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MONASH UNIVERSITY
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REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 7 December 2001 .....
.....
Sec. Research Graduate School Committee

Alterations

- **Page 6, Line 5 should read:**
  In most mammalian species, spermatozoa release hyaluronidase upon contact with the COC, to facilitate digestions of the hyaluronic acid matrix binding the cumulus cells (Lin *et al.*, 1994). In the cow, however, the oocyte is denuded rapidly in vivo, thus the sperm are not likely to encounter a cumulus mass (Lorton and First, 1979).
- **Page 8, Line 17 should read:**
  The cow embryo has a Na^+-K^+ ATPase pump on both the basolateral and apical membranes of the TE cells, generating ion gradients, which result in the movement of fluid into the embryo to form a blastocoel (Watson et al., 1999). Interestingly, the mouse blastocyst has only a basolateral Na^+-K^+ ATPase pump (Benos and Biggers, 1981).
- **Page 12, Line 19 should read:**
  "....and significantly increased the proportion of oocytes reaching MII..."
- **Page 28, Table 1.2:** Grippo et al. (1992) reported the concentration of ions in oviduct fluid of the cow to be as follows: Na^+ (ampulla 140.9 mM, isthmus 159.3 mM); K^+ (ampulla 4.53 mM, isthmus 4.24 mM); Ca^{2+} (ampulla 1.83 mM, isthmus 2.46 mM); Mg^{2+} (ampulla 0.662 mM, isthmus 0.685 mM). The concentrations of Na^+ reported by Grippo et al. (1992) were slightly lower than that reported by Olds and VanDemark (1957) (see Table 1.2), while the concentration of K^+ was almost twelve fold lower than that reported by Olds and VanDemark (1957). These discrepancies may be due to different techniques used to measure ionic concentrations. Olds and VanDemark (1957) collected oviduct fluid for assaying by passing whole oviducts through a clothes wringer, while Grippo et al. (1992) collected oviduct fluid daily from indwelling catheters.
- **Page 38 and in general throughout the whole thesis:**
  Unless otherwise indicated, lactate refers to D.L-lactate.
- **Page 60, Line 3:**
  "Thompson *et al.*, 1992a" should be included in the brackets as a reference for the use of amino acids for culture of sheep embryos.
- **Page 67, Line 10 should read:**
  There is recent evidence, however, to show that the antiporter is in fact present in mouse, human, hamster and cow cleavage stage embryos (Gibb *et al.*, 1997; Lane *et al.*, 1998.....).
- **Page 93, Line 5:**
  Add "Brison and Leese, 1994" to references in brackets.
- **Page 95, Line 8 and page 392:**
  Date of publication of Crabtree reference should read "1929" and not "1998".
- **Page 132, Figure 2.7:**
  Reviewer commented that the Hoechst stained cells appear smudged and blurred and thus suggests that calculations for the inner cell mass cell numbers (blue) can only be estimates. The images in the photographs unfortunately do not reflect the same clarity that is viewed under the microscope for two reasons. Firstly, the blue stained cell nuclei are overexposed which creates a blurred image. Secondly, the photographed image is only of one focal plane. The individual cell nuclei (both TE and ICM) are much clearer when one is able to view several different focal planes. For each embryo, both TE and ICM cells were counted three times to verify the cell numbers. The technique used in the present study is a published technique for determining TE and ICM cell numbers (Hardy *et al.* 1989a).

