THE SHEEP EGG: ENZYMATIC REMOVAL OF THE ZONA PELLUCIDA AND CULTURE OF EGGS *IN VITRO*

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Summary. A total of 429 eggs was used to investigate procedures both for the removal of the zona pellucida and for the culture of sheep eggs *in vitro*.

The effect of pronase, papain, bromelain, trypsin and α -chymotrypsin, on the zonae pellucidae of fertilized and unfertilized eggs was studied in the first series of experiments. All the enzymes were effective in removing the zonae pellucidae from unfertilized eggs. Pronase was the only enzyme that had a significant lytic effect on the zonae of fertilized eggs denuding 34% of eggs in less than 5 min and almost all the other eggs within 90 min.

Investigation of some of the factors involved in the successful culture of normal sheep eggs *in vitro* constituted the second part of the study. Eggs of both the eight- to sixteen-cell and morula stages were cultured for 72 hr in either sheep serum, enriched tissue culture medium 199 or Brinster's medium. The development and viability of the eggs after culture was determined by transferring some eggs to recipient sheep and making accurate cell counts on the remainder. Eggs recovered at the eight-cell stage underwent only one or two cleavage divisions during the 72-hr culture period. These eggs did not develop into foetuses when transferred to the uteri of recipient sheep. On the other hand, the majority of morulae cultured for 72 hr in enriched M199 or sheep serum underwent cleavage and blastulation *in vitro*. That the cultured blastocysts were normal was demonstrated by their implantation and growth into foetuses when transferred to the uteri of suitable recipients.

INTRODUCTION

The removal of the zona pellucida and the culture of the eggs *in vitro* are important steps in procedures such as the production of mouse chimeras by egg fusion (Tarkowski, 1961; Mintz, 1962a). There have, however, been no reliable methods described for the enzymatic removal of the zona pellucida or for the successful culture of sheep eggs *in vitro*.

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The zona pellucida of the mouse egg can be removed either by mechanical means (Tarkowski, 1961) or by exposing the egg to one of a number of chemical agents, the most effective of which is the proteolytic enzyme pronase (Smithberg, 1953; Mintz, 1962b; Gwatkin, 1964). This enzyme is not, however, an effective means of removing the zonae from the eggs of rats and sheep (Toyoda & Chang, 1968; Pighills, Hancock & Hall, 1968). Different proteolytic enzymes have been used in the present study in an attempt to find a suitable chemical means of removing the zona pellucida from the sheep egg.

Most of the work on the culture of mammalian eggs *in vitro* has concerned mouse eggs. In this species, cultured eggs will develop readily into blastocysts which will then continue to term if transferred to a suitable host animal (see Brinster, 1968). By contrast, sheep eggs develop poorly under conditions *in vitro*. Eggs placed in culture before the sixteen-cell stage undergo a very limited number of divisions before becoming degenerate (Robinson, 1951; Wintenberger, Dautier & Thibault, 1953; Hancock, 1963; Kraemer, 1966; Tervit & McDonald, 1969; Moore, 1970). Embryos recovered at the morula stage do, however, develop more readily *in vitro* and some apparently normal blastocysts have been obtained in culture (Wintenberger *et al.*, 1953).

The experiments described in this paper are concerned with the removal of the zona pellucida and the culture of sheep eggs *in vitro*.

MATERIALS AND METHODS

The experiments were carried out between October 1968 and March 1969 on eighty Targhee and eighteen Hampshire Down sheep. The occurrence of oestrus was checked once daily using either a vasectomized or a fertile ram according to the experimental group. The day on which oestrus occurred is referred to as Day 0 of the cycle.

All surgical procedures were carried out under sodium pentobarbital anaesthesia, observing aseptic precautions.

Donor sheep

Superovulation was induced in the donor sheep either by the subcutaneous injection on Day 12 of 1500 units PMSG (Ayerst Laboratories) or by the administration of 5 to 15 mg of FSH (Armour Laboratories) given in two to four daily intramuscular injections, starting on the 12th day after oestrus.

