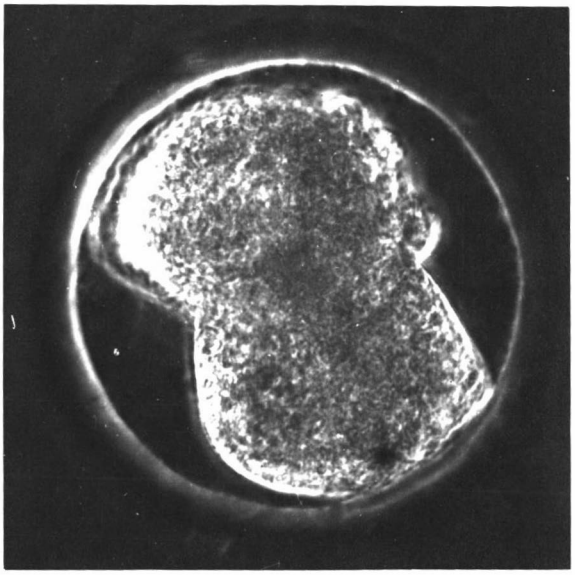


Figure 14 - Fragmenting 2- cell ovum recovered after 48 hours culture. This ovum was fertilised but uncleaved when inserted into the culture chamber.
(Approx. x 400)

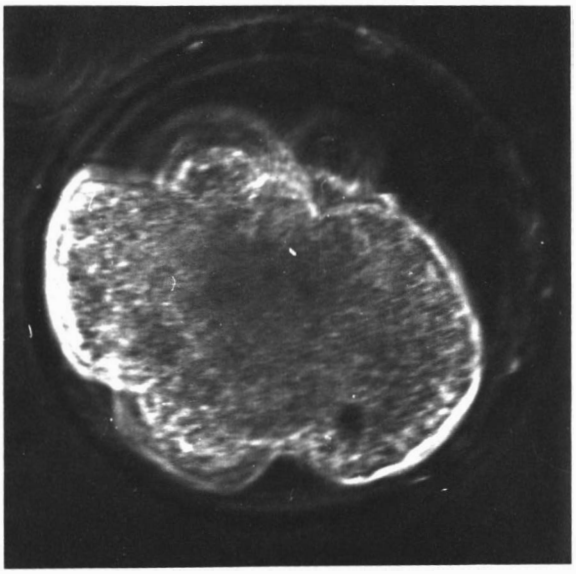


Figure 15 - Fragmenting 4- cell ovum recovered after 48 hours culture. This ovum was a normal 4- cell when inserted into the culture chamber.
(Approx. x 400)

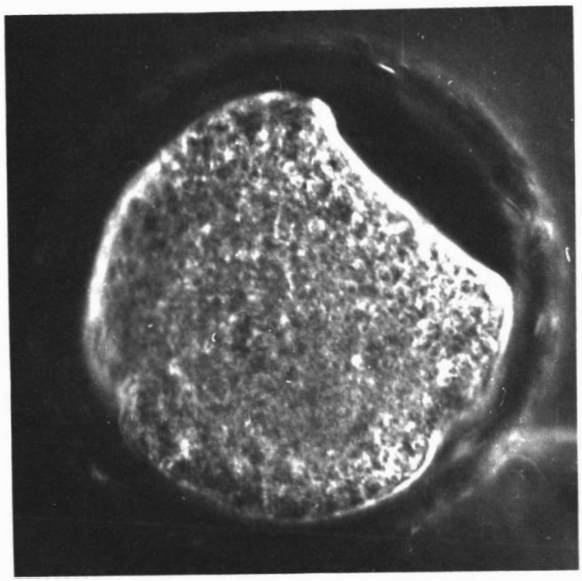


Figure 16 - Involuted, unfertilised ovum beginning to fragment after 48 hours culture. This ovum was involuted when inserted into the culture chamber.
(Approx. x 400)

TABLE 24

DIMENSIONS OF OVA

Cleavage Stage (No. cells per ovum)	No. of Ova	Dimension (microns)		
		T (Mean ± S.E.)	Z (Mean ± S.E.)	b (Mean ± S.E.)
1	38	170.93 ± 1.18	139.91 ± 1.88	120.66 ± 1.89
2	9	172.58 ± 2.95	142.37 ± 2.78	102.49 ± 4.79
4	4	175.80 ± 3.52	147.30 ± 4.26	79.35 ± 3.62
6	4	175.30 ± 2.52	148.55 ± 2.22	62.98 ± 4.44
8	21	174.65 ± 1.57	144.24 ± 1.23	62.87 ± 0.74
8 to 12	13	174.01 ± 1.27	144.86 ± 1.14	57.63 ± 1.46
12 to 16	7	174.94 ± 3.25	145.30 ± 3.21	49.09 ± 2.10
16	3	182.13 ± 1.49	155.07 ± 2.89	47.03 ± 4.14

ANALYSIS OF VARIANCE

Source	d.f.	T (Mean Squares)	Z (Mean Squares)	b (Mean Squares)
Between cleavage stages	7	79.46 (N.S.)	158.62 (N.S.)	12292.24 **
Error	91	50.12	78.54	86.80

N.S. = Not Significant
** = $P < 0.01$
T = Total Ova Diameter
Z = Zonal Cavity Diameter
b = Average Blastomere Diameter

blastomeres in ova at each cleavage stage gave the total volume of ova cytoplasm for that cleavage stage. The results are presented in Table 25 and it can be seen that the blastomere volume was approximately halved with each successive cleavage, and that the total volume of cytoplasm in the ovum decreased from the 2- cell to the 16- cell stage.

TABLE 25

MEAN BLASTOMERE VOLUME AND TOTAL VOLUME OF OVA CYTOPLASM RELATIVE TO THE STAGE OF OVA CLEAVAGE

Stage of Ova Cleavage (No. cells per ovum)	Mean Blastomere Volume (cubic microns)	Total Volume of Ova Cytoplasm (Cubic microns)
1	917,861	917,861
2	562,674	1,125,348
4	261,150	1,044,600
8	129,906	1,039,248
16	54,386	870,176

CHAPTER VII

STUDIES ON THE SURVIVAL OF TRANSFERRED OVA

Chapter VII

STUDIES ON THE SURVIVAL OF TRANSFERRED OVA

In this study, sheep ova were cultured *in vitro* for 24 or 48 hours and then transferred into recipient ewes to test ova viability. In addition, control transfers were conducted where the ova were transferred into recipient ewes almost immediately after recovery from the donor ewes. A total of 29 transfer experiments were conducted and involved the transfer of a total of 58 fertilised ova.

TRANSFERS IN THE 1966 BREEDING AND 1966-67 EARLY ANOESTROUS SEASONS

The number of ewes available for ova transfer experiments in these two seasons was small. This was because ewes became pregnant during the breeding season (as a result of poor recovery of fertilised ova during studies on the *in vivo* cleavage of ova), and also because of unsatisfactory hormonal stimulation of the anoestrous ewes. It was decided that in order to achieve the maximum number of transfer experiments from the available ewes, each ewe would act as donor, and where possible, as recipient of her own ova. Transfer of ova into a ewe was only attempted when all of the ova shed by that ewe (based on the corpora lutea count) had been recovered. Because of poor ova recovery some donors could not act as recipients. All ewes were mated to the entire ram so that each ewe was a potential donor and recipient of her own ova. Thirty of the 36 ova transferred in this study were recovered from, and then transferred into the same ewe.

The success of the ova transfer experiments conducted in these two seasons is shown in Table 26. A transfer experiment was judged to be successful when the recipient ewe became pregnant and therefore one or more embryos were recovered from her genital tract at slaughter.

In these two seasons and also in the late anoestrous and 1967 breeding seasons, all the embryos were viable (as judged by heart activity) when recovered from the slaughtered recipient ewes.

1. Control transfer of ova

Table 26 shows that 4 of the 6 control transfer experiments were successful. In control transfers 1, 2, 3, 5 and 6 (Table 27) the donor and recipient were the same ewe. Therefore the laparotomy for ova recovery from the donor (laparotomy 1) and the laparotomy for ova transfer into the recipient (laparotomy 2), refer to a single laparotomy conducted on each of these ewes. At this laparotomy the ova were recovered and held *in vitro* for a period not exceeding 5 minutes during examination. They were then transferred back into the same ewe and it was assumed that the stage of development of the transferred ova and the recipient genital tract were closely synchronised. Three of these 5 transfer experiments were successful. In transfer number 4, the donor and recipient were different ewes and therefore laparotomy 1 and 2 were conducted on different ewes and at different times relative to the onset of oestrus. Onset of oestrus in the donor was 21.5 hours before that in the recipient and at laparotomy, 2, 6- to 8- cell ova were recovered from the donor and 2, one- cell ova from the recipient. The 2, 6- to 8- cell ova were transferred into the recipient (old ova into young uteri) and both were present as viable embryos at slaughter.

TABLE 26

SUCCESS OF OVA TRANSFER EXPERIMENTS CONDUCTED IN THE BREEDING AND EARLY ANOESTROUS SEASONS

Type of Transfer	No. of Transfer Experiments	No. of Ewes Pregnant after Transfer	No. of Ova Transferred	No. of Viable Embryos at Slaughter
Control	6	4	8	5
After 24 hours culture	8	0	18	0
After 48 hours culture	6	0	10	0

TABLE 27

SYNCHRONISATION OF STAGE OF DEVELOPMENT OF TRANSFERRED OVA AND RECIPIENT GENITAL TRACTS

Type of Transfer	Transfer No.	Time of Lap.1 in Donor (hours from onset of oestrus)	Cell Stages Recovered at Lap. 1 (No. cells per ovum)	Time to Lap.2 or Transfer Lap. in Recipient (hours from onset of oestrus)	No. and Cell Stages of Ova Transferred at Lap. 2	Expected Cell Stages in the Recipient at Transfer Lap. (No. cells per ovum)	Result of Transfer Experiment
Control	1	61.75	2 and 4	61.75	1,2-cell;1,4-cell		N.P.
	2	53.25	2	53.25	1,2-cell		P.
	3	69.75	8	69.75	1,8-cell		P.
	4	* 69.50	6 to 8	48.00	2,6- to 8-cell		P.
	5	53.50	4	53.50	1,4-cell		N.P.
	6	55.00	2	55.00	1,2-cell		P.
After 24 hours culture	1	* 63.50	2 and 4	87.25	1,6-to 8-cell	8 to 12	N.P.
	2	55.50	2	77.00	2,6-cell	8	N.P.
	3	70.25	2	93.25	3,4-cell	8	N.P.
	4	* 63.25	4	87.50	1,6-cell	8 to 12	N.P.
	5	69.75	6 to 8	98.50	4,8-cell	> 8 to 12	N.P.
	6	70.75	4 to 6	95.00	1,4-cell;1,12-cell	> 8 to 12	N.P.
	7	* 80.25	4 to 6	102.50	4,12-to 16-cell	8 to 12	N.P.
	8	53.25	2	77.00	1,4-cell	8	N.P.
After 48 hours culture	1	53.50	1	101.25	1,4-cell	> 8 to 12	N.P.
	2	54.00	2	107.00	1,2-cell;1,4-cell	> 8 to 12	N.P.
	3	62.00	8	109.00	1,8-cell;2,12- to 16-cell	> 8 to 12	N.P.
	4	69.75	4 to 6	117.00	1,6-cell;1,12-cell	> 8 to 12	N.P.
	5	69.00	6	118.50	1,6-cell	> 8 to 12	N.P.
	6	46.00	2	93.00	1,4-cell	> 8 to 12	N.P.

