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Development of Sheep Embryos *in Vitro* in a Medium Supplemented with Different Serum Fractions

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Abstract

Sheep embryos will generally develop into expanded blastocysts *in vitro* only in culture media supplemented with serum or serum components. In order to better understand how serum supports embryo development, a batch of ovine serum was fractionated by (a) ultrafiltration into two components containing substances with molecular weights greater and less than 10 Kd (kilodaltons), and (b) gel filtration into protein fractions 1, 2 and 3, containing groups of proteins with mean molecular weights of about 500, 150 and 65 Kd, respectively. The principal protein in fraction 3 was albumin. Day 6 sheep morulae were cultured *in vitro* for 48 hours in a bicarbonate-buffered salt solution supplemented with various concentrations of ovine serum or of these components or protein fractions of serum.

Morulae could develop to fully expanded blastocysts in medium supplemented with whole serum or with the >10 Kd component or protein fraction 3 only, but could not develop in medium supplemented with the <10 Kd component only or with the <10 Kd component and protein fractions 1 or 2. However, the proportion of embryos that developed fully in medium supplemented with the >10 Kd component or protein fraction 3 was increased by adding the <10 Kd component of serum to the medium. The addition of protein fraction 2 decreased the proportion of embryos that developed to expanded blastocysts in medium containing protein fraction 3 and the <10 Kd component, but not in medium containing whole serum. Since the compositions of different sera may vary markedly, these results suggest (a) reasons why different sera vary in their ability to support embryo development *in vitro,* and (b) factors which may influence development of the sheep embryo in the uterus, where plasma proteins comprise nearly all the protein in the fluid bathing the embryo.

Introduction

After entering the uterus on days 3–4 of pregnancy, the sheep morula lies freely in the uterine lumen surrounded by its zona pellucida and bathed in a small volume of uterine fluid (McLaren 1982). Plasma proteins account for nearly all the protein present in ovine uterine flushings during the first ten days of pregnancy (Roberts *et al.* 1976; Miller *et al.* 1977). Sheep embryos will generally develop *in vitro* to the expanded blastocyst stage only in culture media containing serum or commercial preparations of serum albumin (Tervit and Goold 1978; Wright and Bondioli 1981; Moore 1982; Bunch *et al.* 1984; Quinn *et al.* 1984). Serum contains a heterogeneous mixture of naturally occurring substances, including many different proteins. The composition of different batches of serum from the same species may vary widely (Taylor 1974; Blunt 1975), as may the ability of different batches of serum to support embryo development *in vitro* (Ladd *et al.* 1982; Bates *et al.* 1985; Shirley *et al.* 1985). Some types or batches of serum inhibit embryo development *in vitro* (Caro and Trounson 1984; Shirley *et al.* 1985).

Taken together, these findings suggest that plasma proteins have an important role *in vivo* in the development of sheep embryos through the morula and blastocyst stages, and that embryo survival and development in the ewe may be influenced by variations in the protein composition of the plasma and endocrine and other factors which regulate the transudation of these proteins across the endometrium to the uterine lumen. In order to gain some understanding of how plasma proteins influence embryo development, we have examined the ability of

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day 6 sheep morulae to develop *in vitro* in a basal bicarbonate-buffered medium supplemented with different concentrations of whole serum or several different components of serum.

Materials and Methods

Experimental Animals, Superovulatory Treatments and Collection of Embryos

Mature Merino ewes were run under field conditions at the University of Sydney Farms, Camden. The time of oestrus in groups of ewes was synchronized using intravaginal pessaries impregnated with 60 mg medroxy progesterone acetate (Repromap, Upjohn Pty Ltd, Sydney). These were inserted in ewes for 12–14 days and removed at 0830 h. Superovulation was induced by treatment with pregnant mare serum gonadotrophin (PMSG) alone or with a combination ('cocktail') of PMSG and follicle stimulating hormone of animal pituitary origin (FSH-P; Heriot Agencies Pty Ltd, Ferntree Gully, Victoria). In the case of animals receiving PMSG alone, a single dose was given s.c. at 1700 h, about 40 h before pessary removal. In animals receiving 'cocktail' treatments, six doses of FSH-P were given s.c. at 0830 h and 1630 h on three consecutive days, and a single dose of PMSG was given s.c. at a separate site at the same time as the first injection of FSH-P, about 48 h before pessary removal. Hence the final dose of FSH-P was given to these animals 8 h after pessary removal. The pregnant mare serum gonadotrophin was a preparation of raw serum obtained from a pregnant mare between days 60 and 80 of pregnancy and stored at −20°C in 20 ml aliquots until required.