- **Page 137:**
  There is a typographical error in the formula listed. "(mole of substrate present)" in the numerator should read "(mole of labelled substrate present)". The formula is equivalent to that stated by the reviewer, however, the formula used in the present study is an uncondensed version of the reviewer's formula. All calculations are identical using the condensed and uncondensed versions.
- **Page 167, Line 17:**
  After line 17 add: In addition, pyruvate metabolism is affected by the concentration of pyruvate in the medium (Thompson *et al.*, 1993). In the study by Thompson et al. (1996), pyruvate uptake was measured with pyruvate at 1 mM, which may have affected the rate of pyruvate uptake (Eckert et al., 1998).
- **Page 342, Line 8:**
  Following the sentence, "Other factors could also....." in line 9 add: The concentration of pyruvate (1mM) used in the study by Thompson *et al.* (1996) was approximately three times the concentration of pyruvate used in the present study and could have affected the rate of pyruvate uptake (Thompson *et al.*, 1993; Eckert *et al.*, 1998).
- **Page 168, Line 6:**
  O2 and CO2 should read O_2 and CO_2, respectively.
- **Page 169, Line 10 and Page 344, Lines 1-3:**
  Add: "Butcher et al. (1998) suggested that the embryo may convert pyruvate to alanine, thus having a role in the removal of ammonium. Interestingly, the concentration of alanine does increase in the medium during culture of the cow embryo (Partridge and Leese, 1996).
- **Page 182, Figure 4.1, add to legend:**
  (a,d) pyruvate, (b,e) glutamine and (c,f) glucose.
- **Page 185, Line 12:**
  ".....(46.7% versus 15.2%, respectively, $P < 0.05$).
- **Page 192, Lines 8-9 should read:**
  Rieger and Loskutoff (1994), however, found no detectable oxidation of glucose .....".
- **Page 267, Line 13:**
  "netnil" should read "metnil".
- **Page 339, Line 16:**
  "cow embryos" should read "sheep embryos".
- **Page 342, last paragraph (and elsewhere as well):**
  The term "pyruvate oxidation" as used in the present study refers to the entry of pyruvate into the TCA-cycle.

Additions to reference list:

- Lorton SP and First NL (1979): Hyaluronidase does not disperse the cumulus oophores surrounding bovine ova. *Biol Reprod* 21:301-308.
- Brison DR and Leese HJ (1994): Blastocoel cavity formation by pre-implantation rat embryos in the presence of cyanide and other inhibitors of oxidative phosphorylation. *J Reprod Fertil* 101:305-309.
- Gibb CA, Poronnik P, Day ML and Cook DI (1997): Control of cytosolic pH in two-cell mouse embryos: roles of H^+-lactate cotransport and Na^+/H^+ exchange. *Am J Physiol* 273:C404-C419.
- Grippo AA, Henault MA, Anderson SH and Killian GJ (1992): Cation concentrations in fluids from the oviduct ampulla and isthmus of cows during the estrous cycle. *J Dairy Sci* 75:58-65.
- Watson AJ, Westhusin ME, De Sousa PA, Betts DH and Barcroft LC (1999): Gene expression regulating blastocyst formation. *Theriogenology* 51:117-133.

THE IN VITRO PRODUCED COW EMBRYO: FACTORS AFFECTING DEVELOPMENT AND METABOLISM

by

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DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

Tracey Steeves

ACKNOWLEDGMENTS

Thank you to:

1) My supervisors Dr David Gardner and Professor Alan Trounson. Thanks to Alan for your support, direction and advice and for taking me on as a student half-way through my studies. Thank you to David for your endless encouragement, guidance, assistance and time. Your enthusiasm for your work has been an incredible inspiration. Thank you for opening up the wonderful world of embryology to me.
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5) The staff of the Victorian Institute of Animal Science, Dr Richard Fry, Dr Kurt Zuelke and Terry Squires. Thank you for providing me with the opportunity to obtain research material from pre-pubertal cows.
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SUMMARY

The culture of mammalian embryos in vitro has been associated with retarded development, species-specific blocks to development, morphological abnormalities, metabolic perturbations and a reduction in embryo viability. The suitability of various culture systems for the culture of cow embryos has mostly been assessed with respect to proportions of embryos developing to the blastocyst stage over several days, in a one-step culture system. The aim of the present study was to improve our understanding of the requirements of the cow oocyte and embryo during culture, by assessing the relationship of media components to factors such as maturation, development, cleavage, differentiation, metabolism and pHi, thereby leading to better culture conditions and the maintenance of embryo viability.