N.P. = Recipient not pregnant
* = Donor and recipient were different ewes
P. = Recipient pregnant
Lap. = Laparotomy

2. Transfer of ova after 24 hours culture

Table 26 shows that none of the 8 transfer experiments were successful. The results on synchronisation are presented in Table 27. In transfers 2, 3, 5, 6 and 8 the donor and recipient were the same ewe, and so laparotomy 1 and 2 refer respectively to the laparotomy for recovery of ova for culture, and the laparotomy for the reinsertion of ova into the same ewe. Ova from donors which could not act as recipients were cultured *in vitro* and then transferred into other recipient ewes. Ova had already been recovered from these latter recipients at laparotomy 1, but these ova either were too few to be transferred, or became abnormal during culture; hence at the transfer laparotomy they were replaced or augmented by ova from other ewes. In transfers 1, 4 and 7 the donors and recipients were different ewes for some ova, and so laparotomy 1 and 2 refer to the two laparotomies conducted on the *recipient* ewe.

For each transfer experiment, data on the ovum cleavage stage(s) recovered at laparotomy 1 and the interval between laparotomy 1 and 2, were used in conjunction with the results on cleavage rate *in vivo* (Table 10) to calculate the stage of cleavage ova would be expected to have attained in the recipient at the time of the transfer laparotomy, had they not been recovered at laparotomy 1 (Table 27). It was assumed that the recipient's genital tract would develop at the same rate as these ova *in vivo*. Therefore a comparison of the expected cleavage stages *in vivo* at laparotomy 2 with the actual ova cleavage stages transferred at this time, would indicate whether the ova were transferred into genital tracts which were at a relatively earlier or later stage of development.

Except for 4 ova in transfer number 7 and one ovum in transfer number 6, the ova were assumed to be transferred into more fully developed recipient genital tracts. Neither the transfer of ova into younger nor older recipient tracts was successful in this section.

3. Transfer of ova after 48 hours culture

The data presented in Table 26 show that the 6 transfer experiments were not successful. Unfortunately data were not available from the present experiment to allow prediction of expected cell stages greater than 8- to 12- cells *in vivo* at laparotomy 2. However, when the cleavage stages recovered at laparotomy 1 and the intervals between laparotomy 1 and 2 (recorded in the present experiment) were used in conjunction with the cleavage rate of ova to the 16- to -32 cell stage *in vivo* as reported by Clark (1934), the expected cell stages in the recipients at transfer laparotomy were 16- to 32- cells. Thus it was assumed that in all of the transfers, the transferred ova were less developed than the recipient genital tract (Table 27).

TRANSFERS IN THE 1966-67 LATE ANOESTROUS AND 1967 BREEDING SEASONS

In the last section, use of the same ewe as donor and recipient gave close synchronisation of stage of development of the transferred ova and the recipient genital tract in control transfers. However, because ova cultured *in vitro* usually developed less readily than would be expected if they had been left *in vivo* during the culture period (Table 19), use of the same ewe as donor and recipient usually resulted in cultured ova being transferred into more fully developed genital tracts. This could possibly explain the lack of success following the

transfer of cultured ova in the last section. To avoid this possibility it was decided that the donor and recipient would be different ewes in this section.

In control transfers, the donor and recipient ewes were required to have approximately synchronised onset of oestrus. In the transfer of cultured ova, the donor was required to exhibit oestrus a period approximately equal to the culture length prior to the exhibition of oestrus by the recipient. This latter technique was an attempt to ensure that the ova transferred after culture, were at least as well developed as the recipient tract.

Because the donors were required to exhibit oestrus approximately 24 or 48 hours before the recipients (in cultured ova transfers), the M.A.P. impregnated sponges were withdrawn from approximately 60% of the treated ewes one day before those from the remaining ewes (Chapter III). This caused onset of oestrus in the progestagen - treated ewes to be spread over two or more days, and the first ewes to exhibit oestrus could be mated to the entire ram (donor ewes) and the later ewes to the 'teaser' (recipient ewes).

1. Control transfer of ova

The one control transfer experiment conducted was successful (Table 28). In this transfer, onset of oestrus in the donor and recipient ewes was synchronised (Table 30) and from the calculated expected cell stages *in vivo* for the recipient at transfer (had its own ova been fertilised - Table 29), it was assumed that the transferred ova were slightly more advanced in development than the recipient tract. (It was again assumed that the expected ova cleavage stage *in vivo* at transfer laparotomy would indicate the stage of development of the recipient tract at this time.) In these

TABLE 28

SUCCESS OF OVA TRANSFER EXPERIMENTS CONDUCTED IN THE LATE ANOESTROUS AND BREEDING SEASONS

Type of Transfer	No. of Transfer Experiments	No. of Ewes Pregnant after Transfer	No. of Ova Transferred	No. of Viable Embryos at Slaughter
Control	1	1	2	1
After 24 hours culture	7	1	17	2
After 48 hours culture	1	0	3	0

TABLE 29

SYNCHRONISATION OF STAGE OF DEVELOPMENT OF TRANSFERRED OVA AND RECIPIENT GENITAL TRACTS

Type of Transfer	Transfer No.	Time to Transfer Lap. in Recipient (hours from onset of oestrus)	No. and Cell Stages of Ova Transferred at Lap.	Expected Cell Stages in Vivo at Transfer (No. of cells per ovum)	Result of Transfer Experiment
Control	1	56.50	1,2-cell; 1,4-cell	1	P.
After 24 hours culture	1	71.00	1,2-cell; 1,4-cell; 1,6-cell	6	N.P.
	2	54.25	2,2-cell; 2,8-cell	1	N.P.
	3	61.50	3,8-cell	4	P.
	4	45.25	1,2- to 3- cell	1	N.P.
	5	61.25	1,6- cell	4	N.P.
	6	53.25	1,4-cell; 1,4- to 6- cell	1	N.P.
	7	52.75	3,6-cell	1	N.P.
After 48 hours culture	1	63.75	2,4-cell; 1,8-cell	4 to 6	N.P.

N.P. = Recipient not pregnant P. = Recipient pregnant
Lap. = Laparotomy

TABLE 30

SYNCHRONISATION OF ONSET OF OESTRUS IN DONOR AND RECIPIENT EWES AND OVARIAN RESPONSE OF RECIPIENT EWES

Transfer Type	Transfer No.	Interval Between Onset of Oestrus in Donor and Recipient Ewes (hours)	Ovarian Response of Recipient: No. of Corpora Lutea	Ovarian Response of Recipient: No. of Corpora Lutea and Large Follicles	No. of Ova Transferred	Result of Transfer Experiment
Control	1	0	1	1	2	P.
After 24 hours culture	1	8	0	2	3	N.P.
	2	24	0	3	4	N.P.
	3	16	2	3	3	P.
	4	32	0	1	1	N.P.
	5	24	2	2	1	N.P.
	6	24	1	1	2	N.P.
	7	24	2	2	3	N.P.
After 48 hours culture	1	44	2	2	3	N.P.

N.P. = Recipient not pregnant

P. = Recipient pregnant

two seasons one laparotomy was conducted on each of the recipient ewes, this being for the transfer of ova. Because of the large variation in the interval from onset of oestrus to the recovery of each cleavage stage in vivo (Table 10), the estimated ova cleavage stages in these recipients at transfer laparotomy were not very accurate.

2. Transfer of ova after 24 hours culture

From Table 28 it can be seen that one of the 7 egg transplantation experiments conducted was successful. In this transfer (number 3 - Table 30) the onset of oestrus in the donor was 16 hours before that in the recipient. At laparotomy, the donor yielded 1, 2- cell and 2, 4- cell ova. These ova cleaved during the 24 hours of culture so that as shown in Table 29, 3, 8- cell ova were transferred into a recipient whose own ova would have been expected to be about the 4- cell stage had they been fertilised. Two of these transferred ova were present as viable embryos at slaughter and are illustrated in Figure 17.

From Table 29 it can be seen that except for transfer 1, all the transferred ova were estimated to be at a more advanced stage of development than the recipient genital tract.

Table 30 shows that 3 of the recipient ewes had not ovulated by the time of the transfer laparotomy. In these ewes the large follicles were ruptured in an attempt to induce luteinisation, but at slaughter no luteal tissue was observed in their ovaries.

3. Transfer of ova after 48 hours culture

Although the donor's onset of oestrus was 44 hours before that of the recipient, and the transferred ova were estimated to be at approximately the same stage of development as the recipient tract, the

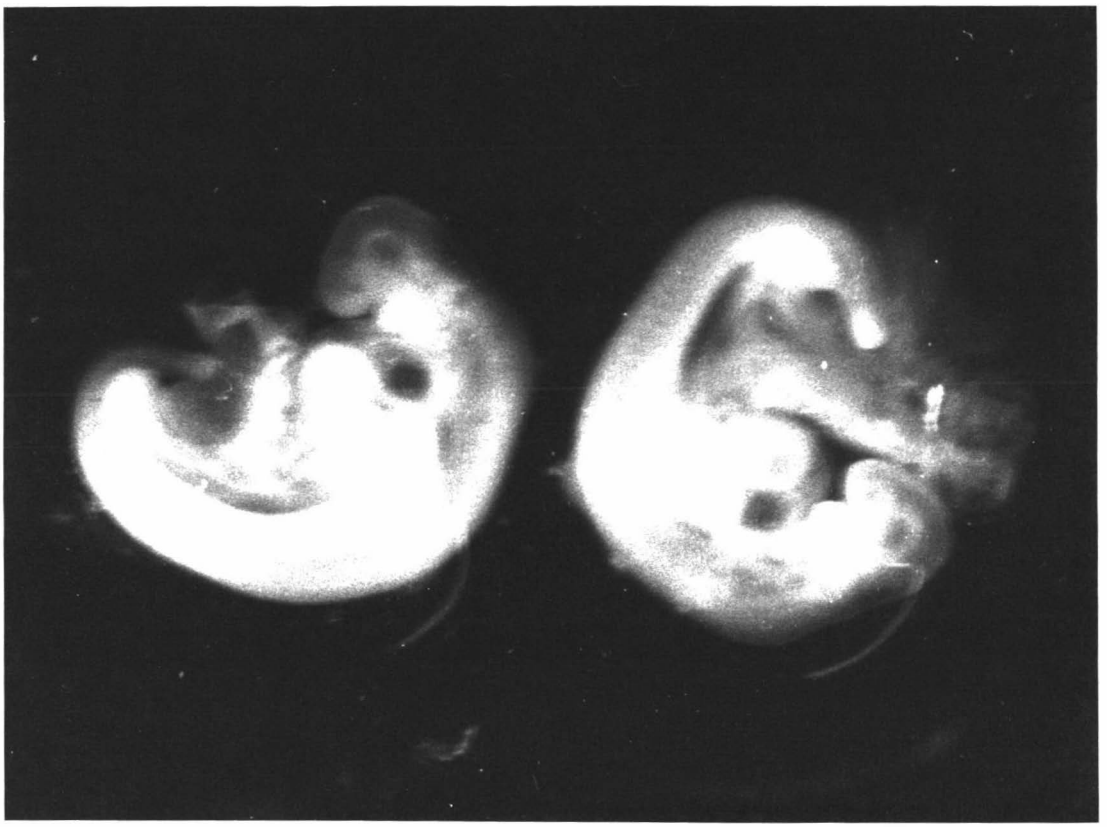


Figure 17 - Two 23 day viable embryos recovered from the recipient ewe pregnant after the transfer of cultured ova (Approx. x 12.5)

one transfer experiment conducted was not successful (Tables 29 and 30).

