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Changes in composition of the porcine zona pellucida during development of the oocyte to the 2- to 4-cell embryo

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SUMMARY

Our objective was to identify any changes that occur in the composition of the porcine zona pellucida during development of the 2- to 4-cell embryo from the oocyte. Oocytes, unfertilized eggs and single and 2- to 4-cell embryos have been recovered surgically and their zonae pellucidae ^{125}I-labelled and analysed individually by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The zonae from ovulated eggs possessed two major glycoproteins M_r 250000 and 90000 which were absent from follicular oocytes but present in the fluid from the oestrus, but not luteal, oviduct. The glycoproteins remained on the zona pellucida of 2- to 4-cell embryos whose analysis showed the presence of additional polypeptides of M_r 150000, 57000, 50000 and 25000. It is concluded (i) that shortly after ovulation, and in spite of the presence around the egg of cumulus oophorus and corona radiata cells, significant amounts of oviducal glycoproteins are able to bind firmly to the zona pellucida, and (ii) that after contact with spermatozoa there is evidence of a limited hydrolysis of the structure by the sperm protease acrosin.

INTRODUCTION

Important postovulatory functions of the zona pellucida, the glycoprotein matrix which surrounds the mammalian egg, are broadly two-fold; first it acts as a selective 'screen' for fertilization and second, as a 'capsule' in which the early embryo is transported to the site of implantation. Specific events identified with the former involve sperm binding to the zona pellucida, species-specific sperm penetration of the investment and establishment of a block to polyspermy. Requirements for the latter are maintenance of the structure's general physiology until the embryo hatches and an involvement in the protection and nutrition of the embryo. The composition, sperm receptor and immunological properties of the zona have been investigated in several species (Dunbar, 1983, review) along with analyses of oviduct and uterine fluids (Edwards, 1980; Lee & Ax, 1984; Sutton, Nancarrow, Wallace & Rigby, 1984). Such studies have shown that the macromolecular composition of the zona pellucida varies from species to species and is quite complex in that the major glycoproteins present consist of families of up to fifteen differently charged subspecies. It has also been shown that oviducal and

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uterine fluids may change their composition at oestrus and a number of oestrus-associated macromolecules have been identified. Differences in the composition of the zona pellucida between the mouse oocyte and early embryo have been detected (Bleil, Beall & Wassarman, 1981) but hitherto the zona pellucida of the domesticated species has not been examined during these stages of development. Previous studies on the structure of the zona pellucida of the domesticated species, mainly the pig, have utilized preparations isolated on a large scale from ovaries. Using the pig as a model we have overcome the difficulty of shortage of material by utilizing the permeant reagent *N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl propionate (Bolton & Hunter, 1973) to analyse individual zonae pellucidae from oocytes, eggs and early embryos following their surgical recovery from the follicle, oviduct and uterus.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest purity available. ^{14}C-methylated molecular weight marker proteins and the reagent *N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl propionate were purchased from the Radiochemical Centre, Amersham, Bucks, UK. Pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) were purchased from Intervet Laboratories Ltd, Cambridge, UK.

Collection of oocytes and eggs

8- to 10-month-old cross-bred (Large White and Landrace) gilts were injected with 1200 i.u. PMSG on day 16 of the oestrus cycle followed by 500 i.u. hCG on day 19·5. At 36 or 42–44 h post hCG the animals were anaesthetized and mature oocytes taken from preovulatory follicles, while ovulated eggs were flushed from the oviducts. In some experiments mature oocytes were taken and immediately transferred to the undisturbed oviduct of the same animal, allowed to incubate there for 0·5 h with the animal remaining anaesthetized, and subsequently collected by flushing. Single-cell embryos were recovered from the oviducts of animals 49 h post hCG that had previously (24–36 h post hCG) been inseminated with 60–70 ml of whole boar ejaculate and 2- to 4-cell embryos from the uterus at 68–70 h post hCG. Using this regime eggs and transferred oocytes recovered from the oviduct were devoid of corona radiata cells but where eggs were collected at 38–40 h post hCG the few that had ovulated were contained in a tight corona radiata and cumulus oophorus. As with oocytes taken from the follicle such cells were removed by gentle suction of the gamete into and out of a fine-bore Pasteur pipette. Embryos were recovered with numerous spermatozoa attached to the zona pellucida and these were removed as thoroughly as possible (as judged by light microscopy) in the same way. The medium used for collection of eggs was phosphate-buffered saline (PBS) containing 4 mg ml^{-1} bovine serum albumin (Brown & Cheng, 1985) but for labelling experiments eggs were washed thoroughly in PBS without albumin.