Groups of ewes receiving superovulatory treatments were run with entire rams fitted with 'Sire-Sine' harnesses and marking crayons in order to detect ewes in oestrus. In most, but not all, ewes the onset of oestrus occurred within 32 h of pessary removal. Intra-uterine laparoscopic inseminations were carried out in all ewes at 24-29 h after pessary removal, using the procedure of Killeen and Caffery (1982). Fresh semen was collected immediately prior to inseminations and assessed for quality, and suitable ejaculates were diluted in 2-3 volumes of Dulbecco's phosphate buffer containing 62·5 mg l^{-1} potassium penicillin and 50 mg l^{-1} streptomycin sulfate and held in a water bath at 30°C for up to 1 hour. Each ewe received 0·1–0·2 ml of diluted semen in each uterine horn. Entire rams remained with the ewes for a further day after the inseminations.

Embryos were collected via mid-ventral laparotomy 6 days after inseminations (day 0 = day of insemination and, in most instances, oestrus) in a flushing medium of Dulbecco's phosphate buffer (pH 7·3, Dulbecco and Vogt 1954) supplemented with 10% ovine serum. The embryos were rinsed in fresh flushing medium and examined under bright field illumination (×10–40 magnification). Those judged morphologically normal day 6 embryos were mostly late morulae, but some early blastocysts which were not yet expanding were included. The normal embryos from all ewes on any day of collection were pooled and held at 30°C in the flushing medium under air until they were placed in appropriate treatment culture media. Presumably there was some variation in the quality of embryos judged morphologically normal between superovulatory treatments and between weeks of embryo collection.

The numbers of recently formed corpora lutea and persistent large follicles in each ovary on day 6 were recorded. These data and those for the total numbers of embryos and unfertilized ova and the number of normal embryos recovered were tabulated against superovulatory treatments. These data are not presented, since the doses of PMSG and FSH-P employed were repeatedly and empirically changed during the course of the study, and even within experiments, in order to maximize the rate of recovery of normal embryos on day 6. Hence, valid dose-response relationships cannot be determined. Further, the potency of the raw PMSG preparation was not determined by any standard bioassay procedure. Nevertheless, the following summary gives some indication of the ovarian responses and embryo recovery rates obtained. On average, ewes treated with PMSG alone received about 4·0 ml of pregnant mare serum and yielded on day 6 about eight corpora lutea and two large follicles, 1·5 unfertilized ova and 3·5 embryos of which 3·0 were considered normal. Ewes given the 'cocktail' treatment received about 1·5 ml of the same pregnant mare serum and 11 mg FSH-P. Decreasing doses of FSH-P were given over the three consecutive days of treatment (e.g. when ewes received a total of 11 mg FSH-P, 2·5 mg, 2·0 mg and 1·0 mg were given twice daily on the 1st, 2nd and 3rd days, respectively). On average, these animals yielded about 12 corpora lutea and one large follicle, 0·8 unfertilized ova and 7·5 embryos of which 6·5 were considered normal. Overall, the 'cocktail' treatments were definitely superior to treatments with PMSG alone, in terms of the mean yield of normal embryos per ewe (Ryan *et al.* 1984) and most ewes in this study received 'cocktail' treatments.

Code of Practice for the Care and Use of Animals

The maintenance and experimental use of sheep in this study was in accordance with the guidelines issued by NH & MRC/CSIRO/ACC in 1985.

Culture of Embryos in vitro

In total, 674 day-6 embryos were cultured *in vitro* in five experiments. The embryos required for each experiment were collected over 2–4 weeks, from 2–4 groups of donor ewes. On each day of collection for each experiment, embryos were allotted at random to the different treatment groups. The basal culture medium used in all experiments was a bicarbonate-buffered salt solution, pH 7·6, supplemented with potassium penicillin (62·5 mg l^{-1}) and streptomycin sulfate (50 mg l^{-1}) (Moore 1982). The sterile preparation of glassware and other culture apparatus was based on the methods described by Moore (1970) and Wittingham (1971). Analytical-grade salts which were known to be non-toxic to embryos were used for the preparation of flushing and culture media. A single batch of serum was used in all experiments. This was prepared by pooling sera obtained from whole blood collected from the jugular veins of several conscious mature ewes and rams. The serum was passed through an asbestos filter (Seitz — Grade 6, Ekwip Filtration Engineers, Milperra, N.S.W.) and stored at −20°C in 10 ml aliquots in plastic vials until required. Experimental culture media were supplemented with serum or various components of serum.

Flushing and culture media were prepared within 48 h of use and sterilized by passage through acetate filters with a 0·45 μm pore size (Millipore Pty Ltd, Lane Cove, N.S.W.). Culture media were equilibrated with a gas phase of 5% CO_2 in air. Embryos were cultured for 48 h at 37°C in sterile disposable polystyrene tubes (12 × 75 mm, Falcon Plastics, Bacto Laboratories Pty Ltd, Liverpool, N.S.W.) containing 1·0 ml of medium under a layer (0·3 ml) of lightweight paraffin oil (weight per ml at 20°C approximately 0·850 g, BDH Chemicals Aust. Pty Ltd, Victoria). During the period of incubation, the tubes were placed upright in a water-jacketed test-tube rack sealed within an anaerobic jar and gassed twice daily with 5% CO_2 in air.