FACTORS AFFECTING SURVIVAL OF OVA IN CONTROL TRANSFERS

Of 7 control ova transfer experiments conducted in this study, 5 (71%) were successful. A total of 10 ova were transferred, 6 (60%) of these being present at slaughter as viable embryos.

Because only 7 control transfers were attempted, the results in this section were insufficient to analyse but are presented as they may indicate some trends.

1. Number of corpora lutea present in the ovaries of the recipient ewe

As shown in Table 31, the number of corpora lutea in the ovaries of the recipient ewes ranged from 1 to 3. An increase in the number of corpora lutea present appeared to result in a decrease in the chance of a recipient becoming pregnant after transfer, and a decrease in the percentage of transferred ova surviving to viable embryos at slaughter.

2. State of recipient genital tract at slaughter

This refers to whether ova were transferred into the flushed side of a recipient genital tract or not. Because the recipient ewes in the 1966 breeding and early anoestrous season transfers were mated to the entire ram, the genital tracts of these ewes had to be flushed before ova transfer. In 5 of these ewes, ova were then transferred into the flushed side of a genital tract. From Table 32 it can be seen that this practice appeared to decrease the chance of a pregnancy resulting, but had no marked effect on the survival of transferred ova to viable embryos at slaughter.

TABLE 31

EFFECT OF NUMBER OF RECIPIENT CORPORA LUTEA ON THE SUCCESS OF TRANSFER EXPERIMENTS

No. of Corpora Lutea	No. of Ewes	Pregnant Ewes after Transfer No.	(%)	No. of Ova Transferred	No. of Viable Embryos at Slaughter	% Transferred Ova Present as Viable Embryos at Slaughter
1	3	3	(100)	4	3	(75)
2	3	2	(67)	5	3	(60)
3	1	0	(0)	1	0	(0)

TABLE 32

EFFECT OF STATE OF RECIPIENT GENITAL TRACT ON THE SUCCESS OF TRANSFER EXPERIMENTS

State of Recipient Genital Tract at Transfer	No. of Ewes	Pregnant Ewes after Transfer No.	(%)	No. of Ova Transferred	No. of Viable Embryos at Slaughter	% Transferred Ova Present as Viable Embryos at Slaughter
Unflushed	2	2	(100)	3	2	(67)
Flushed	5	3	(60)	7	4	(57)

TABLE 33

EFFECT OF NUMBER OF OVA TRANSFERRED ON THE SUCCESS OF TRANSFER EXPERIMENTS

No. of Ova Transferred	No. of Ewes	Pregnant Ewes after Transfer No.	(%)	Total No. of Ova Transferred	No. of Viable Embryos at Slaughter	% Transferred Ova Present as Viable Embryos at Slaughter
1	4	3	(75)	4	3	(75)
2	3	2	(67)	6	3	(50)

TABLE 34

EFFECT OF CLEAVAGE STAGE OF THE TRANSFERRED OVA ON THE SUCCESS OF TRANSFER EXPERIMENTS

Cleavage Stage of Transferred Ova	No. of Ewes	Pregnant Ewes after Transfer No.	(%)	Site of Transfer	No. of Ova Transferred	No. of Viable Embryos at Slaughter	% Transferred Ova Present as Viable Embryos at Slaughter
2-cell	3	2	(67)	Tubal	3	2	(66.6)
4-cell	2	0	(0)	Tubal	2	0	(0)
6-to 8-cell	1	1	(100)	Uterine	2	2	(100)
8-cell	1	1	(100)	Uterine	1	1	(100)

3. Number of ova transferred

Either one or two ova were transferred into the recipient ewes. From Table 33 it can be seen that an increase in one ovum transferred did not appear to markedly affect the chance of a pregnancy resulting, but may have decreased the survival of transferred ova to slaughter.

4. Cleavage stage of transferred ova

In this study 2-, 4-, 6- to 8-, and 8- cell ova were transferred into the recipient genital tracts (Table 34). The age of ova transferred and the site of transfer were confounded, and it was found that uterine transfers were more successful than tubal transfers for both ewes becoming pregnant, and survival of transferred ova to viable embryos at slaughter. The results from one ewe were deleted from these data. In this ewe, 1, 2- cell and 1, 4- cell ovum were transferred into the same Fallopian tube, and although one of these ova were present as a viable embryo in the uterine horn at slaughter, it was not possible to ascertain from which transferred ovum it had developed.

5. Synchronisation of stage of development of transferred ova and recipient genital tract

In this experiment, the transfer of 4 ova into genital tracts assumed to be at an earlier stage of development resulted in the presence of 3 (75%) viable embryos at slaughter, while the transfer of 6 ova into genital tracts at a similar developmental stage resulted in the presence of 3 (50%) viable embryos at slaughter.

6. Period ova held in vitro before transfer

In 6 control transfers the ova were held *in vitro* in recovery dishes at room temperature for approximately 5 minutes before transfer.

In the seventh transfer (Control transfer 1 - Table 30), the ova were held in a dialysis chamber at 36°C for 40 minutes, before transfer. The percentage of the transferred ova surviving to viable embryos at slaughter after these two methods of storage was 62.5% and 50% respectively. This indicated that the storage of ova for 40 minutes in dialysis chambers did not markedly affect their chance of survival after transfer.

MEASUREMENT OF EMBRYOS

Twenty-one ewes became pregnant during these experiments. Six of these were pregnant as a result of successful ova transfer experiments and 15 were pregnant from poor recovery of fertilised ova at laparotomy 1 (4 ewes), or laparotomy 2 (11 ewes).

1. Measurement of 23-25 day old embryos

Six 23-25 day old embryos were recovered at the slaughter of 5 of the ewes pregnant from successful ova transfer experiments. The 'C' shape of the embryo as described by Green and Winters (1945) was found to be present in these embryos. All the embryos were judged to be viable at slaughter and since they were not sufficiently translucent to allow accurate measurement of the number of somites present, the only measurement taken was the crown-rump length. These are recorded in Table 35 and it can be seen that each of the twin embryos (from ewe number 4) was approximately half the length of the single embryos (recovered from ewes 1, 2 and 3).

2. Measurement of 44-101 day old foetuses

All the foetuses were classified as viable at slaughter and the crown-rump lengths are recorded in Table 35.

TABLE 35

AGE AND CROWN-RUMP LENGTH OF EACH EMBRYO

Embryo Age*	Ewe No.	Crown-Rump Length (cm.)	Mean Crown-Rump Length for each age (cm.)
23	1	1.10	
	2	1.10	
	3	1.00	
	4	0.45 0.50	0.83
25	5	1.20	1.20
44	6	5.70 5.70	5.70
45	7	6.00 5.40	5.70
46	8	6.30	6.30
47	9	6.60 6.70	
	10	6.20 6.20	6.43
50	11	7.60	7.60
51	12	8.30	8.30
54	13	9.60	9.60
56	14	10.30	
	15	9.70	
	16	9.80	9.93
57	17	9.90	9.90
79	18	21.50	
	19	19.30	20.40
95	20	27.10	27.10
101	21	25.50 27.20	26.35

* Embryo age measured in days from onset of oestrus to slaughter of pregnant ewes. Ewes 1,2,3,4,5 and 21 were pregnant from ova transfer experiments.

The growth in length of the embryos from 23 to 101 days of age is illustrated in Figure 18. From day 23 to approximately day 32 there was a period of relatively slow growth in length, but after this period there was a rapid and relatively uniform increase in embryo length.

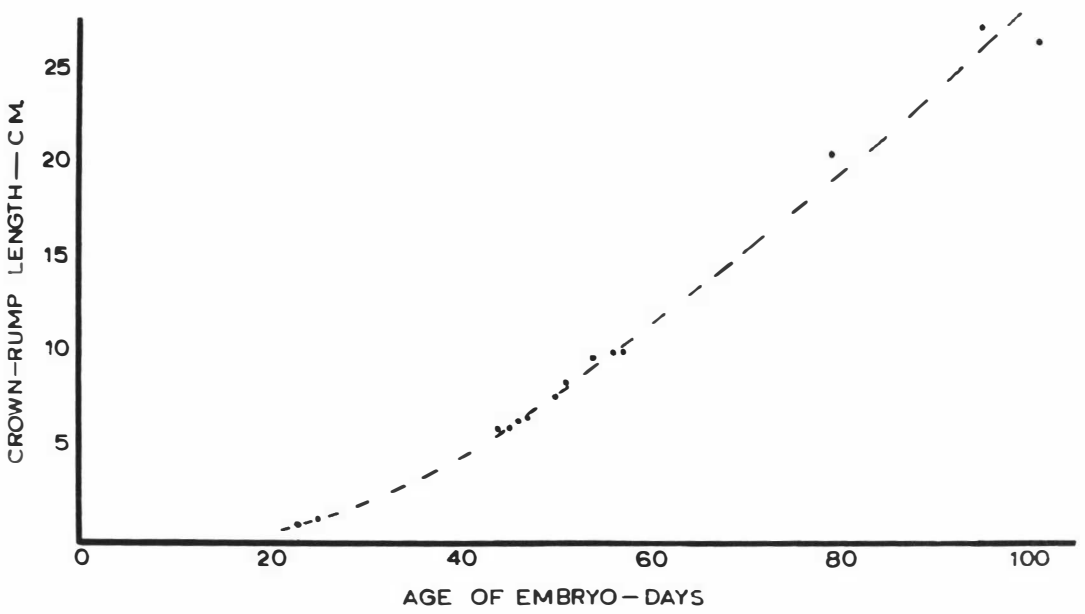


Figure 18 - Mean crown-rump lengths of embryos from 23 to 101 days of age.