Amino acids were found to have a temporal and differential effect on embryo development, cell number and metabolism. The embryo had a requirement for Eagle's non-essential amino acids and glutamine for culture to the 8– to 16-cell stage and showed a further requirement for essential amino acids for development to the blastocyst stage. This resulted in an improved development, total cell number and proportion of cells in the ICM. A reduced concentration of essential amino acids was positively correlated with development. Culture with the non-essential amino acids and glutamine reduced the glycolytic activity and pHi of 8- to 16-cell embryos. Both glycolytic activity and pHi were negatively correlated with development. The present study indicated that, when amino acids were present in the culture medium, the cow embryo utilized amino acids in preference to pyruvate.

MEM vitamins retarded development of cow embryos during the first 72 h of culture. When present for development from the 8- to 16-cell stage, MEM vitamins

increased blastocoel expansion and had a significant effect on blastocyst metabolism. While glycolytic activity was unaffected, glutamine oxidation was significantly higher and pyruvate oxidation was significantly lower in expanded blastocysts, following culture with MEM vitamins. Riboflavin, myo-inositol, pantothenate and a combination of folic acid, nicotinamide and riboflavin all significantly increased glutamine oxidation in expanded blastocysts without affecting pyruvate oxidation. Pantothenate was the only vitamin found to improve blastocyst development.

Linear rates of pyruvate and glucose uptakes by embryos were determined from the zygote to the blastocyst, under conditions similar to those in which embryos were cultured. Both glucose and pyruvate uptakes were low during the early cleavage stages, increasing significantly with blastocyst development. Determinations of the metabolism of oocytes revealed that both glucose and glutamine metabolism increased during oocyte maturation.

Differences were found in the uptake and metabolism of nutrients by embryos and oocytes from pre-pubertal and adult cows during culture. A delay in the increase in glycolytic activity during maturation, a delay in reaching the GVBD stage, a smaller oocyte size and a different pattern of pyruvate uptake by early cleavage stage pre-pubertal embryos, may be factors accounting for poor developmental capacity. With respect to total cell number and the uptake of glucose and pyruvate, the viability of blastocysts developed from pre-pubertal oocytes did not appear to be compromised.

The findings of the present study have made a significant contribution to understanding the physiological and metabolic requirements of the cow embryo during culture and to elucidating factors affecting developmental competence and viability.

PUBLICATIONS

Publications arising from this thesis:

Steeves TE and Gardner DK (1999): Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol Reprod* 61:731-740.

Steeves TE and Gardner DK, Zuelke KA, Squires TJ and Fry RC (1999): In vitro development and nutrient uptake by embryos derived from oocytes of pre-pubertal and adult cows. *Mol Reprod Dev* 54:49-56.

Steeves TE and Gardner DK (1999): Metabolism of glucose, pyruvate and glutamine during the maturation of oocytes from pre-pubertal and adult cows. *Mol Reprod Dev* 54:92-101.

Conference abstracts arising from this thesis:

Steeves TE and Gardner DK (1997): Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol Reprod Suppl* 1:89.

Steeves TE and Gardner DK (1998): Utilization of glucose, pyruvate and glutamine during maturation of oocytes from pre-pubertal calves and adult cows. *J Reprod Fertil Suppl* 54:518.

ABBREVIATIONS

aa	Amino acids
ANOVA	Analysis of variance
AR	Acrosome reaction
BOEC	Bovine oviduct epithelial cells
BME	Basal Medium Eagle
BRL	Bovine rat liver cells
BSA	Bovine serum albumin
COC	Cumulus oocyte complex
CSF	Colony stimulating factor
DMO	5,5-dimethyl-2,4-oxazolidinedione
DNP	Dinitrophenol
E	Epinephrine
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMP	Embden-Meyerhof pathway
FCS	Fetal calf serum
FGH	Fibroblast growth hormone
FSH	Follicle stimulating hormone
GAG	Glycosaminoglycan
GH	Growth hormone
GSH	Glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
H	Hypotaurine
H-199	HEPES-buffered TCM-199 medium
HA	Hyaluronic acid
HECM	Hamster embryo culture medium
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HTF	Human tubal fluid
ICM	Inner cell mass
IGF	Insulin-like growth factor
IVF	In vitro fertilization
IVM	In vitro maturation
KSOM	Potassium simplex optimized medium