The eggs were flushed from the donor's reproductive tract *in vivo* with either sterile sheep serum or tissue culture medium 199 (M199 Microbiological Associates) using the technique of Hunter, Adams & Rowson (1955). The eggs were examined microscopically and then kept under an atmosphere of 5% CO₂ and 95% air in an incubator at 37° C pending further experimental manipulation.

Recipient sheep

Either one or two eggs were transferred to the uteri of forty-eight non-pregnant sheep. The onset of oestrus in the donor and recipient animals was exactly synchronized in every case where eggs were to be transferred directly

without an intervening period in culture. Cultured eggs, on the other hand, were transferred to recipients that had exhibited oestrus 24 hr after the onset of oestrus in the donor animal; this was to compensate for possible retardation in the development of eggs in culture.

Experimental design

The work was carried out in two parts and was designed to provide information on (A) the enzymatic removal of the zona pellucida and (B) the conditions required for successful culture of sheep eggs *in vitro*. The two studies were run concurrently.

A. *Enzymatic digestion of the zona pellucida and the viability of the denuded eggs.* The effectiveness of five proteolytic enzymes in removing the zonae of fertilized and unfertilized eggs, recovered 72 hr after oestrus, was tested in Exp. 1. The enzymes used and their source, together with the concentration, pH and osmolarity at which they were tested, are shown in Table 1.

TABLE 1
ENZYME PREPARATIONS USED FOR THE REMOVAL OF THE ZONA PELLUCIDA
FROM SHEEP EGGS

Enzyme	Concentration*	pH	Osmotic pressure†	Source of enzyme
Pronase	0.5%	6.8 to 7.2	282	Calbiochem B Grade
Papain	0.5%	5.2 to 5.3	300	Sigma Type II
Trypsin	0.5%	6.8 to 7.2	295	Pfanstiehl
α -Chymotrypsin	0.5%	6.8 to 7.2	294	Worthington
Bromelain	0.5%	5.7	297	Sigma Grade II

* In Hanks' solution.

† mosmol; average of four or five determinations; average S.D. = 15 mosmol.

Individual eggs with the appropriate enzyme solution were placed in sterile immunological well-trays (Disposo-Tray, Linboo Chemical Co.) and the eggs were examined repeatedly during the first 15 min of exposure to the enzyme. Thereafter, each egg was examined every 10 to 15 min until the zona was lost or, failing that, until the egg had been in the enzyme solution for 90 min.

The fertilized eggs were removed from the enzyme solution as rapidly as possible after the loss of the zona, washed in four successive wells containing sheep serum and kept in serum at 37° C until required for culture or transfer. The viability of the denuded eggs was determined either under culture conditions *in vitro* or by transferring the eggs to the uteri of recipient sheep. The eggs were fixed and stained after 72 hr in enriched M199 culture medium. (See Study B for details of culture procedure.)

B. *Culture of sheep eggs in vitro.* Two of the conditions affecting the development of sheep embryos *in vitro*, namely the age of the embryo and the nature of the medium used for culture, were investigated in this study. Embryos were recovered from donor sheep at 72 or 120 hr after oestrus and were cultured in micro-drops of medium (100 to 200 μ l) placed under mineral oil according to the technique of Brinster (1963). The culture dishes (Falcon plastic) containing

the embryos were incubated for 72 hr at 37° C in an atmosphere of 5% CO₂ and 95% air.

The three media used in the culture experiments were sheep serum, Brinster's medium (Brinster, 1965) and enriched medium M199. Sheep serum was treated at 56° C for 30 min for complement fixation and then kept frozen at -10° C until required. When at equilibrium under 5% CO₂ in air, the pH of the serum was between 7.85 and 7.90 and the osmotic pressure was 313 mosmol. No osmotic pressure adjustments were made to compensate for the slight hypertonicity of the serum.