CHAPTER VIII

DISCUSSION

Chapter VIII

D I S C U S S I O N

PRODUCTION OF OVA FOLLOWING HORMONAL STIMULATION OF DONOR EWES

Administration of gonadotrophin during the follicular phase of the oestrous cycle hastens the onset of the following oestrus (Warwick and Casida, 1943; Wallace, 1954). Cumming (1965) reported a significant decrease in oestrous cycle length when ewes were injected with gonadotrophin on day 12 rather than on day 13 of the cycle, but the results of Wallace (1954) and the present experiment have shown that oestrous cycle length was not significantly affected by the day of gonadotrophin injection. Wallace, Lambourne and Sinclair (1954) suggested a relationship between the level of P.M.S. and the extent of the shortening of the cycle, but the results of Robinson (1951) and the present experiment showed no such relationship.

A P.M.S. dose level-ovulation response relationship has been reported by Robinson (1951) and Wallace (1954). Both workers observed that the variation in ovarian response between ewes was enormous and that the response in each ewe was unpredictable.

During the breeding season in the present experiment there was a decrease in ovarian response (as indicated by number of corpora lutea and number of corpora lutea plus large follicles), with the increase in dose level from 1,000 i.u. to 1,200 i.u. P.M.S.

However when 1,500 i.u. P.M.S. was injected the ovarian response was greater than that recorded after 1,000 i.u. P.M.S. Cumming (1965) reported a similar result. The P.M.S. administered in the present experiment was assumed to be of correct potency and it was presumed that the methods used did not affect this potency, so that the low ovarian response to 1,200 i.u. P.M.S. was considered to be a true reflection of the variability in response to high levels of P.M.S.

The mean ovulation rate of Romney ewes not treated with gonadotrophin during the breeding season has been reported to be 1.17 ovulations per oestrus (Wallace, 1954). In the present experiment, the mean ovulation rates and ranges of ovulations for the 131 ewes injected with either 1,000, 1,200 or 1,500 i.u. P.M.S. were 3.08 (1-15), 2.48 (1-8), 4.51 (1-19) respectively. These results indicate the marked increase in multiple ovulations (above the level reported by Wallace for untreated ewes) and the individual variation to each dose level of P.M.S.

The incidence of oestrus and ovulation is very variable after the treatment of anoestrous ewes with progesterone and/or gonadotrophin (Raeside and Lamond, 1956; McDonald, 1961). Also the time of treatment within the anoestrous season is important (Grant, 1934; Gordon, 1958).

In the present experiment, 7 of 11 (64%) ewes treated with progestagen-impregnated sponges and 1,200 i.u. P.M.S. during the early anoestrous season (July - August), and 35 of 39 (90%) ewes treated with sponges and 1,000 i.u. P.M.S. during the late anoestrous season (January - February), exhibited oestrus after the hormonal treatment. In the former 7 ewes, the ovarian response after the hormonal treatment was not significantly different to that recorded

in the breeding season for 1,200 i.u. P.M.S. The 39 late anoestrous ewes were injected with either 1,000 i.u. or 1,500 i.u. P.M.S. on the real or estimated day 12 or 13 of the induced cycle. Thirty-one (79%) of these ewes exhibited oestrus after this second P.M.S. treatment and in the 29 ewes which gave information after laparotomy (one ewe was not laparotomised and one was discarded after laparotomy as her ovaries and uterus were infantile), the ovarian response was found to be significantly lower ($P < 0.01$) than that recorded in the breeding season for both these dose levels of P.M.S. These results, although only involving a limited number of sheep, indicate the variability of oestrus and ovulation response obtained with hormonal treatment of ewes during the anoestrous season.

Further analysis of these late anoestrous data revealed that the ewes laparotomised between 10th and 14th February (anoestrous 2- sponges in for 18 or 19 days) had significantly less corpora lutea ($P < 0.05$), but not a significantly different number of corpora lutea plus large follicles, than the ewes laparotomised between the 1st and 2nd February (anoestrous 1- sponges in for 8 or 9 days). To explain these differences it is suggested that some ovulatory factor may have been absent in the ewes laparotomised about the 12th February, and that this could account for the number of large unruptured follicles present in the ovaries of these ewes at this time. These ewes may have had a hormonal imbalance possibly because of photoperiodic stimulation to begin the breeding season, or perhaps because of the long period of progestagen treatment and this imbalance could result in the lack of an ovulatory factor.

Gonadotrophin is usually injected on day 12 or 13 of the oestrous cycle (i.e. approximately 3 or 4 days before onset of oestrus). In the present experiment, the early anoestrous ewes injected with P.M.S. on the day before progestagen-impregnated sponge withdrawal (injected an average of 3 days before onset of oestrus), gave a higher ovarian response than the ewes injected on the day of sponge withdrawal (injected an average of 2 days before onset of oestrus).

Of a total of 436 ova shed by ewes in the breeding season, 342 were recovered. This is a recovery rate of 78% which compares favourably with the 58% reported by Hunter et al. (1955) and the 61% reported by Hancock and Hovell (1961).

The recovery of ova was expressed on a per ewe basis in the present experiment. This was done to remove the bias which exists towards results from ewes in which higher numbers of ova were shed, when the recovery percentage (after a particular treatment) is calculated from the proportion of ova recovered of the total number of ova shed by all the ewes receiving that treatment. For example, although the recovery of ova per ewe was lowest in ewes receiving 1,000 i.u. P.M.S., the dose of P.M.S. had no significant effect on ova recovery per ewe. When the recovery of ova was calculated from the proportion of ova recovered of the total number of ova shed by all the ewes receiving each dose level of P.M.S., significantly less ova were recovered after 1,000 i.u. (63%) than after 1,200 i.u. P.M.S. (84%). It was thought that this low recovery after 1,000 i.u. P.M.S. was due to the fact that only 14 ewes contributed to this result and in one of these ewes, only 5 of the 15 ova shed were recovered.

Hancock and Hovell (1961) and Cumming (1965) reported no significant decrease in the percentage of ova recovered with an increase in the number of ova shed. In the present experiment it was found that the recovery of ova per ewe was not markedly affected by the number of ova shed by that ewe (at least up to 19 ova).

Laparotomies for recovery of ova from the donor ewes were conducted 36 - 101 hours after the onset of oestrus in the present experiment. Cumming (1965) reported no decrease in the recovery of ova when laparotomy was conducted up to 96 hours after the onset of oestrus, but in the present experiment it was found that there was a decrease in the proportion of ova recovered from a ewe, when that ewe was laparotomised 89 or more hours after the onset of oestrus. Sheep ova are reported to enter the uterus on the third to fourth day after the onset of oestrus (Assheton, 1898; Clark, 1934), and so it is likely that the lower ova recovery found after 88 hours from the onset of oestrus was due to ova having passed into the uterus where, because of the large area, the recovery of ova would be expected to be less satisfactory than from the oviduct. Also the ova may have passed into the portion of the uterine horn which was not flushed with the Fallopian tube at laparotomy.

In the present experiment, normal fertilised ova at all stages of cleavage were required for culture and possibly for transfer experiments. To avoid possible adverse effects on ova viability caused by prolonged examination and manipulation of ova after recovery, the ova were examined quickly at low magnification before transfer into culture chambers or recipient ewes. Because of this rapid examination and because the ova were required for culture and transfer, it was not possible to identify fertilised one - cell ova at recovery by the

presence of both polar bodies in the unfixed living ovum (Hancock, (1962) indicated that this method of identification was not reliable as identification of polar bodies was often not possible in fertilised ova) or by cytological criteria after fixing and staining the ova (Chang, 1952). However, rather than classifying one - cell ova as unfertilised solely on the basis that they were uncleaved at recovery, they were first examined at recovery for the presence of spermatozoa adhered to, or embedded in the zona pellucida and then cultured *in vivo* or *in vitro* for 24 hours. Any one - cell ova which developed normally over this culture period were considered to have been fertilised when first recovered.

This method of identifying fertilised one - cell ova was found to have two disadvantages. The first was that the recovery of ova at laparotomy 2 after culture *in vivo* was low (58%). Thus if all of the one - cell ova recovered from a ewe were transferred at laparotomy 1 and not recovered at laparotomy 2, the only information available on the fertilisation of these ova was that they were uncleaved and may or may not have had spermatozoa attached to the zona pellucida at recovery. The second disadvantage was that the ova cultured *in vitro* did not always cleave readily.

The first disadvantage could possibly be solved by ligating the oviduct of the ewe after transfer of the ova for culture *in vivo*. Experimentation with different culture media and techniques should enable sheep ova to be cultured more readily *in vitro* than recorded in the present experiment and so overcome the second disadvantage.

Hart (1956), Laffey and Hart (1959), and Robinson (1961) reported that the presence of numerous spermatozoa adhering to the surface, or embedded in the zona pellucida of one - cell ova was sufficient

evidence to classify these ova as fertilised. Hancock (1962) claimed that there was little direct evidence of the accuracy of this criterion and so in the present experiment, where 27 uncleaved ova cultured *in vivo* to test for fertilisation were not recovered after culture, they were classified as unfertilised regardless of whether spermatozoa were present in the zona pellucida or not at laparotomy 1. Only 6 (22%) of these ova were likely to have been fertilised as judged by the cleavage *in vivo* or *in vitro* of other uncleaved ova recovered from the same ewes.

In the present experiment, all of the 23 one - cell ova which cleaved normally during culture *in vivo* or *in vitro* for 24 hours (and therefore were classified as fertilised), only 5 of the 36 one - cell ova which did not cleave but which remained normal in appearance, and 4 of the 28 one - cell ova recovered abnormal after culture, had numerous spermatozoa attached to the zona pellucida. Using Hart's method of classification, 32 of these one - cell ova would have been classified as fertilised. Since some ova with spermatozoa attached to the zona pellucida may not be fertilised, the classifying of 23 of these 32 ova as fertilised in the present experiment, would tend to indicate that the method of classification of fertilised one - cell ova used in this present experiment was reasonably accurate. When the previously mentioned disadvantages are eliminated, the present method of classification of fertilised one - cell ova may prove to be very accurate.

Wintenberger *et al.* (1953) reported that unfertilised sheep ova were not activated when cultured at 38 - 39°C. There was no evidence from the present work to suggest that activation of one - cell ova had occurred.

If some one - cell ova had not been required for transfer experiments and others had not been required for *in vitro* culture for up to 72 hours (after which time many were abnormal), it would have been of great interest to have fixed and stained all of these ova after the first 24 hours culture *in vivo* or *in vitro*, and then to have examined them all for normal fertilisation and cleavage by cytological studies.

Calculation of the percentage of recovered ova fertilised either on a per ewe basis, or a proportion basis in the present experiment, showed that the lowest fertilisation rate was recorded in the ewes receiving 1,500 i.u. P.M.S. (i.e. in the ewes with the greatest ovarian stimulation). Hancock and Hovell (1961) reported no significant correlation between the numbers of ova shed and the proportion of recovered ova that were fertilised. However, the results of Averill (1958), Cumming (1965) and the present experiment indicate that the fertilisation rate declines at the higher ovulation rates, e.g. in the present experiment, the mean number of corpora lutea in sheep injected with 1,500 i.u. P.M.S. and from which were recovered only fertilised ova (32 ewes), only unfertilised ova (7 ewes), or both types of ova (14 ewes), were 4.5, 8.6 and 7.7 respectively.

