

## The early pregnancy factor of sheep and cattle

C. D. Nancarrow, A. L. C. Wallace and A. S. Grewal

C.S.I.R.O., Division of Animal Production, P.O. Box 239, Blacktown, N.S.W., 2148, Australia

**Summary.** The appearance and production of an early pregnancy factor (EPF) has been studied in sheep and cattle. This factor can be detected in serum and tissues of pregnant animals by its synergistic action with antilymphocyte serum in reducing the number of rosettes formed in a rosette inhibition test. The range of the rosette inhibition titre for serum from non-pregnant animals was 4-10. Values higher than these were considered to indicate the presence of EPF. In one ewe EPF was detected 29.5 h after the onset of oestrus at which time a zygote was recovered. The transfer of sheep zygotes into unmated non-pregnant ewes, and the infusion of extracts of mouse zygotes into the oviducts ipsilateral to the side of ovulation in ewes caused an increase in the titre of serum from the recipient animals. Infusion of extracts of unfertilized mouse ova did not change the titre. Removal of zygotes or embryos resulted in a reduction in the titre within 48 h to non-pregnant values. Extracts of tissues analysed for the presence of EPF gave high titres when taken from early pregnant ewes whereas tissues from non-pregnant animals gave low values. Removal of the ovary containing the corpus luteum from sheep 4-5 days after mating and in which pregnancy was maintained by exogenous progesterone resulted in a decrease in titre during the following 2-4 days. Removal of the contralateral ovary or the induction of luteolysis with a prostaglandin F-2 $\alpha$  analogue did not result in a decrease.

In cattle, pregnancy diagnosis by the rosette inhibition test 4 days after an artificial breeding programme gave good agreement with conventional pregnancy testing methods, e.g. palpation *per rectum* and plasma progesterone assays. From these experiments we suggest that a substance 'zygotin' is released by the fertilized egg and stimulates the production of EPF, most probably from the ovary containing the corpus luteum. Zygotin may act directly on the ovary or via an intermediary substance released by the oviduct. The role of EPF is unknown. It does not appear to be necessary for the maintenance of pregnancy because ovariectomized sheep with no demonstrable EPF remain pregnant when given exogenous progesterone. Routine pregnancy testing in sheep and cattle is not practicable with the present rosette inhibition test.

### Introduction

Life begins at fertilization. Despite much being known of events which follow, little is understood about the intimate relationship between the mother and her developing offspring. At some stage the zygote or developing embryo must communicate with the maternal organism to establish a suitable nutritional, immunological and hormonal milieu. The first observed response of the ovum to penetration by the spermatozoon is the shedding of cortical granules, a response related to the prevention of polyspermy rather than a signal to the mother indicating pregnancy.

The signal that prevents the corpus luteum from regressing at the end of the 16–21-day oestrous cycle must be received by Day 12 (sheep) or Day 16 (cow) (Moor, 1968; Heap, Flint & Gadsby, 1979). It is probable that the humoral factor responsible for this signal is a protein of trophoblastic origin aptly named 'trophoblastin' (Martal, Lacroix, Loudes, Saunier & Wintenberger-Torrès, 1979; Martal, 1981). For some years this antiluteolytic factor was the earliest known message from the embryo to its mother. More recent evidence has shown that the zygote may signal its presence to the mother shortly after fertilization and before the need for an antiluteolysin (Morton, Nancarrow, Scaramuzzi, Evison & Clunie, 1979b). A substance termed 'early pregnancy factor' (EPF) is present in maternal blood and can be detected by a rosette inhibition test. When embryos are removed at 19–21 days of pregnancy the value for the rosette inhibition titre falls, within 48 h, into the range for non-pregnant sheep (Nancarrow, Evison, Scaramuzzi & Turnbull, 1979).

In this paper we will present further evidence of the appearance of EPF in maternal serum in response to a humoral signal from the zygote, discuss the interrelationships of these agents with the reproductive system of ruminants and suggest possible future applications of the work.