Collection of zonae pellucidae

A Leitz micromanipulator was used to carefully remove zonae pellucidae from individual oocytes, eggs and embryos.

Collection of oviduct fluid

The oviducts of animals in oestrus (day 1) and the luteal phase (days 7 and 14) of the cycle were flushed with 1·5–2 ml PBS without bovine serum albumin. Tissue debris was removed by centrifugation (2000 *g* for 15 min).

^{125}I-labelling of zonae pellucidae and oviduct fluid protein

Zonae pellucidae of the same classification were labelled in groups of 5–15 depending upon numbers of oocytes and eggs recovered from each animal. To each group in 10 μl PBS was added 2·5 μCi of reagent in 20 μl 0·1 M-borate buffer pH 8·5 (Bolton & Hunter, 1973). After 15 min at 25 °C the zonae were washed three times in PBS and dissolved individually in sample buffer for electrophoresis (see below). Labelling of 0·05 ml oviduct fluid was carried out using the same concentration of reagent as for zonae pellucidae, after which protein was precipitated with nine volumes of cold ethanol and dissolved in electrophoresis buffer.

Electrophoresis

Labelled zonae pellucidae and ethanol-precipitated oviduct fluid were dissolved in sample buffer consisting of 62·5 mM-Tris–HCl pH 8·6, 2% w/v SDS and 10% (w/v) glycerol; 5% v/v 2-mercaptoethanol was included when reduction of samples was required. Samples were separated on 5–20% (w/v) polyacrylamide gradient slab gels (80 mm × 100 mm × 0·5 mm) containing 0·1% w/v SDS after which ^{125}I-labelled proteins were detected by autoradiography on Kodak X-Omat film (Brown & Cheng, 1985). Periodic acid-Schiff (PAS) staining of gels for carbohydrate was carried out using the method of Fairbanks, Steck & Wallach (1971). For two-dimensional electrophoresis the method of O'Farrell (1975) was used with samples focused for 4000 v.h. on a pH 3–10 Pharmalyte (Pharmacia) gradient followed by second dimensional separation on SDS–PAGE employing an 8–16% gradient of polyacrylamide.

RESULTS

Comparison of zonae pellucidae from oocytes and ovulated eggs

Non-reducing SDS–PAGE of zonae from oocytes showed that label had been incorporated into protein occupying a relatively diffuse area of the gel corresponding to an M_r of approximately 79000, with a more diffuse area of label spreading towards the anode showing a lower molecular weight limit of M_r 55000 (Fig. 1A, track i). Under reducing conditions the polypeptide of M_r 79000 was considerably diminished but there was heavy labelling of a polypeptide of M_r 69000; the diffuse region of label down to 55000 was still present (Fig. 1A, track ii). This pattern of labelling was very consistent from oocyte to oocyte and from animal to animal. Non-reduced zonae from freshly ovulated eggs showed a different labelling pattern. Labelling of the polypeptide of M_r 79000 occurred with improved resolution although the labelled material below this remained diffuse. The most striking difference was that in addition to the polypeptide of M_r 79000 there was labelling of two other polypeptides with M_r values of 250000 and 90000 (Fig. 1B); to emphasize the consistency of labelling pattern from zona to zona, four are shown but, in fact, zonae from more than 100 eggs gave an identical pattern of ^{125}I-labelling. When zonae pellucidae were analysed under reducing conditions the M_r 250000 polypeptide was absent and each zona pellucida resolved into three major polypeptides with M_r values of 90000, 79000 and 69000 (Fig. 1C). With the loss of the polypeptide of M_r 250000 there was a consistent concomitant intensity of labelling at M_r 90000 and diminution in labelling of the polypeptide of M_r 79000 (Fig. 1C, 2) together with a trace of material of M_r 46000. As was observed with oocytes (Fig. 1A) the species of M_r 69000 appears to be derived from that of M_r 79000 which diminishes after reduction.