Robinson (1951) reported in sheep injected with high doses of P.M.S. (1,000 - 2,000 i.u.), that a large number of ova were released, accelerated tubal transport occurred and the proportion of ova fertilised was reduced. The results of the present experiment gave no evidence of rapid tubal transport of ova, since the recovery rate of ova did not decline until the ova were expected to be in the uterus. Therefore another explanation had to be found for the reduced fertilisation rate of ova in ewes showing great ovarian stimulation (i.e. >12 corpora lutea and >12 corpora lutea plus large follicles).

Poor spermatozoa transport or survival has been implicated as a likely explanation, since only 48 of 105 (46%) ova recovered from ewes with greater than 12 corpora lutea had spermatozoa attached to the zona pellucida. Heap and Lamming (1962) have shown in the rat and rabbit that the ovarian hormones affect the composition of uterine washings, and have suggested that the composition of the uterine fluid may be under ovarian hormonal control according to the needs of spermatozoa metabolism, fertilisation, and embryonic development. Thus in sheep showing great ovarian stimulation, the ovarian hormones may affect the uterine and tubal fluids in such a way that these fluids are unsuitable for spermatozoa transport or survival; this explaining why a low proportion of the ova recovered from these ewes are fertilised.

Ovulation is assumed to occur near the end of oestrus in the ewe (24 - 36 hours after the onset - McKenzie and Terrill, 1937) and fertilisation to occur soon after ovulation (Chang and Rowson, 1965). Therefore in the present experiment, the low fertilisation rate of ova recovered from ewes laparotomised prior to 53 hours after the onset of oestrus would not seem normal, but can be explained. Only 8 ewes contributed ova which were considered in the calculation : of these, 2 were laparotomised 36 hours after the onset of oestrus and here only one of the 7 recovered ova were fertilised; 5 were laparotomised 40 to 52 hours after the onset of oestrus and here 75% of the recovered ova were fertilised; but the average fertilisation rate was greatly reduced by the remaining ewe (laparotomised 49 hours after the onset of oestrus) since none of the 13 ova recovered from this ewe were fertilised.

IN VIVO CLEAVAGE OF FERTILISED OVA

The time of ovulation in the ewe is variable and difficult to assess (Green and Winters, 1945; Chang and Rowson, 1965). The former workers considered that ovulation occurred approximately as the ewe was passing from oestrus (Shropshire sheep) and the latter workers that it occurred 30-40 hours after the onset of oestrus (Dorset Horn sheep). In the present experiment no data were available on the time of ovulation in the Romney ewe, but results for the shortest and longest interval from the onset of oestrus to the recovery of each ovum cleavage stage suggest that the time of ovulation and also fertilisation may be very variable in the Romney ewe.

In the present experiment, 23 fertilised but uncleaved ova were recovered at an average of 56 hours after the onset of oestrus. If it is assumed that ovulation occurs near the end of oestrus (24-36 hours after the onset - McKenzie and Terrill, 1937) and that first cleavage occurs 19-24 hours after ovulation (Dziuk, 1965), then the finding of fertilised but uncleaved ova at the above time would seem normal. However the finding of ova at this time does not indicate the time of fertilisation of ova in the Romney ewe.

The first 2- cell ova were recovered by Clark (1934) 39⅓ hours post coitum and by Green and Winters (1945) 39 hours post coitum. Both these workers allowed their ewes to be mated as close as possible to the end of oestrus and so if oestrus is assumed to be of approximately 24 hours duration, then these 2- cell ova were recovered about 63 hours after the onset of oestrus. In the present experiment the first 2- cell ova were recovered 40 hours after the onset of oestrus. The mean interval after the onset of oestrus to the recovery of 2- cell

ova was 58.3 hours, this being later than the 47.8 hours reported by Chang and Rowson (1965) and earlier than the 79 hours reported by Cumming (1965).

Clark (1934), Green and Winters (1945), Chang and Rowson (1965) and Cumming (1965) all reported rapid segmentation of sheep ova from the first to the third or fourth division. The results of the present experiment confirm this and also the observation of Cumming (1965) that each cleavage stage can be recovered over a wide time range.

In general the times to the recovery of the various cell stages in the Romney ewe are similar to those reported for other breeds (Clark, 1934; Green and Winters, 1945; Hancock and Hovell, 1961; Chang and Rowson, 1965).

The dose level of P.M.S. appeared to affect the interval from onset of oestrus to recovery of 2- to 6- cell ova in the present experiment. However it is thought that the variation in interval to recovery of these cleavage stages after different doses of P.M.S. was largely due to the small numbers of ewes contributing to each result (average was 6), and the large variation between individuals in the interval from onset of oestrus to the recovery of each cleavage stage. Further experimentation with control and P.M.S. treated ewes is needed to indicate whether P.M.S. treatment has any significant effect on the interval to recovery of ova at various cleavage stages.

When ova were recovered from 76 ewes with multiple ovulations, variations in the cleavage stages of the recovered fertilised ova were observed in 23 ewes. However in agreement with the observations of Averill (1958) that the cell stages of cleaving eggs in individual

ewes seldom exceeded one-and-a-half complete cleavages, it was found that only 2 of these 23 ewes yielded ova which varied by more than one-and-a-half cleavages.

It was found that at any given time after the onset of oestrus, both Fallopian tubes within ewes usually contained ova at comparable cleavage stages.

Averill (1958) reported no evidence to suggest that some eggs cleaved more rapidly than others within breeds. In the present experiment, the cleavage rate of ova in the Fallopian tube unflushed at laparotomy 1 but flushed at laparotomy 2 (experimental tube), and of ova transferred into a Fallopian tube at laparotomy 1, was in many cases slower than the control cleavage rate. The results were few, but indicated that either the cleavage rate of fertilised ova varied between ewes, or that both treatments may have in some way slowed the cleavage rate of ova. The experimental tube was not deliberately manipulated at laparotomy 1 and so it is difficult to envisage how the manipulation of the rest of the genital tract at this time may affect the cleavage rate of ova in this tube. Indeed the results from this Fallopian tube hint that the variation in ova cleavage rate between individual ewes may be greater than previous workers have suggested.

One possible explanation for a slowed cleavage rate of ova in the experimental tubes and in the Fallopian tubes after transfer, could be that the tubal secretion in these tubes is affected by manipulation and/or transfer in such a way that it does not favour ova cleavage. Perhaps the metabolic state of the tubal environment is altered, or perhaps the rate of tubal secretion is increased so that the ova are carried to a locality in the reproductive tract which is unsuitable for the cleavage stage of these ova. Some ova were transferred into a

Fallopian tube which had already been flushed at laparotomy 1. Since the Fallopian tube is manipulated with the fingers during flushing to remove the ova and recovery serum, the secretory cells may be damaged in these tubes and so the tubal environment may be adversely affected and not support ova cleavage at the normal rate.

CULTURE, ABNORMALITIES AND DIMENSIONS OF OVA

1. Culture of Ova

No fertilised one-cell sheep ova cultured *in vitro* (for 48 to 140 hours) by Wintenberger *et al.* (1953) cleaved to a stage greater than 9 blastomeres. Segmentation usually stopped at the 4- to 6- cell stage after which the ova began to degenerate. In the present experiment, 14 uncleaved but fertilised ova cleaved normally during a culture period of 24 hours and, in close agreement with the above workers, none had cleaved to a stage greater than 8 blastomeres, segmentation usually stopping at the 2- to 6- cell stages. Thus it appears that one- cell sheep ova will not develop readily when cultured *in vitro* in sheep serum.

Ova at the 2- cell to 6- to 8- cell stages were not cultured by Wintenberger *et al.* (1953). In the present experiment a high proportion of the ova cultured at these cell stages cleaved normally during culture for 24 hours, but few ova completed two divisions.

Wintenberger *et al.* (1953) reported very poor success with the culture of 8- to 12- cell ova. Their ova underwent only a small number of divisions and most degenerated quickly. In the present experiment it was found that the proportion of 8- cell and 8- to 12- cell ova which cleaved normally during culture was lower than that recorded

for ova cultured at most other cleavage stages, and that segmentation of these ova usually stopped at the 12- cell to 12- to 16- cell stages. The ova did not degenerate very rapidly over the first 24 hours of culture, but as the culture length increased the 8- to 12- cell ova degenerated rapidly. Wintenberger et al. (1953) suggested that since ova at the 8- to 12- cell stages in vivo would be expected to be changing from Fallopian tube to uterine media, this change could be critical to the ova and so ova cultured at these cell stages in vitro would not readily develop. However against this theory it should be mentioned that sheep ova cultured in vivo are not too critical about their development site up to the blastocyst stage (e.g. will develop readily in the ligated oviduct of the sheep and within the rabbit genital tract). Also in the early attempts to culture mouse ova (Hammond, 1949; Biggers and McLaren, 1958) it was found that these ova developed most readily in vitro when cultured at or beyond the 8- cell stage. However with the development of new culture media and techniques, mouse ova can now be successfully cultured from the 2- cell to the blastocyst stage in vitro (Brinster, 1963). Thus the author believes that the critical factors limiting the culture of one- cell to 8- to 12- cell sheep ova, at present, are the media and techniques used for culture rather than the cleavage stages of the cultured ova.

Wintenberger et al. (1953) observed that when 15- to 20- cell sheep ova were cultured, they would cleave readily and some would develop to the blastocyst stage. In the present experiment ova at these stages were not cultured. Therefore further studies will need to be undertaken to observe the development of sheep ova cultured

at more advanced stages of cleavage (i.e. > 8- to 12- cells) and also to accurately determine the cleavage rate of ova cultured *in vitro*.

Hancock and Hovell (1961) considered that the increased ova survival reported by Averill and Rowson (1958) after storage of ova in dialysis chambers, was due to the protection afforded against increasing osmotic concentration rather than to the removal by dialysis of harmful metabolic products. The former workers chose to store ova in conventional tubes from which the recovery of ova seemed less difficult, and their results suggested that storage of ova in dialysis chambers conferred no real advantage over their method at least for storage up to 5½ hours. In the present experiment the recovery of ova from the culture chambers was very high and it was felt that the use of dialysis chambers was justified because the ova were to be cultured for up to 72 hours.

The serum used for recovery, culture *in vitro*, and for transfer of ova in the present experiment was not heat treated before use. This was neglected because it has been suggested that heat treatment is not essential in seitz-filtered serum since the heat-labile factor in serum said to be injurious to heterologous ova is removed by passage through bacterial filters (Chang, 1949).

2. Abnormalities of ova

Hart (1956) attempted to define and classify normal and abnormal sheep ova. Using his system, a single cell ovum was deemed to be normal up to its present stage of development if it was fertilised, or able to be fertilised when recovered. Dividing ova were deemed to be normal at recovery unless obvious signs of abnormalities were

present. Hart identified fertilised ova by the numerous surplus spermatozoa found adhering to, or embedded in the zonae pellucidae of these eggs and also by certain changes taking place in the nucleus of fertilised one- cell ova.