### Materials and General Methods

#### *Animals*

The experiments were carried out during the period of seasonal anoestrus for sheep. Merino ewes were kept indoors and induced to ovulate synchronously by a progesterone–PMSG regimen (Sil-estrus: Abbott Laboratories, Greece; Folligon: Intervet International, Holland). Rams, either vasectomized or entire and bearing raddle harnesses, were placed with small groups of ewes within 24 h of progestagen removal. Checks for oestrus were made three times daily. More frequent observations were sometimes carried out to determine the onset of oestrus more precisely. When fertilization was required, oestrous ewes were placed with entire rams until mating was observed. When possible pregnancy was confirmed by recovery of the zygote or embryo from the reproductive tract. In experiments in which the corpus luteum was removed by ovariectomy or luteolysis, pregnancies were maintained with Sil-estrus implants.

#### *Rosette inhibition test*

The collection and processing of blood samples and the preparation of assay materials have been described previously (Morton *et al.*, 1979b; Nancarrow *et al.*, 1979). In the present series of experiments two anti-ovine lymphocyte sera (Nos. 4037 and 4188) and an anti-bovine lymphocyte serum were used. To aid counting, the lymphocytes were stained by adding 50  $\mu$ l 0.04% acridine orange in 0.9% (w/v) saline to the tubes before resuspension of the formed rosettes. The mixture was subsequently examined by simultaneous fluorescence and light microscopy. Dilutions of antilymphocyte serum are expressed as  $\log_2$  (reciprocal dilution  $\times 10^{-3}$ ) (Morton *et al.*, 1979b). The mean ( $\pm$ s.e.m.) values of the rosette inhibition titres for sera taken from ewes before mating or experimentation, i.e. the non-pregnant range, were  $6.8 \pm 0.4$ ,  $n = 17$  (Antiserum 4037) and  $5.0 \pm 0.4$ ,  $n = 7$  (Antiserum 4188). The upper 95% confidence limits for the non-pregnant range of these antisera were 9.7 and 7.0, respectively.

### Role of the embryo in the production of early pregnancy factor in sheep

In the following experiments we have examined the role of the conceptus in the continued production of EPF by using embryo transfer techniques, by investigating the temporal relationship between fertilization and appearance of EPF and by infusing cell-free homogenates of ova into metoestrous ewes.

*Embryo transfer*

Blood samples were taken from synchronized recipient ewes on the day of embryo transfer (Day 3 of the cycle, 1 or 3 embryos), and 1 and 12 days thereafter. The ewes were slaughtered 9 weeks later, and the number of fetuses was recorded (Table 1). There were increased titres in sera from all ewes by 1 day after embryo transfer and also at 12 days, although a considerable decrease in the titre did occur for 1 ewe (Table 1). Only 2 of these 4 ewes were still pregnant at 9 weeks.

**Table 1.** Changes in the rosette inhibition titres for sera of recipient ewes after the synchronous transfer of embryos on Day 3 of the oestrous cycle

No. of embryos transferred	Fetuses present at 9 weeks	Rosette inhibition titre* after transfer		
		0 days	1 day	12 days
1	0	4	20	20
1	1	6	16	14
3	0	8	20	12
3	3	6	26	26

\* Antiserum 4037 was used.

*Response to fertilization*

The relationship between the appearance of EPF and the time of fertilization was examined in more detail by sampling ewes hourly after they had mated with fertile rams, beginning 25 h after the onset of oestrus. These ewes normally ovulate 27–33 h after the onset of oestrus. During this period each ewe was anaesthetized and laparotomized and the ova were flushed from the oviduct or recovered from the preovulatory follicle if ovulation had not occurred. From one such ewe a fertilized egg was recovered 29.5 h after the onset of oestrus. At 28 and 29 h this ewe was diagnosed pregnant, the rosette inhibition titre rising to 12 and 18, respectively. By 3.5 h after removal of the zygote, the titre decreased to 6.

*The signal from the zygote*

The preceding results show that the continued presence of the zygote or embryo is necessary for production and release of EPF. This experiment was therefore designed to test the hypothesis that a humoral factor released from the zygote provides the information for the maternal organism to produce and release EPF.