LH	Leutinizing hormone
LIF	Leukemia inhibitory factor
MDH	Malate dehydrogenase
MEM	Minimum essential medium
MI	Metaphase I
MII	Metaphase II
MIC	Modular incubation chamber
mMTF	Modified mouse tubal fluid
P	Penicillamine
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PEP	phosphoenolpyruvate
PFK	Phosphofructokinase
pHi	Intracellular pH
pHo	Extracellular pH
Pi	Inorganic phosphate
PPP	Pentose phosphate pathway
PVA	Polyvinylalcohol
PVP	Polyvinylpyrrolidone
QC	Quality control
RO	Reverse osmosis
ROS	Reactive oxygen species
SNARF-1	Carboxy-seminaphthorhodafluor-1-acetoxy-methylester
SOD	Superoxide dismutase
SOF	Synthetic oviduct fluid
SOM	Simplex optimized medium
TALP	Tyrodes albumin lactate pyruvate
TCA	Tricarboxylic acid
TCM-199	Tissue culture medium 199
TE	Trophectoderm
TGF	Transforming growth factor
TVR	Transvaginal oocyte recovery

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

As early as 1890, man realized the potential benefits of assisted reproductive technologies (Heape, 1890). The ability to mature and fertilize an oocyte, nurture the embryo in vitro for several days, then transfer it to the uterus of a recipient animal and produce a live offspring, opens many doors and creates new challenges for scientific, medical and agricultural advancement.

In domestic livestock species the benefits of assisted reproductive technologies are numerous. Multiple ovulation and the subsequent transfer of embryos to recipients offer a means of producing more offspring from genetically elite cows and bulls in a shorter period of time. Offspring can also be produced from genetically elite animals following the removal of oocytes from the ovaries of the cow immediately post-mortem. The benefits of the production of both transgenic and cloned animals to agricultural practices have recently raised a great deal of interest. A transgenic animal is one in which desirable recombinant DNA molecules have been inserted into the animal's genome at the embryonic stage. The production of transgenic animals involves strategies to increase the protein content of milk in dairy cows, to enhance the quality of sheep's' wool, to improve resistance of livestock to disease and to enhance growth of beef cows. Cloning offers the potential to produce unlimited numbers of genetically identical animals from one livestock animal with genetically elite traits.

Large numbers of embryos are required to develop the specific technologies to the point of being commercially viable. While donor cows can be superovulated and mated for the non-surgical retrieval of embryos, embryo recovery rates are low and, as a result, the procedure is costly on a per embryo basis. The use of ovaries from cows

slaughtered at abattoirs, however, has provided a viable alternative for producing large numbers of embryos. Unlike the human, cow embryos will not survive in the uterus when transferred prior to compaction. Therefore, the production of live offspring from in vitro produced embryos requires the maintenance of embryos in vitro up to the morula and blastocyst stages. As a result, there has been a tremendous focus on the establishment of procedures for the in vitro maturation, fertilization and culture of embryos from a variety of livestock species.

Despite the successful production of live offspring from in vitro produced embryos, maturation, fertilizability and subsequent embryo development and viability are often poor in in vitro production systems compared with in vivo matured oocytes and in vivo produced embryos. A great deal of research during the past two decades has focused on the optimization of maturation, fertilization and culture systems for the production of large numbers of viable blastocysts. It is only through an improved understanding of the requirements of the oocyte as it matures and of the embryo as it develops from the zygote to the blastocyst stage that in vitro production systems for mammalian oocytes and embryos will be optimized.

1.2 DEVELOPMENT OF OOCYTES AND EMBRYOS IN VIVO

1.2.1 Maturation

Oocytes are formed in the fetal ovary from primordial germ cells which differentiate to form oogonia. The number of oogonia in the fetal ovary continues to increase through mitotic divisions, reaching a maximum of two to three million by 110 days of gestation in the cow (Erickson, 1966a). When oogonia lose the ability to