In the present experiment both single cell and cleaved ova were classified as abnormal on the basis of the presence of obvious morphological deviations. These deviations have been described by Hart (1956), Laffey and Hart (1959), Braden (1964), and Allison (1967). Hart (1956) and Allison (1967) classified one- cell ova as involuted if the vitellus was greatly reduced in size and had crater indentations evident. In the present experiment, the one- cell ova classified as involuted had crater indentations in the vitellus but the vitellus was seldom as reduced in size as depicted by Hart and Allison.

Using this basis for classification of abnormal ova it was found that 11.5% of the ova recovered in the present experiment were abnormal and that 72.1% of these were involuted. Hart (1956) reported that 17.3% of the ova recovered from basically Corriedale type ewes were abnormal and that 63.2% of these ova were involuted.

Involution has been reported to only affect one- cell ova and to prevent these ova from being fertilised (Hart, 1956). However, evidence from the present experiment has made the author suspect that involution does not prevent fertilisation in the one- cell ovum. To support this argument it was found that 7 (23%) of the involuted ova recovered in the present experiment were fertilised. It is suggested that fertilisation may have been delayed in these ova so that they were involuted before or about the time of fertilisation. Six of these 7 fertilised involuted ova were cleaved : these consisted of

1,2- cell; 3,4- cell; 1,6- cell, and 1,8- to 12- cell ovum. In these ova the blastomeres were normal in appearance except at the involuted area where, because the vitellus was markedly indented, they assumed different shapes the most common of which was a kidney shape. The remaining fertilised involuted ovum was uncleaved at recovery but cleaved during culture in vitro so that a normal 4- cell ovum was recovered from the chambers. All (4) fertilised involuted ova which were cultured in vitro appeared to lose their involution as they continued to cleave.

Of the 24 unfertilised involuted ova recovered at laparotomy, 18 (75%) were recovered from one ewe. Because the fertilisation rate of ova has been found to decline at higher ovulation rates, it is considered that these 18 ova were likely to be unfertilised because of the high ovarian response of the donor and were likely to be involuted because they were unfertilised.

Thus the author's evidence suggests that involution is not an abnormality which will prevent an ovum from being fertilised and developing normally, and that it is a natural consequence of the lack of fertilisation of an ovum.

The incidence of ova with fragmented cytoplasm and ova with abnormally thick zonae pellucidae was low in the present experiment and all the ova exhibiting these abnormalities were uncleaved and unfertilised.

Braden (1964) reported no marked seasonal variation in the incidence of abnormal ova, but Laffey and Hart (1959) reported that the incidence of abnormal ova in the late breeding season was much higher than that reported by Hart (1956) for the main breeding season. In the present experiment it was found that the incidence of abnormal

ova decreased from the early to late breeding season. The greater incidence of abnormal ova in the early breeding season was mainly due to one ewe which contributed 18 abnormal ova (56% of all the abnormal ova recovered at this time). These 18 ova were involuted. If the involuted ova were deleted from both the early and late breeding seasons, the level of abnormalities in these two seasons was not marketly different (5.8% and 2.9% respectively). The high level of abnormal ova recovered in the late breeding season by Laffey and Hart (1959) could be partly due to the fact that their tupped ewes were only slaughtered twice weekly. This practice meant that the post-ovulation age of many unfertilised ova at recovery was sufficient for these ova to have undergone normal degenerative changes and thus be classified as abnormal. Also almost half of their abnormal ova had broken zonae. In the present experiment, only 2 ova were recovered with this abnormality and since these ova had been transferred into a Fallopian tube at laparotomy 1 and recovered with their zonae broken at laparotomy 2, it was considered that they may have been abnormal because of the recovery techniques used and so were deleted from the calculations. As recovery of ova from the Fallopian tube entails flushing with recovery media under pressure, it is possible that some zonae are broken in the process (Braden, 1964). However Laffey and Hart (1959) maintain that their egg handling techniques resulted in no observable damage to the ovum and so it is possible that the reason for their high level of ova with broken zonae was as explained by Braden (1964), i.e. that eggs may vary in their sensitivity to mechanical rupture and that this sensitivity may be increased under certain conditions such as increasing time after ovulation. This latter condition was acting in Laffey and Hart's (1959) experiment because of their killing procedure.

Braden (1964) reported that the incidence of morphologically abnormal unfertilised ova increased significantly with increasing interval from onset of oestrus up to 3-4 days. He pointed out that most of these abnormalities developed 1-3 days after ovulation and represented degenerative changes that might well be expected in ageing unfertilised ova. In the present experiment the incidence of abnormal unfertilised ova also increased with increasing intervals from onset of oestrus to recovery. However, although 29 of the 30 abnormal ova recovered 0-3 days after onset of oestrus had abnormalities which represented degenerative changes that might well be expected in ageing unfertilised ova (24 were involuted and 5 had fragmented cytoplasm - the remaining ovum had an abnormally thick zona pellucida), the 6 ova recovered 3-4 days after the onset of oestrus all had abnormally thick zonae pellucidae, an abnormality which did not represent normal degenerative changes expected at this time.

It was found that as the period of culture <u>in vitro</u> increased, there was a marked increase in the incidence of abnormal ova. The incidence of involuted ova decreased markedly over the first 24 hours of culture, but was opposed by a marked increase in the incidence of fragmented ova. After the first 24 hours of culture, the incidence of involuted ova slowly decreased while the incidence of fragmented ova slowly increased. Nine (90%) of the unfertilised involuted ova inserted into the chambers fragmented over the first 24 hours of culture and so it would appear that fragmentation may follow involution as a natural consequence in the degeneration of unfertilised ova. In addition, 8 normal uncleaved and 7 normal cleaved ova fragmented during the first 24 hours of culture. The incidence of vacuolated

ova increased as the culture period increased, but neither this abnormality nor the thick zona pellucida abnormality were very prevalent in the present experiment.

Some of the ova inserted into the culture chambers at most cleavage stages became abnormal during culture for up to 72 hours. Few ova at some cleavage stages were cultured and, except for the observation that within cleavage stages the incidence of abnormal ova usually increased with increasing culture length, no regular trends were observed. The high incidence of abnormal ova after the culture of one- cell ova can largely be explained on the basis of fertilisation : all of the 23 abnormal ova recovered after 24 hours culture, 15 of the 16 abnormal ova recovered after 48 hours culture, and 5 of the 7 abnormal ova recovered after 72 hours culture were unfertilised and therefore would be expected to undergo ageing and degenerative changes as the culture period lengthened. Some of the fertilised ova at most cleavage stages (especially the 6- to 8- cell and 8- to 12- cell stages) became abnormal during culture. Thus it appears that the culture medium and culture technique used in the present experiment did not readily support normal development of fertilised ova.

3. Dimensions of ova

The average diameter of the zonal cavity of sheep ova has been reported to be 0.147 mm. and the average zona pellucida thickness to be 0.014 mm. (Clark, 1934). These dimensions were found to be similar in the present experiment, the values obtained being 0.146 mm. and 0.0146 mm. respectively. The average total ovum diameter was found to be 0.175 mm. Seven ova were recovered with abnormally thick zonae pellucidae (average thickness 0.028 mm.) and although 6 of these

ova had much spermatozoa attached to the zona pellucida, all were classified as unfertilised since they did not cleave during culture in vitro. Presumably the spermatozoa were unable to penetrate the thick zona pellucida and so fertilisation did not occur.

Austin (1961) reported that the total mass of ova cytoplasm actually decreased during ovum cleavage and that with each successive cleavage, the size of the blastomeres was approximately halved. In agreement with these observations, it was found in the present experiment that the total volume of ova cytoplasm decreased from the 2- cell to the 16- cell stage and that the blastomere volume was approximately halved with each successive cleavage.

SURVIVAL OF TRANSFERRED OVA

Synchronisation of transferred ova and recipient genital tract stages of development is thought by many workers to be an important factor governing the success of egg transfer experiments in sheep (e.g. Hancock and Hovell, 1961). Most workers have based their synchronisation of transferred ova and recipient genital tract stages of development on the synchronisation of the onset of oestrus in the donor and recipient ewes. They have assumed that if the onset of oestrus is closely synchronised, then at transfer laparotomy, the stage of development of the transferred ova and recipient genital tract should also be quite closely synchronised. However, because of the marked between-ewe variation in the time of ovulation and fertilisation in relation to the onset of oestrus, it is unlikely that the transferred ova and recipient genital tract stages of development are closely synchronised even if the onset of oestrus in the donor and recipient ewes are exactly synchronised. This could explain why workers have

reported some unsuccessful transfer experiments when the onset of oestrus in the donor and recipient ewes was closely synchronised (Moore and Shelton, 1964; Shelton and Moore, 1966).

Successful egg transfer experiments have been reported where the onset of oestrus in the donor and recipient ewes were closely synchronised (± 12 hours - Shelton and Moore, 1966), and also where they were not closely synchronised (± 48 hours - Averill, 1956). It would be of great interest to know how closely synchronised the stage of development of the transferred ova and recipient genital tracts actually were in these successful transfers, i.e. whether the transfer of ova into older, younger, or closely synchronised tracts gave most success.

In the present experiment, data were available to calculate the stage of development the recipient's ova would have been expected to have attained *in vivo* at the time of the transfer laparotomy (had they not been recovered for culture at laparotomy 1 or had they been fertilised). These expected cell stages could be quite accurately determined in the breeding and early anoestrous seasons since data were available from each recipient on the cleavage stage of ova recovered at laparotomy 1, and on the interval between laparotomy 1 and laparotomy 2 (transfer laparotomy). These data were used in conjunction with data on the cleavage rate of ova *in vivo* to calculate the expected cell stages at laparotomy 2. It was assumed that the cleavage rate of ova did not vary between ewes, but as previously discussed, there was a suggestion that there may have been slight variation in cleavage rate between ewes. In the late anoestrous and 1967 breeding seasons, only one laparotomy was conducted on each recipient (for the transfer of ova) and so the expected cell stages were estimated from the interval from onset of oestrus to this

laparotomy. Because of the large variation in the interval from onset of oestrus to the recovery of each cleavage stage *in vivo*, the estimated cleavage stages at transfer laparotomy in these recipients may not have been very accurate. It was assumed that the recipient's genital tract would have developed at the same rate as these ova and so a comparison of the expected cleavage stages *in vivo* at transfer laparotomy with the actual cleavage stages transferred at this time, would indicate whether the ova were transferred into genital tracts which were at the same, or a relatively earlier, or later stage of development. In the control transfers (Table 27), the cleavage stages of ova in the recipients at transfer laparotomy were known because fertilised ova were recovered from these ewes immediately before ova were transferred into their exposed genital tracts.

Because the culture technique used may have affected ova viability and hence the chance of a successful ova transfer experiment, the results of the control transfers and transfers of cultured ova are discussed separately.

1. Control Transfers

It must be remembered that only 7 control transfer experiments were conducted and so the interpretation of these results may not be reliable.