Four unmated ewes were anaesthetized and laparotomized on Day 1 of the oestrous cycle. The infusates consisted of the 39 000 g supernatants of frozen-thawed mouse eggs (fertilized or unfertilized) or the fertilization medium in which these ova had been incubated for 8 h (Hoppe & Pitts, 1973). Each ewe received a 1-ml infusion over 2.0–2.5 h via Silastic (Dow Corning) cannulae placed in the oviduct ipsilateral to the ovulation. In one preparation, ova had been fertilized *in vitro* by capacitated mouse spermatozoa. Jugular blood samples were taken at intervals over a 3-h period and the following day and rosette inhibition titres were estimated (Table 2). Neither of the two ewes given material prepared from the unfertilized ova gave titres outside the non-pregnant range. However, the infusate prepared from fertilized mouse ova increased the titre to the pregnant range at 1 h. The titre was further increased at 3 h but had returned to non-pregnant values at 22 h. The medium in which the ova were fertilized caused a small rise in the titre at 2.5 and 3 h. Titres for the infusates prepared from the fertilized or unfertilized ova were in the non-pregnant range (4–6).

**Table 2.** Appearance of early pregnancy factor in ovine maternal serum during and following infusion of aqueous extracts of unfertilized or fertilized mouse ova or of their respective media into the ipsilateral oviduct of four non-pregnant ewes

Sample (h after beginning of infusion)	Unfertilized		Fertilized	
	Ova	Medium	Ova	Medium
0	—	4	8	6
0.5	4	8	8	6
1.0	6	4	16	5
2.5	8	4	18	10
3.0	6	4	24	10
22.0	6	6	4	6

Values are rosette inhibition titres using Antiserum 4188.

### Source of the early pregnancy factor in sheep

#### Distribution

As it appeared unlikely that the zygote itself synthesized the EPF, we investigated possible maternal sites of production. Initially we attempted to locate the source of EPF by examining the immunosuppressive activity of extracts of tissues taken from early pregnant and non-pregnant sheep (Nancarrow & Wallace, 1980). Values for the rosette inhibition titres obtained for tissues investigated appear in Table 3. With only one exception, titres for tissues from pregnant ewes up to 8 days *post coitum* were in the range 12–22 while those for the wether and non-pregnant ewes remained in the range 5–8. While these data fail to show a specific source of EPF they do indicate that it is distributed throughout the animal.

#### Ovarian involvement

The role of the ovary in the production of EPF was examined because the rosette inhibition titre obtained for extracts of the ovary containing the corpus luteum was consistently higher than for extracts of the contralateral ovary (Table 3).

**Table 3.** Rosette inhibition titres for homogenates of tissues obtained from 2 non-pregnant and 5 pregnant ewes and one castrated male

Tissue	Non-pregnant ewe		Pregnant ewe				Castrated male
	1 day	6 days	2 days	3 days	4 days	8 days	
Endometrium							
Body	8	—	18	20	20	—	—
Horns	6	—	16	18	18	—	—
Oviduct							
Ipsilateral to CL	—	—	20	20	8	—	—
Contralateral to CL	—	—	—	18	12	—	—
Ovary							
With CL	6	—	22	20	22	—	—
Without CL	—	—	20	18	18	—	—
Hypothalamus	—	6	—	18	—	18	6
Pituitary	—	8	—	22	—	—	8
Brain cortex	—	5	—	—	—	20	—
Kidney	—	6	—	—	—	18	8
Gracilis muscle	—	6	—	—	—	18	8

Antiserum 4037 was used. Tissues were homogenized in 3 volumes of Hanks basic salt solution and centrifuged at 39 000 g for 30 min. The supernatants were assayed as for serum.

Ewes at 4–5 days *post coitum* were laparotomized and the numbers and sites of ovulations were recorded. At this time, unilateral ovariectomy was carried out on a total of 11 ewes, 5 contralateral and 6 ipsilateral to the site of ovulation, while 5 other ewes received two doses of 100 µg cloprostenol (I.C.I., Aust. Ltd) 24 h apart to induce luteolysis. The ewes were bled at regular intervals before slaughter at 4 or 16 days after surgery (Table 4). Examination of the reproductive tracts confirmed that the prostaglandin treatment had induced luteolysis. Morulae or blastocysts were not obtained from 2 ewes but the titres indicated that they had been pregnant at the time of blood sampling. There were no discernible decreases in the titres during the first 2–4 days after contralateral ovariectomy (2 ewes) or cloprostenol-induced luteolysis (2 ewes). However, when the ovary containing the corpus luteum was removed, there was a fall in titre from that at surgery,  $20.5 \pm 1.0$  (s.e.m.), to  $14.5 \pm 2.8$  ( $P < 0.1$ , paired t-test) at 24 h and to  $12.0 \pm 2.4$  ( $P < 0.01$ ) at 48 h.