Experiment 1

The aim of this experiment was to determine the relationship between concentration of serum and rate of embryo development. Embryos were allotted to incubation tubes containing bicarbonate buffer supplemented with serum at concentrations of 0, 1·25, 2·5, 5·0, 10·0 and 20·0% (v/v) (Table 1).

Experiment 2

The aims of this experiment were to determine (a) whether high and/or low molecular weight substances in serum were necessary for embryo development and (b) whether freezing and thawing of serum components reduced their biological activity in the culture system. Aliquots of serum were fractionated into two components containing substances above and below a molecular weight of approximately 10 Kd (kilodaltons), using an Amicon Model 8050 Ultrafiltration Cell and Diaflo Ultrafiltration Membrane type YM10 (Amicon Corporation, Scientific Systems Division, Danvers, Maryland, U.S.A.). The volumes of both components were adjusted back to the starting volume of serum with basal culture medium, so that the concentrations of substances in each component should have been essentially the same as their corresponding concentrations in the serum. Aliquots of the serum components were stored at −20°C and 5°C for two days. Whole serum or components of serum were incorporated into the culture medium at a concentration of 2·5% in all treatment groups (Table 2).

Experiment 3

The aim of this experiment was to determine the approximate molecular weight of those proteins in serum which support full embryo development. Aliquots of serum were fractionated as shown in Fig. 1. Three well separated serum protein fractions were 'cut' from the eluant from the column. Protein fractions 1, 2 and 3 contained proteins with mean molecular weights of approximately 500, 150 and 65 Kd, respectively. The reference proteins used to calibrate the column (Fig. 1) were purchased from Sigma, St. Louis, Missouri, U.S.A. The volume of each protein fraction was adjusted with basal culture medium to the starting volume of serum applied to the column, so that each fraction contained similar concentrations of those proteins present in the fraction as were present in whole serum. Protein concentrations were determined by the method of Hartree (1972). Embryos were cultured in one of nine treatment media (Table 3).

Experiment 4

The aims of this experiment were to determine (a) whether protein fractions 1 and/or 2 could interfere with the ability of protein fraction 3 to support embryo development and (b) whether the presence of the serum component of <10 Kd mol. wt increased the rate of embryo development in a medium containing fraction 3. Embryos were cultured in one of six treatment media (Table 4).

Experiment 5

The aim of this experiment was to determine whether additional fraction 2 protein could inhibit embryo development in the presence of whole serum. Embryos were cultured in one of four treatment media (Table 5).

Assessment of Embryo Development after Culture in Vitro

Embryos were scored promptly after the end of the incubation period for stage of development and probable viability. All embryos were examined and scored under bright field illumination (×40 magnification) and without knowledge of the treatment received. The criteria for scoring were as follows:

Full development and viable: full or near full expansion of the blastocyst, with marked thinning of the zona pellucida, translucent trophoblast cells and a clearly visible inner cell mass. The zona pellucida may remain intact, or embryos may be hatching or hatched. In the latter cases the embryo has sometimes collapsed.

Partial development and questionable viability: limited expansion (<50%) of the blastocyst and a little thinning of the zona pellucida. Cells are often darkened and degenerating.

No development and non-viable: degenerating morulae or early blastocysts with no expansion or thinning of the zona pellucida and darkened and poorly defined cells.

The actual viability of embryos in each of these categories at the end of the culture period was not determined by transfer to suitable recipent ewes.

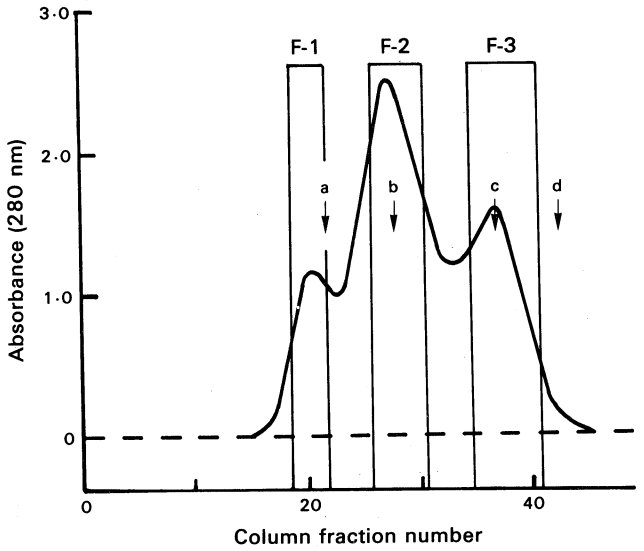

Fig. 1. Preparation of protein fractions 1, 2 and 3 from ovine serum by gel filtration. Serum was fractionated by molecular sieving on a 90 cm × 17 mm column of Sephadex G-200 gel. The column fractions collected to form protein fractions 1, 2 and 3 (F-1, F-2 and F-3) are indicated. The elution positions and molecular weights of four marker proteins were as follows: *a*, apoferritin, 443 Kd; *b*, γ-globulins, bovine, 158 Kd; *c*, serum albumin, bovine, 67 Kd; *d*, chicken egg albumin, 45 Kd.