Brinster's medium was prepared just before use, the calcium and magnesium salts being added last to minimize carbonate formation. The enriched M199 medium was prepared by adding 2.0 ml of complement-fixed foetal bovine serum (Microbiological Associates), 0.7 ml of 0.154 M-sodium lactate (1 mg of lactate per ml of final solution) and 1.1 ml of distilled water to 6.2 ml of M199 to give 10 ml of medium. Both the Brinster's and enriched M199 media were isosmotic (290 mosmol) and under a 5% CO₂ in air atmosphere, had a pH of 7.2.

TABLE 2
ENZYMATIC DIGESTION OF THE ZONAE PELLUCIDAE OF FERTILIZED SHEEP
EGGS RECOVERED FROM DONOR ANIMALS 72 HR AFTER OESTRUS

Enzyme preparation	No. of eggs tested	No. of donor sheep	No. of donors whose eggs were denuded in:		
			< 15 min	15 to 90 min	> 90 min
Pronase	70	38	13	23	2
Papain	7	6	1	0	5
Bromelain	8	6	1	0	5
Trypsin	9	7	0	1	6
α-Chymotrypsin	8	5	0	2	3

A careful microscopic examination was made of all the eggs after 72 hr in culture. Those that had undergone cleavage *in vitro* were either fixed and stained and the number of nuclei in each egg counted, or else the eggs were transferred to the uteri of recipient ewes. Normal (uncultured) eggs as controls were likewise transferred to recipient animals and the percentages of eggs developing in each group were compared.

RESULTS

A total of 351 fertilized eggs and seventy-eight unfertilized eggs were recovered from the donor animals.

A. Enzymatic digestion of the zona pellucida and the viability of denuded eggs

The effectiveness of various proteolytic enzymes in removing the zona pellucida of the fertilized sheep egg is shown in Table 2. Of the five proteolytic enzymes used, only pronase consistently removed the zona pellucida from the eggs. However, it was in only 34% of the donors in the 'pronase group' that

the fertilized eggs were denuded relatively rapidly: the zonae were removed from the eggs of 26% of the donors in less than 3 min and from the eggs of 8% of the donors in 5 to 15 min. An exposure to pronase of more than 45 min was required to denude the eggs of the other donors in this group. Even after 90 min, most of the eggs exposed to papain, bromelain, trypsin or α -chymotrypsin still had intact zonae.

Perhaps the most striking feature of this experiment was the consistent manner in which all the eggs from the same donor responded to pronase. Either all the eggs from one animal lost their zonae very rapidly or they all required a considerable period in pronase before becoming devoid of their zonae. The importance of maternal factors in determining the speed with which pronase removed the zona pellucida was examined further in a second trial. Four sheep whose eggs had been denuded rapidly in the first experiment were again injected with PMSG and then used as donors for a second time. As

TABLE 3

ENZYMATIC DIGESTION OF THE ZONAE PELLUCIDAE OF UNFERTILIZED SHEEP EGGS RECOVERED FROM DONOR ANIMALS 72 HR AFTER OESTRUS

Enzyme preparation	No. of eggs tested	No. of donor sheep	No. of donors whose eggs were denuded in:		
			< 15 min	15 to 90 min	> 90 min
Pronase	16	8	7	1	0
Papain	16	9	5	2	2
Bromelain	16	8	3	3	2
Trypsin	15	6	1	3	2
α -Chymotrypsin	15	7	0	6	1

before, all the eggs from three of these donors were denuded very rapidly (1 to 3 min) while the eggs from the fourth donor were denuded in 15 to 25 min. This trial suggests that the zonae of eggs from some donors are less resistant to the action of pronase than eggs from other sheep.

It will be seen from Table 3 that unfertilized eggs, flushed from the uteri of sheep 72 hr after oestrus, are highly susceptible to rapid enzymatic digestion of the zona pellucida. Unfertilized eggs were denuded fairly rapidly and consistently by all five enzymes, whereas fertilized eggs were little affected by bromelain, papain, trypsin and α -chymotrypsin.

In an attempt to test the viability of the eggs after removal of the zona, fifteen denuded eggs were transferred to the uteri of recipient ewes and two groups of fourteen eggs were cultured for 72 hr, one group in homologous sheep serum and the other in enriched M199 medium. Cleavage did not occur in any of the denuded eggs in culture and none of the eggs transferred to the uteri of recipients developed into foetuses. The structural integrity of the blastomeric mass was retained in only twelve of the twenty-eight cultured eggs; in the other sixteen denuded eggs, the blastomeres became dissociated within 48 hr.