**Table 4.** Rosette inhibition titres obtained from sera of pregnant sheep after following unilateral ovariectomy or cloprostenol-induced luteolysis

Sample time	Ipsilateral ovariectomy				Contralateral ovariectomy		Cloprostenol luteolysis	
Before mating	6	4	5	10*	4	NA	6	NA
At surgery (4–5 days <i>post coitum</i> )	18	18	20	26	28	12	16	18
Days after surgery								
1	20	12	8	18	20	16	18	20
2	12	6	12	18	30	14	NA	16
3	4	8	10	12	NA	12	14	10
4	6	10	10	10	NA	14	20	16
16	6	8	NA	NA	NA	12	14	NA

\* Antiserum 4037; other titres were obtained with Antiserum 4188. NA = not available.

### Pregnancy testing in cattle

A group of 10 cows was randomly selected from a herd of Hereford cattle and used to compare various methods of pregnancy diagnosis and the detection of fertilization by the rosette inhibition test. The cows were given two injections of 500 µg cloprostenol 12 days apart then inseminated twice with frozen semen, at 72 and 96 h after the second injection of cloprostenol. Pregnancies were identified at 10 weeks by palpation *per rectum* and the calving performance recorded. Blood

**Table 5.** Comparison of the rosette inhibition test for the early detection of pregnancy in cattle with other established methods of pregnancy testing

Rosette inhibition titre at Day 4	Progesterone (log ng/ml)*			Pregnant by rectal palpation	Calf born
	Day 20	Day 22	Day 25		
16	>1	>1	>1	+	+
18	>1	>1	>1	+	+
16	>1	>1	>1	+	+
16	>1	>1	0.95	+	–
16	0.99	>1	>1	+	+
18	0.72	1.0	>1	+	+
12	0.92	0.83	0.91	+	+
6	0.15	0.04	0.83	–	–
6	0.97	0.15	–0.10	–	–
10	0.26	–0.40	–0.22	–	–

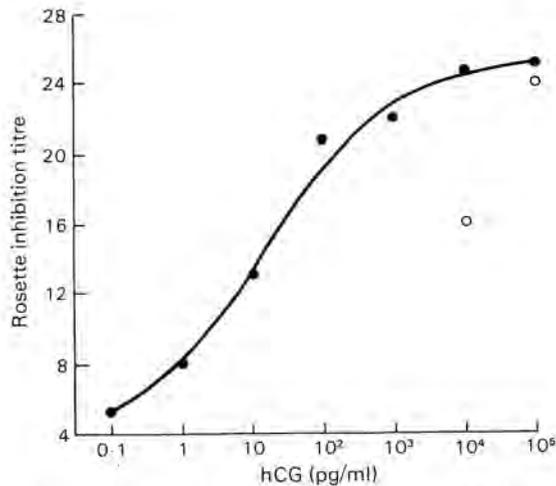
\* Discriminating value 0.30.

samples were taken at 4 days after the first insemination for estimation of the rosette inhibition titres and at 20, 22 and 25 days for measurement of progesterone values by radioimmunoassay. When the log values of the lowest progesterone concentration obtained for each animal were plotted they formed two distinct groups. Cows were judged to be pregnant if this value was in the higher group, i.e.  $>0.30$  (2 ng/ml). The results for the four methods are given in Table 5.

The non-pregnant range for the rosette inhibition titres was 6–10 and the pregnant range was 12–18. The diagnosis of pregnancy, or fertilization, at Day 4 agrees completely with the other assessments of pregnancy. Only one 'pregnant' animal did not calve, possibly because of a mid-term abortion.