Statistical Procedures

The proportions of embryos showing full development after different treatments were compared, using the χ^2 test (Steel and Torrie 1980). No distinction was made between embryos showing partial development and those showing no development.

Results

Experiment 1

A majority of embryos developed into fully expanded blastocysts in bicarbonate-buffered medium supplemented with 2·5–20·0% serum, whereas none developed when serum was omitted. The concentration of serum at which one-half of embryos showed full development was approximately 2·5% (Table 1). Examples of embryos that underwent no, partial and full development during culture are shown in Fig. 2. The difference in the proportions of embryos

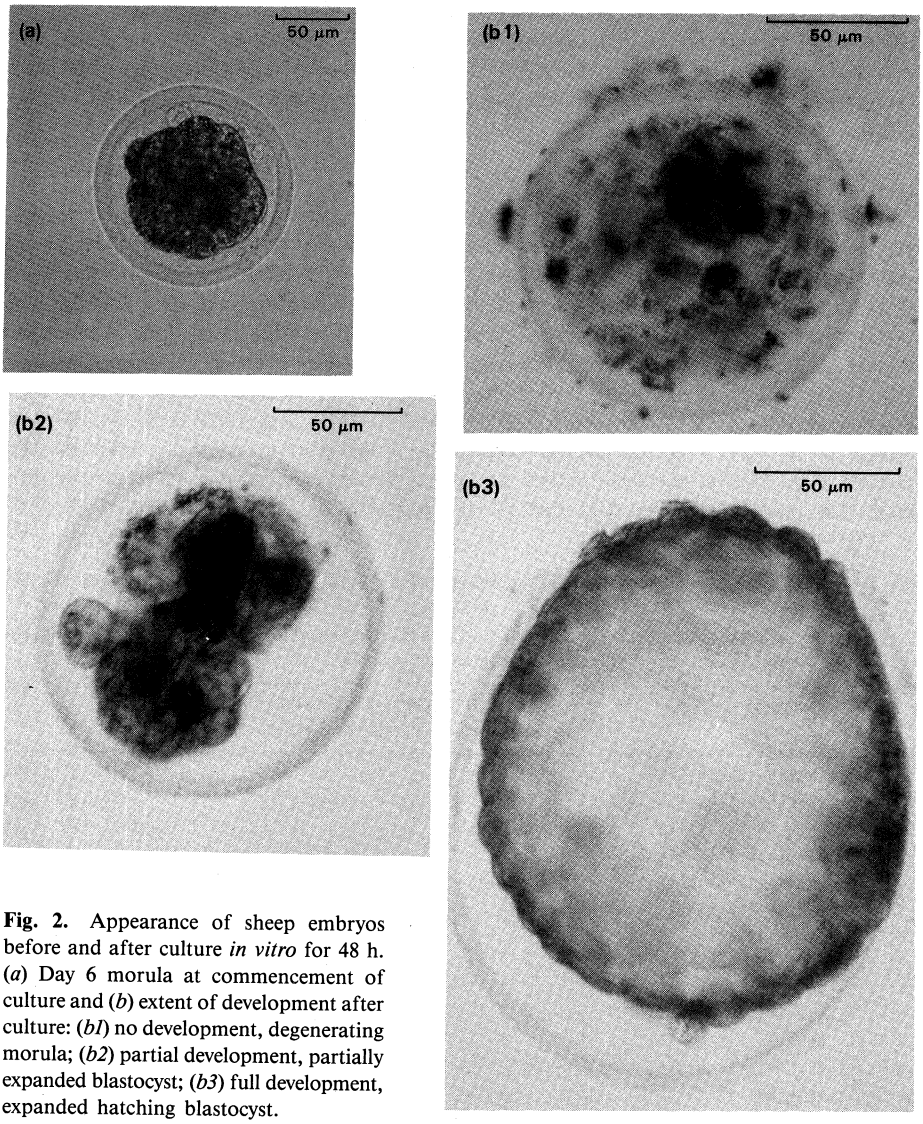

Fig. 2. Appearance of sheep embryos before and after culture *in vitro* for 48 h. (*a*) Day 6 morula at commencement of culture and (*b*) extent of development after culture: (*b1*) no development, degenerating morula; (*b2*) partial development, partially expanded blastocyst; (*b3*) full development, expanded hatching blastocyst.

Table 1. Development of embryos in a medium supplemented with different concentrations of ovine serum

Group	Serum concentration (%)	Number of embryos showing			Total
		full development	partial development	no development	
1	0	0	0	18	18
2	1·25	10	9	11	30
3	2·50	21	7	11	39
4	5·0	20	5	8	33
5	10·0	18	5	7	30
6	20·0	8	1	1	10

developing fully in Groups 2 (10/30) and 4 (20/33) was significant (χ^2 = 4·7, 1 d.f., 0·01 < P < 0·05), but the differences between Groups 2 and 3 and between Groups 3 and 4 were not significant. There was no difference in the proportions of embryos developing fully in Groups 4 (20/33) and 5 (18/30), but the proportion appeared to be highest in Group 6 (8/10), (Group 6 versus Group 4, χ^2 = 1·3, 1 d.f., P > 0·05).