undergo mitosis they are termed primary oocytes. The final mitotic division is followed by a normal interphase during which the entire complement of DNA is replicated. The primary oocyte then enters the first of two meiotic cell divisions, meiotic prophase. This occurs at 75 - 80 days gestation in the cow (Erickson, 1966a). Meiotic prophase is characterized by five distinct cytological stages: leptotene, zygotene, pachytene, diplotene and diakenesis. At birth, the majority of germ cells are at diplotene and remain arrested at this stage until a few hours before ovulation when they progress to diakenesis. During arrest at meiotic prophase, the oocyte undergoes considerable growth (Eppig *et al.*, 1996) as well as synthesis of RNA and proteins (Schultz and Wassarman, 1977; Van Blerkom and McGaughey, 1978; Motlik and Fulka, 1986; Fair *et al.*, 1995). These activities have all been linked with the acquisition of meiotic competence and/or subsequent developmental competence (Blondin *et al.*, 1995; Fair *et al.*, 1995; Arlotto *et al.*, 1996). Some of the proteins and RNA synthesised during this period are required for development of the cleavage stage embryo prior to activation of the embryonic genome (Van Blerkom and McGaughey, 1978; Howe and Solter, 1979; Schultz, 1986). Proteins are also secreted to form the zona pellucida, a glycoprotein shell surrounding the oocyte (Eppig *et al.*, 1996). The zona pellucida consists of three major glycoproteins, ZP1, ZP2 and ZP3 (Greve and Wassarman, 1985). Oocytes are surrounded by several compact layers of differentiated granulosa cells known as cumulus cells. The oocyte and cumulus cells are referred to as the cumulus oocyte complex (COC). The innermost layers of cumulus cells, the corona radiata, form cytoplasmic projections that penetrate the zona pellucida and contact the vitelline membrane of the oocyte (Moor *et al.*, 1980).

The resumption of meiosis is triggered by the preovulatory surge of gonadotrophins (Channing *et al.*, 1978). Meiotic resumption is characterized by GVBD, when the nuclear envelope surrounding the nuclear material disappears. Meiosis then progresses through MI to MII. With progression to MII one set of haploid chromosomes is retained in the oocyte while the second set, termed the polar body, is extruded from the oocyte. The resumption of meiosis and progression to MII is termed meiotic maturation. During maturation of the oocyte the compact cumulus cells surrounding the oocyte secrete hyaluronic acid, forming a three dimensional matrix and enlarging the distance between the cells. This is termed cumulus cell expansion [illegible] associated with the disconnection of cell-cell communication between th[illegible] cells and the oocyte. After reaching MII, just prior to ovulation, the oocyte ha[illegible]mpleted the first meiotic division. Meiosis is again arrested and the second meiotic division does not occur until after fusion of the sperm with the cytoplasmic membrane of the oocyte.

1.2.2 Fertilization

Upon ovulation, the COC is picked up by the fimbria of the oviduct and epithelial cilia facilitate the movement of the COC through the oviduct. Fertilization of cow oocytes occurs on Day 1 of oestrus in the ampullary-isthmic junction of the oviduct (Hawk, 1987). Spermatozoa are held in a reservoir in the caudal region of the isthmus and do not enter the ampulla until ovulation (Hunter and Wilmut, 1984; Hawk, 1987). Consequently, spermatozoa are able to penetrate the oocyte within 2 h of ovulation (Hytell *et al.*, 1988a). Capacitation of spermatozoa occurs in the maternal reproductive tract and involves a series of biochemical and physiological events

rendering the sperm competent to fertilize (Yanagimachi, 1994). Events occurring during capacitation include hyperpolarization of the sperm plasma membrane (Zeng *et al.*, 1995), changes in membrane lipid fluidity (Davis *et al.*, 1979), an increase in glycolysis and oxygen consumption (Talbot and Franklin, 1978) and an increase in protein tyrosine phosphorylation (Carrera *et al.*, 1996). Upon contact with the COC, spermatozoa release hyaluronidase to facilitate digestion of the hyaluronic acid matrix binding the cumulus cells (Lin *et al.*, 1994). Spermatozoa are thus able to move through the layers of cumulus cells surrounding the oocyte. Spermatozoa bind to the zona pellucida, specifically to sperm receptors on ZP3 (Florman and Wassarman, 1985). Once bound to the zona pellucida, spermatozoa undergo an acrosome reaction (AR) resulting in the release of proteases, facilitating penetration of the zona pellucida (Edwards, 1977). The sperm then enters the perivitelline space within the oocyte and the equatorial segment of the sperm fuses with the oolemma (Piko, 1979). The fusion of the two gametes leads to a series of events which result in activation of the oocyte. Cortical granules lying beneath the oolemma are released, changing the electrical potential of the oocyte membrane and preventing penetration and fusion of other sperm (Sun *et al.*, 1994). The sperm nucleus then moves into the cytoplasm of the oocyte, initiating the release of calcium within the cytoplasm of the oocyte (Sun *et al.*, 1994; Swann, 1996). The second meiotic division is initiated in the oocyte, resulting in the formation of the female pronucleus and the extrusion of a second polar body. The sperm nucleus decondenses to form the male pronucleus.