#### Evidence of immunoregulatory activity of fetus-specific proteins

Human chorionic gonadotrophin (hCG) preparations have been shown to augment the rosette-inhibiting properties of species-specific antilymphocyte serum when used with mouse lymphocytes (Morton, Hegh & Clunie, 1976) or with human lymphocytes (Morton, Rolfe, Clunie, Anderson & Morrison, 1977). We have tested both hCG (Sigma Lot 88C-0254, 2750 i.u./mg) and ovine alpha-fetoprotein preparations for their immunoregulatory activity in the sheep pregnancy test using Antiserum 4037. A titre curve was obtained for hCG diluted in Hanks basic salt solution tested over the range 0.1  $\mu\text{g/ml}$  to 100 ng/ml and for two concentrations (10 and 100 ng/ml) of alpha-fetoprotein (Text-fig. 1). Both preparations enhanced the action of antilymphocyte serum.



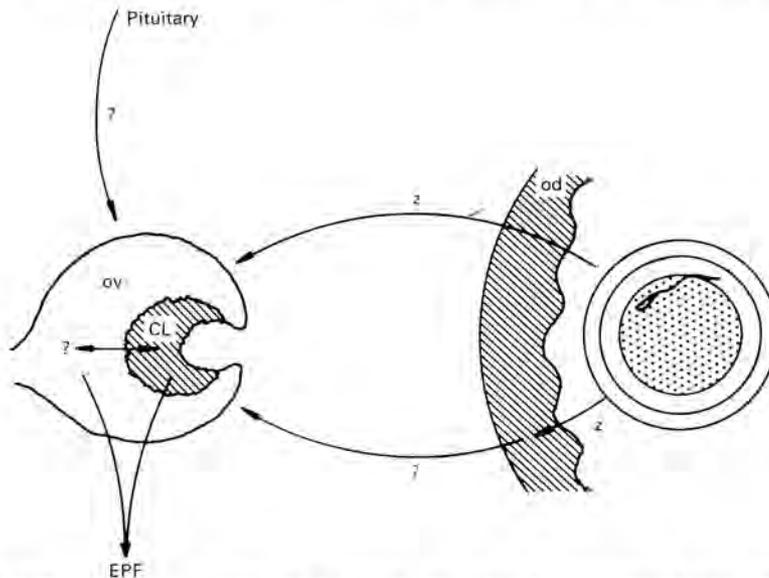
**Text-fig. 1.** Titre curve for hCG (●) when assayed in the rosette inhibition test and comparison with 2 concentrations of ovine alpha-fetoprotein (○). Number of replicates for each point = 2–4.

#### Discussion

It has been shown that almost from the time of fertilization there appears in the maternal blood a substance (EPF) that is detectable by its synergism with the action of antilymphocyte serum in inhibiting the formation of rosettes *in vitro*. The interval between fertilization in the oviduct and the appearance of EPF is no greater than 1 h. Even when embryos were transferred to synchronized recipient sheep on Day 3 of the cycle the rosette inhibition titre was elevated the following day. Likewise, when the conceptus was removed from the oviduct or the uterus (Nancarrow *et al.*, 1979) a rapid decline in the titre followed.

The sudden appearance of EPF in maternal serum suggests that either the egg releases EPF when fertilization occurs or stimulates release from a maternal organ. A rapid release of EPF from the zygote could be explained by exocytosis of cortical granules as occurs from the fertilized eggs of sea urchins and mice. We have observed cortical granules in unfertilized sheep eggs. If this theory is correct, then appropriate homogenates of unfertilized and fertilized eggs should show immunosuppressive activity in the rosette test. Our results do not support this view. The second hypothesis, that the zygote signals the mother to produce EPF, was tested with extracts of fertilized and unfertilized mouse eggs. A response equivalent to that found in early pregnant sheep was obtained only with the zygote extracts. It therefore appears that there is a humoral signal from the zygote to the mother which is translated into production of EPF. This putative substance which we will term 'zygotin' may represent the first signal of pregnancy.

Our results show that EPF pervades the whole body. When the tissues in close proximity to the zygote were assessed, the highest values were obtained for the ovary containing the corpus luteum. Removal of the contralateral ovary does not affect the production of EPF, but when the ipsilateral ovary was removed, the inhibition titres declined within 4 days. However, chemically induced luteolysis failed to lower the rosette inhibition titre, suggesting that it is not the corpus luteum *per se* but the rest of the ovary on the side of ovulation which is responsible for EPF production (Text-fig. 2); the source may be the preovulatory follicle, perhaps supported by some component of the corpus luteum that is not affected by luteolysis. Zygotin may have a direct local effect with a mechanism of transport similar to that proposed for prostaglandin F-2 $\alpha$ . If so, it is likely that zygotin is a small molecular weight molecule, perhaps similar to the prostaglandins or to the tetrapeptide isolated from hamster embryos by Kent (1975).