The zonae pellucidae of oocytes which had been taken from mature follicles and incubated for 0·5 h in the oestrus oviduct (see Materials and Methods) showed an identical pattern of labelling to those of naturally ovulated eggs (Fig. 1D, track v). To determine whether the extrazonal investments which surround the very newly ovulated egg form a barrier to the polypeptides of M_r 250000 and 90000 we timed a

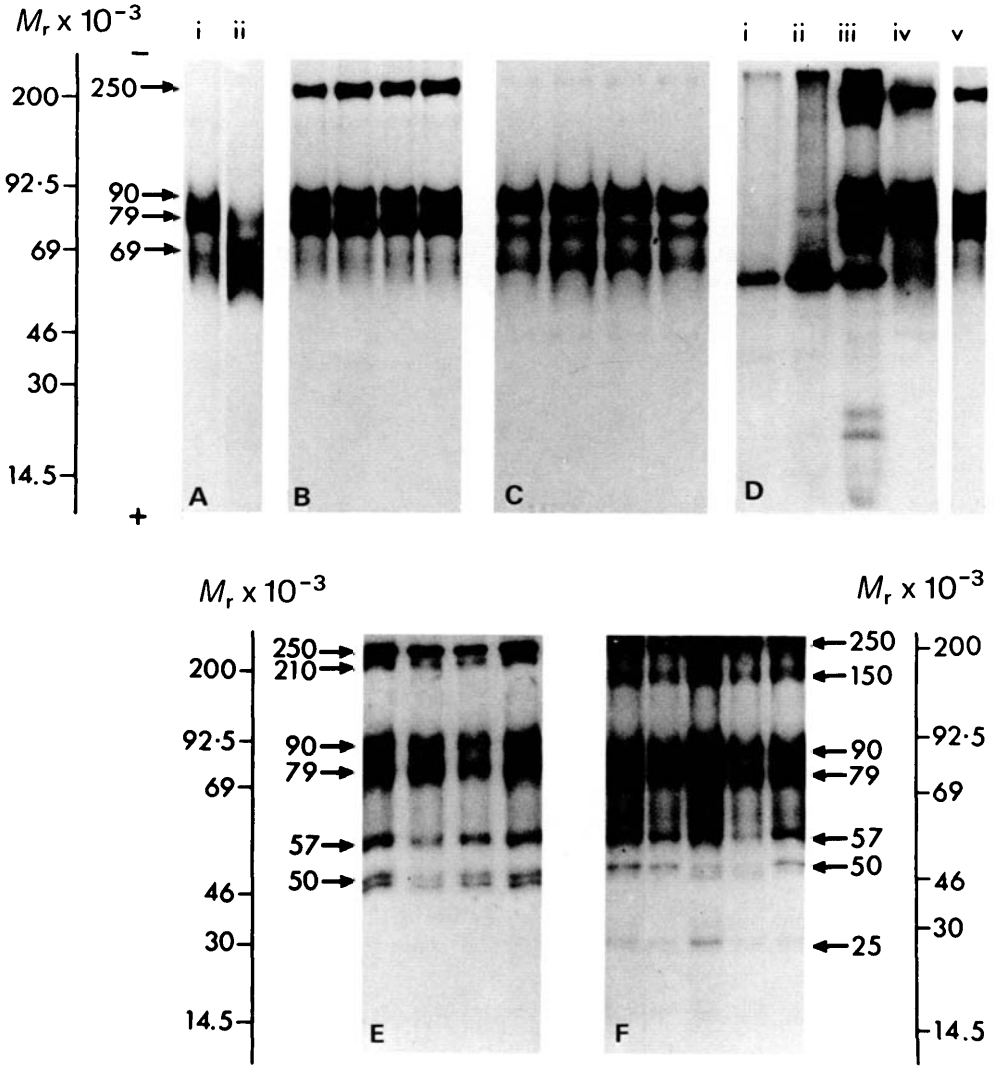

Fig. 1. One-dimensional SDS–PAGE of individual ^{125}I-labelled zonae pellucidae and oviduct fluid. (A) non-reduced (i) and reduced (ii) zonae pellucidae from oocytes; (B) non-reduced zonae pellucidae from four ovulated eggs; (C) reduced zonae pellucidae from four ovulated eggs; (D) oviduct fluid from the luteal (i and ii) and oestrus (iii) phase of the cycle against a zona pellucida from an ovulated egg (iv) and the zona pellucida from an oocyte incubated for 0·5 h in the oestrous oviduct (v); (E) non-reduced zonae pellucidae from four single cell embryos; (F) non-reduced zonae pellucidae from five 2- to 4-cell embryos. Tracks of zonae pellucidae received approximately 200000 c.p.m. generally representing 0·5 of a zona pellucida.

superovulation such that eggs were harvested when about only one-quarter of the stimulated follicles had ruptured (38–40 h post hCG) yielding eggs enclosed in a tight corona radiata and cumulus oophorus (see Materials and Methods). These were washed well, their investments removed down to the zona pellucida and the latter labelled in the usual way. It was found that both polypeptides were present in levels found in zonae pellucidae denuded *in vivo*.