Experiment 2

As in Experiment 1, media supplemented with whole serum or with serum reconstituted from the >10 Kd and <10 Kd components at a concentration of 2·5% allowed full development in aproximately one half of embryos (Groups 1, 4 and 7, Table 2). Some embryos showed full development in medium supplemented with the >10 Kd component only (26% and 35% in Groups 2 and 5, respectively), but no embryos developed fully in medium supplemented with the <10 Kd component only. However, the data suggest that the inclusion of the <10 Kd component enhanced the ability of the >10 Kd component of serum to support full development (Groups 2 and 5 versus Groups 4 and 7, 10/33 versus 17/33 χ^2 = 3·07, 1 d.f., P <0·1 but >0·05). Freezing and thawing of the >10 Kd component did not diminish its ability to support embryo development. In subsequent experiments, serum components and protein fractions were routinely stored at −20°C.

Table 2. Development of embryos in media supplemented with components of ovine serum stored at 5°C or −20°C

Ovine serum was fractionated into >10 Kd and <10 Kd components by ultra-filtration. Whole serum or components of serum were incorporated in the media at 2·5% concentration (v/v)

Group	Component	Number of embryos showing			Total
		full development	partial development	no development	
1	Whole serum	12	4	4	20
2	MW >10 Kd, stored at 5°C	5	7	7	19
3	MW <10 Kd, stored at 5°C	0	2	8	10
4	MW > & <10 Kd, stored at 5°C	9	2	7	18
5	MW >10 Kd, stored at -20°C	5	0	9	14
6	MW <10 Kd, stored at -20°C	0	0	10	10
7	MW > & <10 Kd, stored at -20°C	8	1	6	15

Experiment 3

High proportions of embryos showed full development in media containing 5% reconstituted serum (Group 1, 12/15) or media containing 5% of the <10 Kd component and protein fraction 3 from serum at concentrations of 5·10, 0·90 and 0·45 mg ml^{-1} (Groups 4, 7 and 8; 9/10, 12/14 and 8/12, respectively) (Table 3). The concentration of fraction 3 protein in Group 8 (0·45 mg ml^{-1}) was approximately the same as the concentration of fraction 3 protein in the media in Group 3, Experiment 1 and Groups 1, 4 and 7, Experiment 2, which contained 2·5% whole or reconstituted serum. All these five treatment groups in Experiments 1–3 supported full development in a similar proportion of embryos (50–66%), suggesting that fraction 3 proteins are of primary importance in this *in vitro* culture system. Neither fraction 1 nor fraction 2 proteins could support full embryo development, even when present in media at a concentration (5·10 mg ml^{-1}) substantially in excess of their concentrations in a medium containing 5% serum.

Table 3. Development of embryos in media supplemented with different protein fractions from ovine serum
See Fig. 1 for definition of protein fractions 1, 2 and 3. In Group 1, the >10 Kd and <10 Kd mol. wt components of serum were remixed to provide a medium containing the equivalent of 5% serum. The media for Groups 2–9 were supplemented with the <10 Kd component of serum at the same concentration as in Group 1. The amounts of each protein fraction in the media for Groups 2–4 were adjusted to give the same total protein concentration (5·10 mg ml^{-1}) as was present in Group 1. The amounts of each protein fraction in the media for Groups 5–7 were adjusted to give similar concentrations of those proteins present in each fraction as were present in Group 1 (i.e. these media were approximately 5%, in respect of the concentration of the proteins present in each fraction)

Group	Protein fraction	Concentration of protein in the medium (mg ml^{-1})	Number of embryos showing full development	partial development	no development	Total
1	5% reconstituted serum	5·10	12	0	3	15
2	1	5·10	0	0	10	10
3	2	5·10	0	1	11	12
4	3	5·10	9	0	1	10
5	1 (5%)	0·24	0	1	12	13
6	2 (5%)	1·15	0	4	8	12
7	3 (5%)	0·90	12	1	1	14
8	3 (2·5%)	0·45	8	1	3	12
9	3 (1·25%)	0·23	0	0	12	12

Experiment 4

As in Experiment 3, approximately 60% of embryos cultured in a medium containing 2·5% of fraction 3 and 5% of the <10 Kd component of serum showed full development (Group 2, Table 4). When the <10 Kd component was omitted from this medium, the proportion of embryos showing full development decreased (Group 3 versus Group 2, 10/36 versus 23/37, $\chi^2 = 8{\cdot}7$, 1 d.f., $P < 0{\cdot}01$).