**Text-fig. 2.** Schematic representation of the production of early pregnancy factor. CL = corpus luteum; od = oviduct; ov = ovary; z = zygotin.

A physiological mechanism involving zygotin and EPF could be basic to most mammalian reproduction systems, with these compounds possessing a similar structure in all species. Perhaps an analogy with the hypothalamic releasing hormones is reasonable. However, it is possible that zygotin is a component of EPF and acts as a prosthetic group to a molecule produced in the ovary. Conversely, EPF may be small but associated with larger molecular weight proteins (Clarke, Morton & Clunie, 1978; Noonan, Halliday, Morton & Clunie, 1979).

such as hCG, alpha-fetoprotein or other membrane-associated proteins. However, because EPF is present very early during pregnancy then a role in fetal immunological development as suggested by Noonan *et al.* (1979) is difficult to envisage.

We have shown that when the ipsilateral ovary is removed the rosette inhibition titre decreases rapidly and this is interpreted as removal of EPF from the serum. Whether there is a prolonged action of EPF adsorbed onto the lymphocytes is unknown. However, pregnancy in sheep was maintained with progesterone replacement therapy so that if EPF is secreted solely by the ovary, then it is not fundamental to the maintenance of pregnancy unless it functions as a luteotrophin.

An investigation of the action of EPF on lymphocytes *in vitro* may unravel the mysteries of its function. We have observed that the rosettes in this particular test do not form in the absence of complement and that the numbers formed decrease as the complement preparation ages or as the complement-incubated lymphocytes stand. Human red blood cells and sheep lymphocytes have complement receptors, and we suggest that the synergistic action of EPF and antilymphocyte serum is mediated through either a reduction of the number of complement binding sites on the lymphocytes or an inhibition of the complement pathway to the C<sub>3</sub> configuration. We found that all lymphocytes forming rosettes have complement bound to them as demonstrated by the sandwich technique using rabbit anti-guinea-pig serum and FITC-labelled anti-rabbit- $\gamma$ -globulin.

Other fetal proteins appear to be immunoregulatory in the rosette inhibition test; hCG preparations are active in the mouse, human and sheep tests (Clarke *et al.*, 1978; Morton *et al.*, 1976, 1977; Text-fig. 1) while an ovine alpha-fetoprotein preparation behaved similarly. Whether the other proteins associated with pregnancy in ruminants, trophoblastin (Martal *et al.*, 1979), chorionic somatomammotrophin (Martal & Djiane, 1977) and those proteins obtained from bovine uterine flushings and endometrium (Roberts, 1977; Laster, 1977) or the embryo (Findlay, Cerini, Sheers, Staples & Cumming, 1979), have immunoregulatory potential still has to be determined. If so, it would be interesting to speculate that some common substance may be present in preparations of all these proteins which confers upon them this immunological character.

In practical terms the assay for EPF is a most powerful tool for detecting fertilization and pregnancy up to implantation in sheep, and for at least the first 4 weeks of pregnancy in cattle. Because zygotin is necessary for the continuation of EPF production, the occurrence of embryo mortality can be easily detected within 48 h (Nancarrow *et al.*, 1979). Suspected embryo mortality has been reported (Morton, Clunie & Shaw, 1979a; Morton *et al.*, 1979b): in both cases the increased rosette inhibition titre indicated that fertilization had taken place, but the embryo was not maintained past Day 12 because these ewes returned to service at normal time. Morton *et al.* (1979a) have followed the progress of ovine pregnancy through to term and have shown that false negative results may be obtained from about 2 months *post coitum*. The titres for sera taken from this stage of pregnancy do not reflect the increasing concentrations of alpha-fetoprotein which occur in the fetal circulation.

Although the rosette inhibition test could be of use in pregnancy diagnosis in the first month after mating, the test is currently too time consuming and variable to permit its practical use at present. However, the development of better tests based on radioimmunoassay or radioreceptor assay of EPF and zygotin, and of alternative immunological tests based on the mode of action of antilymphocyte serum and complement, could make this a practicable approach to pregnancy diagnosis.