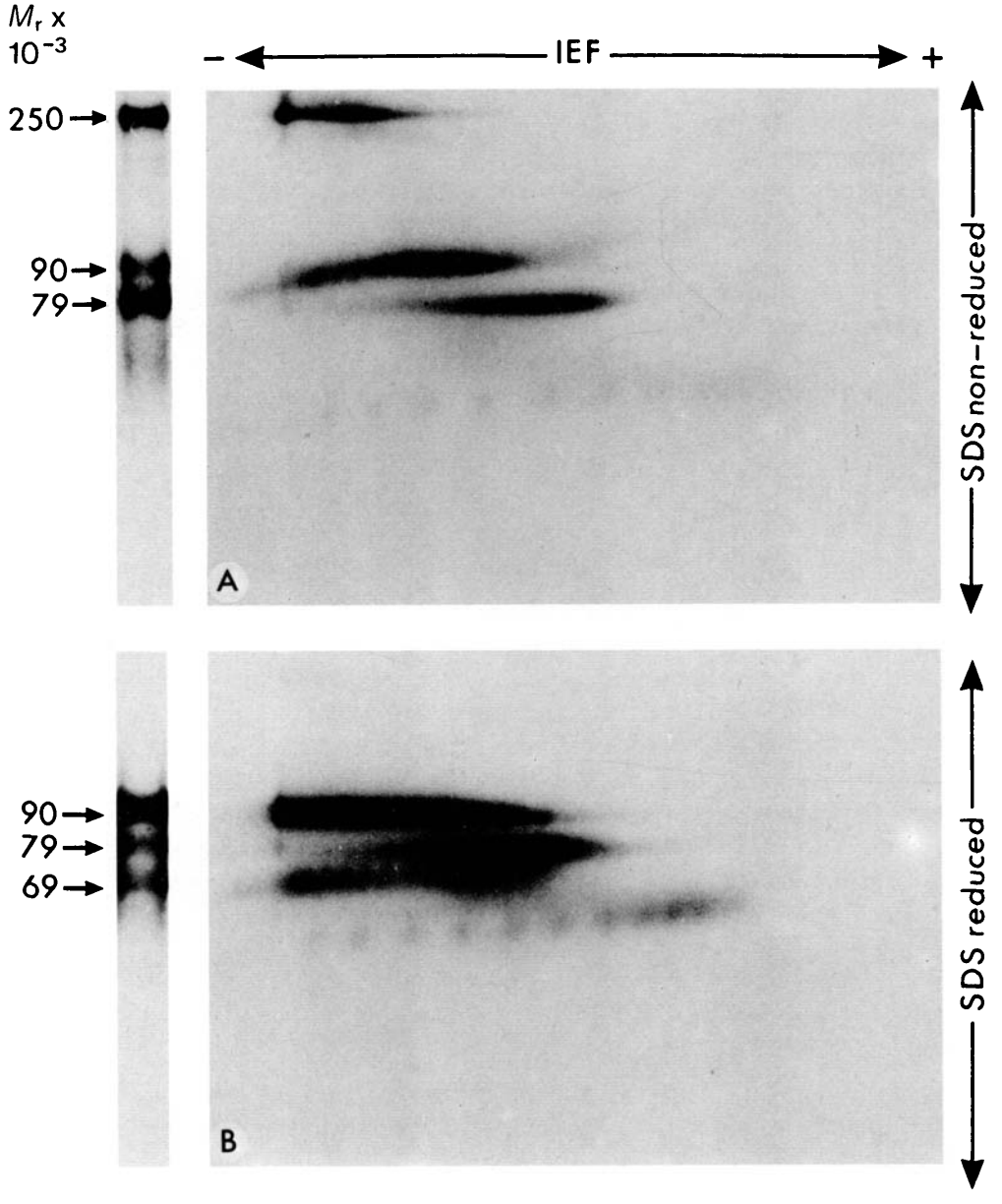

Fig. 2. Two-dimensional SDS–PAGE of the zona pellucida of an unfertilized egg. (A) Second dimension run non-reduced and (B) reduced. Each received approximately 400000 counts representing one zona pellucida.