Table 4. Development of embryos in media supplemented with different protein fractions from ovine serum; interactions between fractions
See Fig. 1 for definition of protein fractions 1, 2 and 3. In Group 1, the >10 Kd and <10 Kd mol. wt components of serum were remixed to provide a medium containing the equivalent of 5% serum. The media for Groups 2 and 4–6 were supplemented with the <10 Kd component of serum at the same concentration as in Group 1

Group	Protein fraction (concentration)	Concentration of protein in the medium (mg ml^{-1})	Number of embryos showing full development	partial development	no development	Total
1	5% reconstituted serum	5·10	22	3	11	36
2	3 (2·5%)	0·45	23	6	8	37
3	3 (2·5%)[A]	0·45	10	7	19	36
4	1 (10%) plus 3 (2·5%)	0·93	18	6	14	38
5	2 (10%) plus 3 (2·5%)	2·75	11	9	17	37
6	1 (10%) plus 2 (10%) plus 3 (2·5%)	3·25	12	4	21	37

[A]Not supplemented with the <10 Kd component of serum.

The addition of 10% of fraction 1 caused a small but insignificant fall in the proportion of embryos showing full development (18/38 in Group 4 versus 23/37 in Group 2, $\chi^2 = 1{\cdot}7$), whereas the addition of 10% of fraction 2 reduced the proportion markedly (11/37 in Group 5 versus 23/37 in Group 2, $\chi^2 = 8{\cdot}3$, 1 d.f., $P < 0{\cdot}01$). There was no interaction between the effects of fractions 1 and 2 (35/74 in Groups 2 plus 6 versus 29/75 in Groups 4 plus 5, $\chi^2 = 1{\cdot}1$).

Experiment 5

About 68% and 47% of embryos developed fully in media containing 2·5% and 1·25% serum, respectively (13/19 in Group 1 and 9/19 in Group 3, Table 5). Unlike the effect of

Table 5. Development of embryos in media supplemented with whole serum and protein fraction 2 from serum
See Figure 1 for definition of protein fraction 2

Group	Composition of medium	Concentration of protein in the medium (mg ml^{-1})	Number of embryos showing full development	partial development	no development	Total
1	2·5% whole serum	2·55	13	1	5	19
2	2·5% whole serum *plus* 10% fraction 2	4·85	12	2	5	19
3	1·25% whole serum	1·28	9	2	8	19
4	1·25% whole serum *plus* 10% fraction 2	3·58	8	4	8	20

addition to a medium containing 2·5% of fraction 3 (Experiment 4), the addition of 10% of fraction 2 to media containing 2·5% and 1·25% whole serum did not significantly decrease the proportion of embryos showing full development (20/39 in Groups 2 plus 4 versus 22/38 in Groups 1 plus 3, $\chi^2 = 0{\cdot}3$).

Discussion

Morulae were cultured *in vitro* for 48 h in this study. The sheep embryo increases from approximately 32–64 blastomeres at the morula stage on day 6 to about 128–256 blastomeres at the expanded blastocyst stage (Green and Winters 1945; Harper 1982). The specific cultural requirements for sheep embryos during this stage of development remain poorly defined. In the simple bicarbonate-buffered medium used in these experiments, the added serum may have one or more of several possible functions, including; (a) providing energy sources, such as glucose, pyruvate, lactate, α-ketoglutarate and fatty acids (Tervit *et al.* 1972; Boone *et al.* 1978); (b) providing amino acids or other 'fixed' nitrogen sources (Kane and Foote 1970*a*, 1970*b*); (c) providing vitamins, minerals, trace elements and growth factors; (d) serving as a source of macromolecules, whose physicochemical properties prevent embryos from adhering to each other and to glass surfaces, stabilize cell membranes and minimize leakage of endogenous amino acids into the medium (Brinster 1965, 1971; Cholewa and Whitten 1970); (e) chelating toxic metal ions such as copper and zinc which may contaminate the medium (Cholewa and Whitten 1970); and (f) providing specific embryotropic factors which stimulate cell division (Kane 1985).

Although sera are widely used to supplement basal culture media, there have been few determinations of the dose-response relationship between concentration of serum in the basal medium and rate of embryo development. As little as 0·5% ovine serum is able to support

the development of some sheep morulae into expanded and hatched blastocysts in a bicarbonate-buffered medium (Moore 1982). Approximately 80% of sheep morulae develop into expanded blastocysts in a bicarbonate-buffered synthetic oviductal fluid supplemented with 10% ovine serum (Quinn *et al.* 1984), whereas not more than 60% of sheep morulae develop into expanded blastocysts in Dulbecco's phosphate-buffered medium supplemented with 20% of foetal calf and some other types of sera (Bunch *et al.* 1984). In Experiment 1 about 50% of morulae developed into expanded blastocysts in the presence of only 2·5% serum and the results suggest that there may be little advantage in supplementing basal media with more than 5% of this batch of serum.

The substances in serum which were necessary for the full development of morulae in this study are found in the >10 Kd component of serum. Either proteins or small molecules bound to proteins are required, and these substances are not inactivated by storage at −20°C. The >10 Kd component presumably provides energy sources, but may also have some of the other functions listed above. Within the >10 Kd component of serum the active proteins or protein-bound substances are clearly located in fraction 3, which comprises a family of proteins with a mean molecular weight of about 65 Kd. In further studies (unpublished data) the composition of fraction 3 was examined by analytical polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. The major protein in fraction 3 is albumin. However, smaller amounts of at least three other proteins of higher molecular weight are present. Attempts to separate albumin from the other proteins in fraction 3 have been unsuccessful, and hence we have so far been unable to culture embryos in a medium containing albumin alone or the proteins other than albumin in fraction 3.