The authors are most grateful for the assistance given by R. Newman, N. Rigby and P. Connell in carrying out the rosette inhibition tests and progesterone assays. We are indebted to Dr B. M. Bindon, C.S.I.R.O., Armidale, who kindly carried out the embryo transfer studies, and Dr P. J. Quinn, University of Newcastle, who supplied the mouse ova.

## References

- Clarke, F.M., Morton, H. & Clunie, G.J.A. (1978) Detection and separation of two serum factors responsible for depression of lymphocyte activity in pregnancy. *Clin. exp. Immunol.* **32**, 318–323.
- Findlay, J.K., Cerini, M., Sheers, M., Staples, L.D. & Cumming, I.A. (1979) The nature and role of pregnancy-associated antigens and the endocrinology of early pregnancy in the ewe. In *Maternal Recognition of Pregnancy*. (Ciba Fdn Symp. No. 64, N.S.), pp. 239–255. Excerpta Medica, Amsterdam.
- Heap, R.B., Flint, A.P.F. & Gadsby, J.E. (1979) Role of embryonic signals in the establishment of pregnancy. *Br. med. Bull.* **35**, 129–135.
- Hoppe, P.C. & Pitts, S. (1973) Fertilization *in vitro* and development of mouse ova. *Biol. Reprod.* **8**, 420–426.
- Kent, H.A., Jr (1975) The two to four-cell embryos as source tissues of the tetrapeptide preventing ovulations in the hamster. *Am. J. Anat.* **144**, 509–512.
- Laster, D.B. (1977) A pregnancy-specific protein in the bovine uterus. *Biol. Reprod.* **16**, 682–690.
- Martal, J. (1981) Trophoblastin and the control of luteal function during early pregnancy in sheep. *J. Reprod. Fert., Suppl.* **30**, 201–210.
- Martal, J. & Djiane, J. (1977) The production of chorionic somatomammotrophin in sheep. *J. Reprod. Fert.* **49**, 280–289.
- Martal, J., Lacroix, M.-C., Loudes, C., Saunier, M. & Wintenberger-Torrès, S. (1979) Trophoblastin, an antiluteolytic protein present in early pregnancy in sheep. *J. Reprod. Fert.* **56**, 63–73.
- Moor, R.M. (1968) Effect of embryo on corpus luteum function. *J. Anim. Sci.* **27**, Suppl. 1, 97–118.
- Morton, H., Hegh, V. & Clunie, G.J.A. (1976) Studies of the rosette inhibition test in pregnant mice: evidence of immunosuppression? *Proc. R. Soc. B* **193**, 413–419.
- Morton, H., Rolfe, B., Clunie, G.J.A., Anderson, M.J. & Morrison, J. (1977) An early pregnancy factor detected in human serum by the rosette inhibition test. *Lancet* **i**, 394–397.
- Morton, H., Clunie, G.J.A. & Shaw, F.D. (1979a) A test for early pregnancy in sheep. *Res. vet. Sci.* **26**, 261–262.
- Morton, H., Nancarrow, C.D., Scaramuzzi, R.J., Evison, B.M. & Clunie, G.J.A. (1979b) Detection of early pregnancy in sheep by the rosette inhibition test. *J. Reprod. Fert.* **56**, 75–80.
- Nancarrow, C.D. & Wallace, A.L.C. (1980) Detection of fertilization in sheep and cattle: serological estimation and description of properties of an early pregnancy factor. *Proc. 9th. Int. Congr. Anim. Reprod. & A.I. Madrid* **3**, 85. Abstr. 2–23.
- Nancarrow, C.D., Evison, B.M., Scaramuzzi, R.J. & Turnbull, K.E. (1979) Detection of induced death of embryos in sheep by the rosette inhibition test. *J. Reprod. Fert.* **57**, 385–389.
- Noonan, F.P., Halliday, W.J., Morton, H. & Clunie, G.J.A. (1979) Early pregnancy factor is immunosuppressive. *Nature, Lond.* **279**, 649–651.
- Roberts, G.P. (1977) Inhibition of lymphocyte stimulation by bovine uterine proteins. *J. Reprod. Fert.* **50**, 337–339.