Analysis of zonae pellucidae from single cell and 2- to 4-cell embryos

On non-reducing SDS–PAGE zonae pellucidae from single-cell embryos recovered from the oviduct showed a more complex labelling pattern than those from unfertilized eggs (Fig. 1E). Additional species of polypeptide were present with values for M_r of 210000 and 57000 with a doublet of approximately 50000. Zonae pellucidae of 2- to 4-cell embryos recovered from the uterus labelled similarly except that additional polypeptides of M_r 150000 and 25000 were present (Fig. 1F). Again, to emphasize the consistency of labelling profile from zona to zona, several tracks of each category are shown.

Electrophoretic analysis of oviduct fluid

^{125}I-labelling of oviduct fluid collected at different stages of the oestrus cycle gave the results shown in Fig. 1D. Material from oviducts in the luteal phase (days 7 and 14) showed labelling restricted largely to one polypeptide of approximate M_r 55000 (tracks i and ii). By contrast, material from the oestrus oviduct (track iii) contained several additional polypeptides that labelled including one heavily labelled species which comigrated with the polypeptide of M_r 250000 present on the zonae pellucidae of an ovulated egg (track iv). Another heavily labelled polypeptide migrated with an approximate M_r of 90000 while a less-heavily labelled species possessed an M_r slightly lower than 79000. Polypeptides of M_r 250000 and 90000 both stained with PAS (results not shown).

Two-dimensional SDS–PAGE of the zonae pellucidae of unfertilized eggs

Isoelectric focusing followed by non-reducing SDS–PAGE (Fig. 2A) showed the polypeptide of M_r 250000 to be less acidic than those of M_r 90000 and 79000 and the lower molecular weight spread of material. All species showed some charge heterogeneity, especially the lower molecular weight material which exhibited ten or more clearly defined subspecies. When the second dimension was run under reducing conditions (Fig. 2B) an increase in material of M_r 90000 was seen, clearly contributed by at least some of the polypeptide of M_r 250000. The polypeptide of M_r 69000 when reduced also showed considerable charge heterogeneity whereas reduction of the lower molecular weight spread of material gave a similar separation to the non-reduced form.

DISCUSSION

A major finding from our experiments is that development of the zona pellucida from within the follicle to the early embryo includes acquisition by the structure of two glycoproteins which are present in the oestrus oviduct. The binding of these glycoproteins is sufficiently firm to prevent their removal by washing and their association with the zona pellucida is maintained until at least the 2- to 4-cell embryo stage.

Results from studies of the major polypeptides of porcine zonae pellucidae prepared on a large scale from ovaries have varied. Menino & Wright (1979) reported four polypeptides with M_r values of 85000, 66000, 57000 and 17000, whereas Noda *et al.* (1983) detected two with values of 96000 and 60000, and Gwatkin, Anderson & Williams only one of M_r 62000. Using two-dimensional electrophoresis Dunbar, Liu & Sammons (1981) resolved three primary components with M_r values ranging from 110000 to 79000, each consisting of up to 15 differently charged subspecies. In all these cases SDS–PAGE was carried out with reduced zonae pellucidae. Using non-reduced material Yurewicz, Sacco & Subramanian (1983) resolved a polypeptide of M_r 58000 from two of M_r 87000. In reduced zonae from mature oocytes we find a major polypeptide of M_r 69000, a minor one of M_r 79000 and a diffuse area of material down to M_r 55000; in non-reduced zonae the polypeptide of M_r 79000 is predominant with the lower molecular weight spread persisting. Our results clearly show that there is a striking difference between the zonae of eggs recovered from the oviduct and zonae from oocytes in that, in the non-reduced form, major polypeptides of M_r 250000 and 90000 are present. When electrophoresed under reducing conditions zonae from ovulated eggs resolved into three polypeptide species of M_r 90000, 79000 and 69000 with, again, the diffuse lower molecular weight material. From both one-dimensional and two-dimensional electrophoresis it appears that reduction of the glycoprotein of M_r 250000 results in one or more subunits of M_r 90000. We thought it possible that the polypeptide of M_r 250000 might have been produced as a consequence of a structural reorganization of the zona, e.g. crosslinking, associated with the final stages of maturation of the oocyte or with ovulation. We tested the first of these possibilities by maturing oocytes in culture to the stage at which they become fertilizable, but subsequent analysis of the zonae showed polypeptides of M_r 250000 and 90000 to be absent. However, it seemed more likely that these polypeptides are acquired from the oviduct and, indeed, the presence of major polypeptides, which stained positively with PAS, of M_r 250000 and 90000 in oestrus oviduct fluid is consistent with this. It is of interest that these macromolecules are able to permeate the cumulus oophorus and corona radiata and thus are likely to establish contact with the zona pellucida before the spermatozoon. From this it would seem that the integration of such molecules with the zona pellucida should be taken into account when sperm receptor properties of zona pellucida glycoproteins isolated from ovaries are evaluated (Dietl, Czuppon, Weichert & Mettler, 1983).