B. Culture of sheep embryos *in vitro*

The degree to which sheep eggs develop after culture *in vitro* for 72 hr is shown in Table 4.

TABLE 4

THE INFLUENCE OF THE AGE OF THE EGG AND THE TYPE OF CULTURE MEDIUM ON THE DEVELOPMENT OF EGGS AND MORULAE CULTURED FOR 72 HR *in vitro*

Interval from oestrus to egg recovery (hr)	Culture medium	No. of eggs cultured	Embryonic development after 72 hr <i>in vitro</i>		
			No development	Arrested development	Normal development
72	M199	82	31	51	0
72	Brinster's medium	30	16	14	0
72	Sheep serum	15	6	9	0
120	M199	48	12	8	28
120	Brinster's medium	15	13	2	0
120	Sheep serum	22	7	3	12

None of the 127 fertilized eggs recovered 72 hr after oestrus underwent normal cleavage and blastulation in culture. Development was arrested after one or, at most, two cleavage divisions in seventy-four eggs; no development occurred in the other fifty-three cultured eggs. There was no indication that the three culture media differed in the degree to which they could support the early development of the eight- to sixteen-cell sheep egg.

Morulae recovered on Day 5 underwent apparently normal development both in enriched M199 and in sheep serum but not in Brinster's medium. Clear disc and cavity formation occurred in 73% (fifty-one out of seventy) of the morulae cultured in M199 or sheep serum. Because of an apparent underdevelopment of the embryonic disc, eleven of the cultured blastocysts were classified as having suffered 'arrested development'. Morulae recovered on Day 5 and placed in Brinster's medium did not undergo more than one cleavage division during the 72 hr in culture.

The shedding of the zona pellucida in culture occurs 24 to 48 hr later than *in utero* and in an apparently different manner; the characteristic hatching of the blastocyst *in vitro* and the shedding of the zona *in vivo* is illustrated in Pl. 1, Figs 1a and b and Fig. 2. An inability to shed the zona probably contributed

TABLE 5

DEVELOPMENT OF CULTURED AND NON-CULTURED EGGS FOLLOWING TRANSFER TO THE UTERI OF RECIPIENT SHEEP

Treatment of eggs before transfer	Interval from oestrus to egg recovery (hr)	Culture medium	Total no. of recipients	No. of eggs transferred	Results of embryo transplantation	
					Recipients pregnant	No. of foetuses
Not cultured	72	—	20	30	8	10
72 hr culture	72	M199	7	15	0	0
72 hr culture	120	M199	9	14	3	3
72 hr culture	120	Sheep serum	4	8	1	1

PLATE 1

FIG. 1a, b. Two morulae recovered from the uterus of a sheep on the 6th day after oestrus. Notice the characteristic rupture of the zona pellucida which precedes the shedding of the zona *in utero*.

FIG. 2. The hatching of a blastocyst from its zona pellucida during development *in vitro*. In culture, the zona cracks slightly and the blastocyst is extruded through the resultant opening. This method of zona shedding is slower and contrasts markedly with the loss of the zona *in vivo* (shown in Fig. 1).

FIG. 3. A thinned zona pellucida is visible surrounding a fully expanded blastocyst.

FIG. 4. An early stage in the collapse of a blastocyst that had undergone blastulation and expansion but had failed to shed its zona *in vitro*.

largely to the degeneration of most of the '5-day' morulae that failed to develop into blastocysts during culture in enriched M199 medium or in sheep serum. Before degeneration, these embryos underwent blastulation and expanded to fill the entire intrazonal space (Pl. 1, Fig. 3). After about 48 hr in culture, the early blastulae collapsed, leaving a thick zona and a contracted cell mass in the centre of the egg (Pl. 1, Fig. 4).