Commercial serum albumin preparations are also widely used in embryo culture media. These preparations are heterogeneous and ill-defined. Preparations of bovine serum albumin (BSA) contain several other proteins in addition to albumin. Further, significant quantities of fatty acids, steroids and other substances of low molecular weight are bound to the albumin in BSA preparations (Kane and Headon 1980; Kane 1985). The amount of bound fatty acid varies substantially between different batches of BSA (Chen 1966) (and presumably also between different batches of ovine serum albumin and fraction 3 from ovine serum), and in the absence of an added energy source the albumin-bound fatty acid promotes the growth of one-cell rabbit embryos into blastocysts (Kane and Headon 1980). BSA also contains a protein-bound embryotropic growth factor of low molecular weight which stimulates cell division in rabbit morulae. This unidentified factor is distinct from known vitamins and growth factors (Kane 1985). Hence it is unclear whether the activity of fraction 3 in the present culture system is attributable to the albumin itself, substances bound to albumin, or other proteins.

The <10 Kd component of ovine serum was not essential for the full development of morulae to expanded blastocysts in this study, but it did appear to enhance the rate of development of morulae into expanded blastocysts in the presence of the >10 Kd component (Experiment 2) and significantly increased the rate of development in the presence of fraction 3 of ovine serum (Experiment 4). The <10 Kd component presumably contains amino acids, energy substrates, vitamins, minerals, trace elements, growth factors and other substances and the identity of the supportive substance(s) in the present culture system is unknown. The low molecular weight component of foetal calf serum similarly supports the development of porcine embryos from the premorula stage to expanded blastocysts in a modified Krebs-Ringer bicarbonate medium containing dialysed foetal calf serum and pyruvate, lactate and glucose (Meyen and Davis 1983).

The predominant proteins in fractions 1 and 2 of ovine serum are macroglobulins and γ-globulins, respectively, but several other species of protein are present in each of these fractions (Blunt 1975). Neither fraction stimulated blastocyst expansion in this study and the fraction 1 proteins could not influence embryo development in the presence of fraction 3. However, the apparently conflicting results for the effects of fraction 2 proteins on the development of morulae in the presence of fraction 3 and the <10 Kd component of serum (Experiment 4) and whole serum (Experiment 5) are puzzling. Fraction 2 (10%) inhibited blastocyst expansion

in the presence of 2·5% fraction 3 and 5% of the <10 Kd component of serum but not in the presence of 2·5% or 1·25% whole serum. Clearly the presence of 10% of fraction 2 in the medium *per se* is not harmful, since 60% of embryos developed into expanded blastocysts in a medium supplemented with 10% serum (Experiment 1). Presumably the differing results in these experiments are related to both the absolute and relative amounts of albumin, other proteins and low molecular weight serum components present. Why fraction 2 proteins can inhibit the development of morulae to expanded blastocysts in some situations is unknown. Since both the absolute and relative amounts of different proteins and substances of low molecular weight may vary widely in different samples of ovine serum (Blunt 1975), the present results suggest reasons why different sera may vary in their ability to support embryo development. It must be acknowledged that, since a single batch of ovine serum was used throughout the present study, it is not altogether clear that the same results would have been obtained with different batches of serum.

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References

Bates, R. D., Kontio, J. A., and Dukelow, W. R. (1985). Chemically defined and serum supplemented media effects on mouse embryo development. *Theriogenology* **23**, 697–700.

Blunt, M. H. (1975). 'The Blood of Sheep — Composition and Function.' (Springer-Verlag: Berlin.)

Boone, W. R., Dickey, J. F., Luszcz, L. J., Dantzler, J. R., and Hill, J. R. (1978). Culture of ovine and bovine ova. *J. Anim. Sci.* **47**, 908–13.

Brinster, R. L. (1965). Studies on the development of mouse embryos *in vitro*. III. The effect of fixed nitrogen source. *J. Exp. Zool.* **158**, 69–78.

Brinster, R. L. (1971). *In vitro* culture of the embryo. In 'Pathways to Conception'. (Ed. A Sherman.) pp. 245–7. (Thomas and Springfield: Illinois.)

Bunch, T. D., Foote, W. C., Call, J. W., and Clarke, W. F. (1984). *In vitro* culture and maintenance of ovine embryos transported in Dulbecco's enriched phosphate-buffered saline. *Theriogenology* **21**, 981–7.

Caro, C. M., and Trounson, A. (1984). The effect of protein on preimplantation mouse embryo development *in vitro*. *J. in vitro Fertilis. Embryo Transf.* **1**, 183–7.

Chen, R. F. (1966). Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**, 173–81.