We have found clear and very consistent differences in the polypeptide profile between zonae from unfertilized eggs and single-cell and 2- to 4-cell embryos in that as the investment develops an increase occurs in the number of polypeptides detectable. These could be derived from the egg itself or from remnants of spermatozoa left on the embryo in spite of our efforts to remove the latter before labelling. However, we believe that the additional polypeptides are a result of proteolysis of the zona pellucida by the sperm acrosomal protease, acrosin, because their appearance, both in the sequence in which they are produced and in

their molecular weights, is characteristic of the initial stages of the limited proteolysis of the ovulated zona pellucida by this protease (Brown & Cheng, 1985). We do not consider the observed proteolysis to have been caused by the fertilizing spermatozoon but by the large numbers of spermatozoa which bind to the zona pellucida at and following the time of fertilization. This need not be physiologically significant but it remains possible that, after the acrosome reaction, interaction between acrosin and molecules at the zona surface following the acrosome reaction could consolidate sperm attachment to this investment by either creating or exposing binding sites. Indeed, it is noteworthy that inhibitors of trypsin-like enzymes (a class in which acrosin falls) will prevent spermatozoa from binding to the zona (Saling, 1981).

Recent studies have shown that the sheep oviduct contains an oestrus-associated glycoprotein with a subunit molecular weight of 80000–90000 (Sutton *et al.* 1984), and in the rabbit there is evidence for the acquisition of oviduct glycoproteins by early embryos (Shapiro, Brown & Yard, 1974; Hanscom & Oliphant, 1976). Our results, which have utilized this phase of development in the pig, confirm the concept that oestrus-induced oviducal glycoproteins do interact with the egg zona pellucida, and we have observed a similar phenomenon in the sheep (unpublished results). At this stage it is not possible to assign a biological role to such interactions but it is possible that the acquisition by the egg of oviduct components such as we have described is important in the control of development of the early embryo where, in fact, embryo culture techniques have met with limited success. For instance, in the pig the 2- to 4-cell embryo obtained by oocyte maturation and fertilization *in vitro* has an absolute requirement for the oviduct for further development. One important role for oviduct components related to survival and development of the early embryo has already been indicated. It has been shown that the rabbit oviduct contains an oestrus-dependent factor which will inhibit complement activity, thus providing a mechanism by which complement-antibody destruction of the embryo can be avoided (Oliphant, Cabot, Ross & Marta, 1984). Their results suggest that the inhibitory activity lies in a family of sulphated glycoproteins with a subunit molecular weight of 72000 and a native molecular weight as high as 500000. Such glycoproteins appear to form part of the morphologically noticeable mucin coat which is laid around the rabbit egg shortly after ovulation and which continues to exist around the early embryo (Hanscom & Oliphant, 1976). To our knowledge, the rabbit is the only species of mammal in which this has been observed but it now appears that the same phenomenon occurs in the pig, though binding of the glycoproteins of M_r 250000 and 90000 to the porcine zona pellucida does not result in the formation of such a morphologically discernible mucin coat. Nonetheless these glycoproteins could be performing a similar immunoprotective role and it remains possible that their acquisition by the zona pellucida forms part of the mechanism by which the oviduct provides nutrition for the development and growth of the early embryo.

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