The transfer of ova into less developed genital tracts was found to be more successful for both the proportion of ewes becoming pregnant and the survival of transferred ova to viable embryos at slaughter, than transfer of ova into tracts at a similar developmental stage. This may indicate that the examination of ova before transfer

retarded their development for a short period after transfer, so that the greatest transfer success was achieved with ova which were transferred into less developed tracts, since these ova may have recovered from any adverse effects by the time their stage of development was closely synchronised with the recipient genital tract.

Averill and Rowson (1958) found a consistent but non-significant increase in the ability of ova to develop in the presence of two or more corpora lutea as opposed to a single corpus luteum in the ovaries of the recipient ewes. Moore et al. (1960) and Cumming (1965) reported no relationship between the number of corpora lutea in recipient ewes and the survival of transferred ova, but in the present experiment, an increase in the number of corpora lutea present appeared to result in a decrease in both the chance of a recipient becoming pregnant and the survival of transferred ova.

The results of the present experiment confirmed the observations of Moore et al. (1960) and Cumming (1965) that an increase in the number of ova transferred did not markedly affect the proportion of ewes which became pregnant. It would seem that, as proposed by Moore et al. (1960), the success or failure of any transfer was more dependent on the inherent ability of the ewe to support a pregnancy than on the number of ova transferred. Their results led Moore et al. (1960) to suggest that a number of ewes at each oestrous period were suffering from some form of transient infertility which could have been of endocrine origin.

The observation of Moore et al. (1960) and Cumming (1965) that an increase in the number of ova transferred lowered the ovum survival rate was also confirmed in the present experiment. Brambell (1948) suggested that prenatal loss in sheep would be distributed at random between ewes with one, two or three ovulations. However the results

of Moore et al. (1960), Cumming (1965) and the present experiment showed that the proportion of ova suffering prenatal mortality increased as the number of ova transferred increased. Insufficient data were available from the present experiment to indicate the importance of the maternal, foetal and intermediate factors (as proposed by Brambell, 1948) in causing prenatal mortality.

In the present experiment, as in the work of Moore et al. (1960), Moore and Shelton (1962b) and Shelton and Moore (1966), the site of transfer and age of the transferred ova were confounded, most success being reported with uterine transfers. However Moore and Shelton (1964) did not confound these two factors and reported that tubal transfers were more successful than uterine transfers. Thus it is clear that both the age of ova and the site of transfer are important, and since workers (Averill and Rowson, 1958; Moore and Shelton, 1964) have shown that the survival of transferred ova increases significantly with increasing age, the supremacy of the uterine transfers in the present experiment (and in other experiments where the two factors were confounded), was probably due not to the site of transfer but to the fact that older ova were transferred into the uterus.

Alliston and Ulberg (1961) reported that 75% of their "within animal" transfers were successful in animals held at 70°F, but did not indicate whether the ova were transferred back into a flushed or unflushed side of the recipient genital tract. In the present experiment, the transfer of ova into a flushed side of a recipient genital tract appeared to decrease the chance of that recipient becoming pregnant after transfer. Although few results were obtained they suggest that the flushing of a side of a genital tract may affect the internal environment, or the transport of ova in that side, in such a way that none of the transferred ova are present as viable embryos at slaughter.

2. Transfer of cultured ova

Averill (1956) reported that 47% of sheep ova stored for 24 hours, 25% stored for 48 hours, and 22% stored for 72 hours at 5 - 8°C in serum, were viable as tested by ova transfer. Buttle and Hancock (1964) found that the storage of sheep ova for 24 or 48 hours at room temperature was not very successful and in the present experiment it was found that only 2 (6%) of 35 ova cultured for 24 hours, and none of 13 ova cultured for 48 hours at 35 - 36°C, were present as viable embryos at slaughter after ova transfer. Several factors were thought to contribute to the low transfer success in the present experiment and are discussed below.

(a) Synchronisation of transferred ova and recipient genital tract stages of development

In the transfer experiments conducted in the 1966 breeding and early anoestrous seasons, the ova were usually transferred into recipient genital tracts which were assumed to be at a more advanced stage of development. The donor and recipient of transferred ova were, where possible, the same ewe in these seasons and, since the ova cultured *in vitro* usually developed less readily than they would have *in vivo*, the ova transferred after culture for 24 hours were usually slightly less developed than the recipient tract while ova cultured for 48 hours were often much less developed than the recipient tract. The limited evidence available from the control transfers in the present experiment indicated that the transfer of ova into less developed tracts was more successful than the transfer of ova into tracts at a similar developmental stage. Hunter *et al.* (1955) and Hancock and Hovell (1961) suggested that the transfer of ova into less

developed tracts was generally more favourable to success than the transfer of ova into more fully developed tracts. Averill and Rowson (1958) suggested that the sheep ovum showed a considerable degree of tolerance for an unsynchronised uterus. They used the interval from onset of oestrus to transfer in the recipient to indicate the stage of development of the recipient tract at this time. Because of the between-ewe variation in the time of ovulation in relation to the onset of oestrus, this does not give an accurate indication of the stage of development of the recipient tract and therefore one does not know whether the ova were transferred into genital tracts at the same, or a relatively earlier or later stage of development in their experiment. Thus although the evidence is not as convincing as would be desired, it is suggested that, in the present experiment, the transfer of ova into more fully developed recipient tracts in 12 of the 14 cultured ova transfer experiments may have been an important factor contributing to the lack of transfer success in these two seasons.

Ova in 6 (75%) of the cultured ova transfer experiments conducted in the late anoestrous and 1967 breeding seasons were transferred into recipient genital tracts which were assumed to be at an earlier stage of development. One of these transfer experiments was successful. It was considered unlikely that the relationship between the transferred ova and recipient genital tract stages of development was an important factor contributing to the lack of success in the other 5 transfers. In the remaining 2 (25%) of the transfer experiments, the ova transferred were of varying cleavage stages and were transferred into tracts which were at a similar, or relatively earlier or later stage of development. These transfer experiments were unsuccessful and this lack of success may be partly due to the transfer of some ova into more

fully developed genital tracts.

(b) Ovarian response of recipient ewes

Three of the 8 recipient ewes in the late anoestrous and 1967 breeding season transfer experiments had not ovulated by the time of the transfer laparotomy. These ewes could not be discarded because of the shortage of suitable recipient ewes. Therefore the large follicles in the ovaries of these ewes were ruptured in an attempt to induce luteinisation, but examination of the ovaries at slaughter showed that this was unsuccessful. This lack of recent luteal tissue may have been an important factor contributing to the failure of the egg transfer experiments in these 3 recipients. All of the recipient ewes in the 1966 breeding and early anoestrous season transfer experiments had ovulated by the time of the transfer laparotomy.

(c) Transfer technique

The transfer of ova into the Fallopian tubes was considered to be the most difficult and hence the most inaccurate part of the transfer technique. The ova were displaced from the transfer pipette in a volume of fluid and it was noted that there was a tendency for this fluid to flow back towards the fimbria (especially as the pipette was withdrawn from the oviduct) and this may well have carried the 'transferred' ova with it. Limited evidence available from the control transfers indicates that the transfer of ova into a flushed side of a genital tract may decrease the chance of a recipient becoming pregnant after transfer. Ova were transferred into the flushed side of a tract in 11 (79%) of the recipient ewes in the breeding and early anoestrous season transfer experiments. This practice may have contributed to the lack of transfer experiment success in these two seasons.

(d) Culture technique

Because the factors already outlined may have affected the success of the transfer experiments and because there have been few investigations with which the results of the present experiment can be compared, it is difficult to ascertain how important the culture technique per se was in limiting the success of the transfer experiments. The fact that 2 of the ova which cleaved normally during culture for 24 hours were present as viable embryos at slaughter (after transfer), indicates that the culture technique used was not completely responsible for the poor success in experiments involving the transfer of cultured ova. In 6 of the unsuccessful experimental transfers of cultured ova (Transfers 6 and 7, after 24 hours culture - Table 27; Transfers 5, 6 and 7, after 24 hours culture and Transfer 1, after 48 hours culture - Table 29) the relationship between the transferred ova and recipient genital tract stages of development seemed optimal for a successful transfer (i.e. some or all of the ova were transferred into genital tracts which were assumed to be at an earlier stage of development), all the recipients had ovulated prior to the transfer laparotomy and in only 2 of the recipients were the ova transferred into a flushed side of the genital tract. This lack of transfer success when most of the known factors which may limit transfer success were thought to be minimised, suggests that other factors (e.g. poor viability of cultured ova) may have been acting. Coupled with the fact that the culture medium and technique used did not readily support normal development of fertilised ova (see discussion on abnormalities during culture), this suggests that the culture technique may have adversely affected the viability of the transferred ova and thus been an important factor contributing to the lack of success in these 6 transfer experiments. This possible effect of the culture technique on ova viability

may have been an important factor contributing to the lack of success in some of the other transfer experiments.

It is felt that more extensive studies on culture media and techniques, and on synchronisation of the stages of development of transferred ova and recipient genital tracts, should enable sheep ova to be cultured more readily *in vitro* than has been found in the present experiment and to be recovered as viable embryos after transfer into recipient ewes.

C H A P T E R I X

S U M M A R Y

Chapter IX

S U M M A R Y

Studies were made on the *in vivo* cleavage and the *in vitro* culture of Romney sheep ova during the 1966 breeding and 1966-67 anoestrous seasons.

Data on ovum cleavage rate *in vivo* were obtained from laparotomies conducted on 101 ewes during these two seasons. One-hundred and sixty-two ova were cultured for 24, 48 or 72 hours in dialysis chambers which contained sheep serum at 35 - 36°C. These ova were examined for normal cleavage, development of abnormalities, and dimensions during culture. To test ova viability after culture 48 cultured fertilised ova were transferred into recipient ewes, and in addition 10 fertilised ova were transferred in control transfers. These transfer experiments were conducted in the 1966 breeding, 1966-67 anoestrous and 1967 breeding seasons. Only 7 donors were available in the 1967 breeding season (and since these ewes were utilised after the results on cleavage rate and culture of ova had been analysed), results from these ewes were only included in the section on the transfer of ova.

The 60 ewes available in the 1966 breeding season were super-ovulated by administration of 1,000, 1,200 or 1,500 i.u. Pregnant Mares' Serum gonadotrophin (P.M.S.) on days 12 or 13 of the oestrous cycle; the 58 ewes available in the anoestrous season were treated with intra-vaginal sponges (each containing 60 mg of 6- methyl 17- acetoxyprogesterone (M.A.P.)) and injected subcutaneously with

P.M.S. at sponge withdrawal; and the 25 ewes available in the 1967 breeding season were all treated with M.A.P. impregnated sponges but only potential donors were treated with P.M.S. at sponge withdrawal. The donor ewes in the 1966 breeding and 1966-67 anoestrous seasons contributed data on the production of ova following hormonal stimulation of these ewes.