Cholewa, J. A., and Whitten, W. K. (1970). Development of 2-cell mouse embryos in the absence of a fixed nitrogen source. *J. Reprod. Fert.* **22**, 553–5.

Dulbecco, R., and Vogt, M. (1954). Plague formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**, 167–82.

Green, W. W., and Winters, L. M. (1945). Prenatal development of the sheep. *Minn. Tech. Bull.* **169**, 3–35.

Harper, M. J. K. (1982). Sperm and egg transport. In 'Germ Cells and Fertilization'. (Eds C. R. Austin and R. V. Short.) pp. 102–27. (Cambridge Univ. Press: Cambridge.)

Hartree, E. F. (1972). Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48**, 422–7.

Kane, M. T. (1985). A low molecular weight extract of bovine serum albumin stimulates rabbit blastocyst cell division and expansion *in vitro*. *J. Reprod. Fert.* **73**, 147–50.

Kane, M. T., and Foote, R. H. (1970*a*). Culture of two- and four-cell rabbit embryos to the blastocyst stage in serum and serum extracts. *Biol. Reprod.* **2**, 245–50.

Kane, M. T., and Foote, R. H. (1970*b*). Fractionated serum dialysate and synthetic media for culturing two- and four-cell rabbit embryos. *Biol. Reprod.* **2**, 356–62.

Kane, M. T., and Headon, D. R. (1980). The role of commercial bovine serum albumin preparations in the culture of one-cell rabbit embryos to blastocysts. *J. Reprod. Fert.* **60**, 469–75.

Killeen, I. D., and Caffery, G. J. (1982). Uterine insemination of ewes with the aid of a laparoscope. *Aust. Vet. J.* **59**, 95.

Ladd, P. C., Plair, A. C., Braidlow, B. K., and Snyder, D. A. (1982). Development of early mouse and sheep embryos in serum-supplemented, phosphate-buffered saline. *Theriogenology* **17**, 94 (abst).

McLaren, A. (1982). The embryo. In 'Embryonic and Fetal Development'. (Eds C. R. Austin and R. V. Short.) pp. 1–25. (Cambridge Univ. Press: Cambridge.)

Meyen, B. A., and Davis, D. L. (1983). Effects of medium and serum on swine embryos. *J. Anim. Sci.* **57** (Suppl.1), 359 (abst).

Miller, B. G., Moore, N. W., Murphy, L., and Stone, G. M. (1977). Early pregnancy in the ewe: effects of oestradiol and progesterone on uterine metabolism and on embryo survival. *Aust. J. Biol. Sci.* **30**, 279–88.

Moore, N.W. (1970). Preliminary studies on *in vitro* culture of fertilized sheep ova. *Aust. J. Biol. Sci.* **23**, 721–4.

Moore, N.W. (1982). Liquid storage and culture of embryos of farm animals. In 'Embryo Transfer in Cattle, Sheep and Goats'. (Eds J. N. Shelton, A. O. Trounson, N. W. Moore and J. W. James.) pp. 51–3. (Aust. Soc. Reprod. Biol., Union Offset Comp: Fyshwich, A.C.T.)

Quinn, P., Warnes, G. M., Walker, S. K., and Seamark, R. F. (1984). Culture of preimplantation sheep and goat embryos. In 'Reproduction in Sheep', (Eds. D. R. Lindsay and D.T. Pearce), pp.289–90, (Australian Academy of Science: Canberra).

Roberts, G. P., Parker, J. M., and Symonds, H. W. (1976). Macromolecular components of genital tract fluids from the sheep. *J. Reprod. Fert.* **48**, 99–107.

Ryan, J. P., Bilton, R. J., Hunton, J. R., and Maxwell, W. M. C. (1984). Superovulation of ewes with a combination of PMSG and FSH-P. In 'Reproduction in Sheep', (Eds. D. R. Lindsay and D. T. Pearce), pp. 338–41, (Australian Academy of Science: Canberra).

Shirley, B., Wortham, J. W., Witmyer, J., Condon-Mahony, M., and Fort, G. (1985). Effects of human serum and plasma on development of mouse embryos in culture media. *Fert. Steril.* **43**, 129–34.

Steel, R. G. D., and Torrie, J. H. (1980). 'Principles and Procedures of Statistics', (McGraw Hill: New York).

Taylor, W. G. (1974). Feeding the baby-serum and other supplements to chemically defined medium. *J. Nat. Cancer Inst.* **53**, 1449–57.

Tervit, H. R., Whittingham, D. G., and Rowson, L. E. A. (1972). Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fert.* **30**, 493–7.

Tervit, H. R., and Goold, P. G. (1978). The culture of sheep embryos in either a bicarbonate-buffered medium or a phosphate-buffered medium enriched with serum. *Theriogenology* **9**, 251–7.

Whittingham, D. G. (1971). Culture of mouse ova. *J. Reprod. Fert.,* Suppl. **14**, 7–21.

Wright, R. W., and Bondioli, K. R. (1981). Aspects of *in vitro* fertilisation and embryo culture in domestic animals. *J. Anim. Sci.* **53**, 702–29.

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