The viability of the cultured embryos was determined by transferring them to the uteri of suitable recipient sheep (Table 5). None of the eggs recovered 72 hr after oestrus, cultured for 3 days and then transferred to host animals, developed into foetuses. On the other hand, four of twenty-two morulae recovered on Day 5 and cultured in enriched M199 or sheep serum for 72 hr developed into normal foetuses after transfer to recipient sheep. This disappointingly low survival of cultured eggs *in vivo* was matched by an almost equally low number of successful transfers of non-cultured eggs; only ten out of thirty eggs developed into foetuses after the direct sheep transfer of eggs from donor to recipient ewes.

DISCUSSION

These experiments highlight some of the major difficulties associated with the enzymatic removal of the zona pellucida and the development of sheep eggs *in vitro*. The only proteolytic enzyme that had a significant lytic effect on the zona pellucida of the fertilized sheep egg was pronase. A 0.5% solution of this enzyme removed the zonae from the eggs of only about one third of the donors within the 3- to 5-min period required to denude the mouse egg. Moreover, the established methods of culturing eggs *in vitro* are not suitable for the normal development of the eight- to sixteen-cell sheep egg, but are suitable for the development of morulae in culture.

In 1956, Chang & Hunt, working with rats, rabbits and hamsters, reported that the zonae pellucidae of unfertilized eggs were much more affected by proteolytic enzymes than were their fertilized counterparts. It is clear from our experiments that the zonae of unfertilized sheep eggs are similarly very much more rapidly denuded by proteolytic enzymes than are the zonae of fertilized eggs. The rapid enzymatic digestion of the zonae of unfertilized eggs and the marked resistance of the zona to the same enzyme after fertilization is probably a reflection of changes in the chemical structure of the zona after sperm penetration. A factor of importance in determining the susceptibility of the zonae of fertilized eggs to digestion by pronase appears to be of maternal origin and influences all the eggs from one donor in a similar manner.

We have observed that the blastomeres often separate in culture after removal of the zona pellucida from the eight-cell egg. This blastomere separation is not necessarily a result of enzyme treatment since Pighills *et al.* (1968) noted similar blastomeric separation of eight- to sixteen-celled eggs after mechanical removal of the zona. In our experiments, it was found that eggs collected at the eight-cell stage, denuded with pronase and immediately transferred to a suitable recipient, failed to develop *in utero*. Morulae which were mechanically denuded before transplantation also failed to develop in the

uteri of recipient ewes (Pighills *et al.*, 1968). However, these authors found that morulae without zonae can survive and develop normally *in utero* if the denuded eggs are first placed in culture for 24 hr before being transferred to host sheep.

It is essential to adopt a critical end-point when assessing embryonic development *in vitro*. We have used two criteria to assess the development of sheep embryos after culture *in vitro*. Firstly, some of the eggs that developed furthest *in vitro* were transferred to suitable recipient sheep and their subsequent development and growth were studied. All the other embryos were fixed, stained and examined microscopically after culture. The accurate nuclear counts made possible by this technique were adopted as a critical second method of assessing embryonic development *in vitro*. It is clear that we, like other workers (Robinson, 1951; Wintenberger *et al.*, 1953; Hancock, 1963; Kraemer, 1966; Tervit & McDonald, 1969; Moore, 1970) cannot sustain the development of eight-cell sheep eggs for 72 hr *in vitro*. The total failure of such eggs to develop normally in culture, despite the use by a number of investigators of a wide variety of techniques and culture media, leads one to suggest that a specific factor might be necessary for the development of the sheep egg at this stage. For example, Krishnan & Daniel (1967) reported that a uterine substance, blastokinin, is required for the normal development of rabbit eggs. However, if a similar substance is necessary for the development of eight-cell sheep eggs then it cannot be a specific uterine factor since these eggs develop normally when placed in the oviducts of rabbits (Averill, Adams & Rowson, 1955). The morula, unlike the eight-cell egg, undergoes rapid cleavage and blastulation *in vitro*. The normality of the blastocysts formed in culture was demonstrated by their ability to implant, and their subsequent growth and development when transferred to the uteri of suitable recipient sheep.

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