The results may be summarized as follows:

PRODUCTION OF OVA FOLLOWING HORMONAL STIMULATION OF DONOR EWES

1. Neither the dose of P.M.S. nor the day of P.M.S. injection had any significant effect on the length of the oestrous cycle.

2. The injection of 1,000, 1,200 or 1,500 i.u. P.M.S. on day 12 or 13 of the oestrous cycle regularly induced multiple ovulations at the ensuing oestrus.

3. There was no significant increase in ovarian response with increasing dose levels of P.M.S. administered. The mean ovulation rates and range of ovulations for ewes injected with either 1,000, 1,200 or 1,500 i.u. P.M.S. were 3.08 (1-15), 2.48 (1-8), 4.51 (1-19) respectively.

4. The incidence of oestrus and ovulation was very variable after the hormonal treatment of ewes during the anoestrous season.

5. The dose level of P.M.S. had no significant effect on the mean percentage of ova recovered per ewe.

6. The mean percentage of ova recovered per ewe was not markedly affected by the ovarian response of that ewe, but declined when laparotomy (for ova recovery) was conducted 89 or more hours after the onset of oestrus.

7. In ewes receiving 1000, 1,200 or 1,500 i.u. P.M.S. the mean percentage of recovered ova fertilised per ewe were 91%, 97% and 84% respectively.

8. The mean percentage of recovered ova fertilised per ewe declined in ewes with greatest ovarian stimulation. The failure of fertilisation in these ewes did not appear to be due to accelerated tubal transport of ova, but rather to abnormalities in spermatozoa transport or survival.

IN VIVO CLEAVAGE OF OVA

9. There was a wide time-range relative to the onset of oestrus during which ova at each cleavage stage could be recovered. These results suggested that the time of ovulation and fertilisation may be very variable in the Romney ewe.

10. Data on the mean intervals after the onset of oestrus to the recovery of each cleavage stage indicated that fertilised ova cleaved at a rapid rate to the 8- to 12-cell stage.

11. The dose level of P.M.S. appeared to affect the mean interval from onset of oestrus to recovery of 2- to 6- cell ova.

12. At any given time after the onset of oestrus, both Fallopian tubes within ewes usually contained ova at comparable cleavage stages.

13. The cell stages of cleaving eggs in individual ewes with multiple ovulations seldom exceeded one-and-a-half complete cleavages.

14. In 23 ewes subjected to two laparotomies, the ova in one Fallopian tube were not recovered until the second laparotomy (approximately 24 hours after the first laparotomy). The cleavage rate of these ova and also of ova transferred into the Fallopian tube(s) of 33 ewes at laparotomy 1 (recovered at laparotomy 2), was often slower than the cleavage rate of ova in the control ewes.

IN VITRO CULTURE OF OVA

15. Ova kept in dialysis chambers seldom cleaved after the first 24 hours of culture.

16. During the first 24 hours of culture, the proportion of uncleaved, 8- cell and 8- to 12- cell ova which cleaved normally was low. A high proportion of the 2- cell to 6- to 8- cell ova cleaved normally during culture but few ova completed two divisions.

17. Further studies will need to be undertaken to observe the development of sheep ova cultured at more advanced stages of cleavage (i.e. > 8- to 12- cells) and also to accurately determine the cleavage rate of ova cultured *in vitro*.

18. The recovery of ova per chamber after 24, 48 and 72 hours culture was 99%, 100% and 100% respectively.

ABNORMALITIES OF OVA

19. Of the ova recovered at laparotomy, 11.5% were abnormal. Of the abnormal ova, 72% were involuted (i.e. had crater indentations in the vitellus), 12% had fragmented cytoplasm and 16% had abnormally thick zonae pellucidae.

20. It is suggested that involution is not an abnormality which will prevent an ovum from being fertilised and developing normally and that it is a natural consequence of the lack of fertilisation of an ovum.

21. The incidence of abnormal ova decreased from the early to late breeding season.

22. The incidence of abnormal unfertilised ova increased with increasing intervals from onset of oestrus up to 3-4 days. Most of the abnormal ova recovered 0-3 days after the onset of oestrus had abnormalities which might be expected in ageing unfertilised ova, but the ova recovered 3-4 days after the onset of oestrus had abnormally thick zonae pellucidae, an abnormality which did not represent a degenerative change expected in unfertilised ova at this time.

23. There was a marked increase in the incidence of abnormal ova as the culture period lengthened. The incidence of involuted ova decreased markedly over the first 24 hours of culture, but was opposed by a marked increase in the incidence of fragmented ova.

24. Within cleavage stages of cultured ova there was usually an increase in the incidence of abnormal ova as the culture period lengthened.

DIMENSIONS OF OVA

25. For ova at all cleavage stages, the average total diameter, the average zonal cavity diameter and the average zona pellucida thickness were found to be 175, 146 and 14.6 microns respectively.

26. The total volume of ova cytoplasm decreased from the 2- cell to the 16- cell stage and the mean blastomere volume was approximately halved with each successive cleavage.

SURVIVAL OF TRANSFERRED OVA

27. In the 1966 breeding and 1966-67 early anoestrous seasons the donor and recipient of transferred ova were where possible the same ewe. All ewes were mated to the entire ram and the ova recovered at laparotomy were either transferred back into same ewe immediately after recovery (Control transfer), or after culture *in vitro*. In the 1966-67 late anoestrous and 1967 breeding seasons the donors and recipients were different ewes, the donors being mated to entire rams and the recipients to 'teasers'.

28. Of 7 control transfer experiments conducted, 71% were successful. A total of 10 ova were transferred, 60% of these being present as viable embryos at slaughter of the recipient ewes.

29. The results from these control transfers were presented to indicate : the effect of the number of recipient corpora lutea; the state of the recipient genital tract at transfer; the number and cleavage stages of the transferred ova; the stage of development of the transferred ova and recipient genital tracts at transfer; and the period the ova were held *in vitro* before transfer, on the success of the transfer experiments.

30. Of 22 transfer experiments involving the transfer of cultured ova, only one was successful. A total of 48 ova were transferred, 2 of these being present at slaughter as viable embryos.

31. Possible caused suggested for this low success following the transfer of cultured ova were : incorrect relationship between the stage of development of the transferred ova and recipient genital tract at transfer; failure of some recipients to ovulate prior to transfer; techniques used in the transfer and culture of ova.

32. Twenty-one ewes became pregnant during the experiment, 6 from successful egg transfer experiments and 15 from poor recovery of fertilised ova at laparotomy. The embryos from these ewes were measured for crown-rump length.

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A P P E N D I X 1 and 2

Appendix 1

BRANDS OF P.M.S. USED IN THE 1966-67 LATE ANOESTROUS SEASON

1. Serum Gonadotrophin B.P. (Organon Laboratories Ltd, London)
2. Serum Gonadotrophin B.P. ('Serogan' - The British Drug House Ltd, London)
3. Serum separated from whole blood collected from three, 2-3 month pregnant mares. The whole blood from these mares was allowed to clot, the serum was drawn off, centrifuged, bottled and then deep frozen.

On 10th January 1967 a bioassay was conducted on the combined serum from these 3 pregnant mares. The bioassay involved 35 immature female white mice (weight range 9 - 12 gms and of approximately 21 days of age). These mice were individually identified and then randomly placed in either of the following 5 treatments.

1. Control - distilled water
2. Standard 1 - 3 i.u. 'Serogan' serum gonadotrophin B.P. in distilled water.
3. Standard 2 - 9 i.u. 'Serogan' serum gonadotrophin B.P. in distilled water.
4. Unknown 1 - 1 volume P.M.S. diluted with 2 volumes distilled water.
5. Unknown 2 - undiluted P.M.S.

Each mouse in each treatment was given an intra-peritoneal injection which contained 0.2 ml. fluid. The mice were slaughtered 48 hours after injection and the ovaries and uterus were dissected

out and weighed. The data were analysed using the method outlined by Finney (1964). The uterine weights could not be used to estimate the P.M.S. potency because they were outside the range for useful bioassay, but by using ovarian weights the P.M.S. potency was found to be 39.8 i.u. per ml. .

Appendix 2

TABLE 1

BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE OF CORPORA LUTEA NUMBER

Dose P.M.S. (i.u.)		$\leq X^2$	n-1	V	(n-1) log V
1,000	(1)	220.86	13	16.99	15.99
	(2)	24.73	10	2.47	3.93
1,200	(1)	103.62	23	4.51	15.05
	(2)	4.00	6	0.67	-1.04
1,500	(1)	1124.25	56	20.08	72.95
	(2)	92.28	17	5.43	12.49

d.f. = 5 chi^2 = 41.47 **

The variance is not homogeneous.

Corresponding values for Bartlett's Test on no. corpora lutea and large follicles were

d.f. = 5 chi^2 = 35.06 **

The variance is not homogeneous.

(1) = Breeding season

(2) = Anoestrous season

** = $P < 0.01$

Appendix 2 cont'd

TABLE 2

BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE OF CORPORA LUTEA NUMBER AFTER SQUARE ROOT TRANSFORMATION

Dose P.M.S. (i.u.)		ΣX^2	n-1	V	(n-1) log V
1,000	(1)	9.12	13	0.70	-2.01
	(2)	6.73	10	0.67	-1.74
1,200	(1)	10.54	23	0.46	-7.76
	(2)	0.54	6	0.09	-6.27
1,500	(1)	54.28	56	0.97	-0.74
	(2)	15.46	17	0.91	-0.70

d.f. = 5 chi^2 = 1.45 (N.S.)

The variance is homogeneous.

Corresponding values for Bartlett's Test on no. corpora lutea and large follicles after square root transformation were

d.f. = 5 chi^2 = 1.35 (N.S.)

The variance is homogeneous.

(1) = Breeding season

(2) = Anoestrous season

N.S. = Not Significant

TABLE 3

DUNCAN'S MULTIPLE RANGE TEST ON SEASONS WITHIN DOSES OF P.M.S. FOR NUMBER OF CORPORA LUTEA

Mean Number of Corpora Lutea for Seasons within Doses of P.M.S.

	1,000 i.u. P.M.S.		1,200 i.u. P.M.S.		1,500 i.u. P.M.S.	
	(B)	(A)	(B)	(A)	(B)	(A)
	3.65	0.92	2.19	1.90	4.54	0.53
1% level	----------		————————		----------	
5% level	----------		————————		----------	

TABLE 4

DUNCAN'S MULTIPLE RANGE TEST ON SEASONS WITHIN DOSES OF P.M.S. FOR NUMBER OF CORPORA LUTEA AND LARGE FOLLICLES

Mean Number of Corpora Lutea and Large Follicles for Seasons within Doses of P.M.S.

	1,000 i.u. P.M.S.		1,200 i.u. P.M.S.		1,500 i.u. P.M.S.	
	(B)	(A)	(B)	(A)	(B)	(A)
	5.02	3.20	3.10	3.10	6.00	3.06
1% level	----------		————————		----------	
5% level	----------		————————		----------	

(A) = Mean ovarian response for anoestrous season

(B) = Mean ovarian response for breeding season

--------- = Significant

———————— = Not significant