Fertilization is considered to be completed at syngamy when the maternal and paternal chromosomes mix to form one diploid nucleus. In many species such as the mouse, this occurs when the oocyte or zygote is still one cell (Schatten *et al.*, 1999).

In the cow, however, the male and female pronuclei remain separate until the breakdown of the pronuclear membranes during the first cleavage division (Betteridge, 1988).

1.2.3 Embryo Development

Development of the cow embryo from the zygote to the blastocyst stage takes approximately six days in vivo (reviewed by Betteridge, 1988). The first cleavage division occurs 24 to 48 h after ovulation (Thibault, 1966). The division of blastomeres within the cleavage stage embryo, typically the 2-cell to 8-cell stages, is asynchronous and uneven, leading to different sized cells (Massip *et al.*, 1983). The blastomeres undergo restrictive mitoses which are termed cleavage divisions and result in no net growth of the embryo. Compared with somatic cells, the mammalian embryo has a very low nucleo:cytoplasmic ratio (Smith and Johnson, 1986) which increases with each cell division. Further, the cell cycle lengths of embryos are different to those found in somatic cells and vary during development through different stages (reviewed by Betteridge, 1988). The blastomeres of the early cleavage stages are unattached, thus homeostatic regulation is not unlike that of unicellular organisms.

Up until the 8- to 16-cell stage the embryo is regulated by the maternal genome and thus, RNA and proteins within the embryo are inherited from the oocyte (De Sousa *et al.*, 1998). The major onset of activation of the embryonic genome in cow embryos has been reported to be at the 8-cell stage (Telford *et al.*, 1990), however, transcriptional activity has been found as early as the 2-cell stage (Viuff *et al.*, 1996). Activation of the embryonic genome is accompanied by destruction of maternal RNA

(Telford *et al.*, 1990). The major onset of embryonic genome activation is concurrent with significant changes in embryo physiology including protein synthesis (Frei *et al.*, 1989; Thompson *et al.*, 1998b), ATP production (Thompson *et al.*, 1996), oxygen consumption (Thompson *et al.*, 1996), nutrient requirements (Rieger *et al.*, 1992b; Thompson *et al.*, 1996) and energy substrate metabolism (Rieger *et al.*, 1992a, 1992b).

Up to the 8- to 16-cell stage, the embryo remains in the oviduct. Around the 32-cell stage, approximately Day 5 after oestrus, cow embryos start to compact and the resultant morulae can be found in the uterus. In mice, the outer blastomeres become polarized just prior to compaction signalling the beginning of cell allocation (Reeve and Kelly, 1983). In contrast, the blastomeres of cow embryos do not polarize prior to compaction (Plante and King, 1994). With compaction, blastomeres lose their spherical shape and begin to adhere to one another. The inner and outer blastomeres of the embryo start to differentiate at compaction to form the trophectoderm (TE) and the inner cell mass (ICM) (Plante and King, 1994). The outer blastomeres, the TE, form a transporting epithelium, with tight junctions between the apposing surfaces of the blastomeres (Plante and King, 1994). Na^+-K^+ ATPase on the basolateral membrane of the TE cells generate ion gradients, resulting in the movement of fluid into the embryo to form a blastocoel (Benos and Biggers, 1981). The TE acts as a barrier to the external environment, regulating the internal milieux of the embryo (Biggers and Borland, 1977). After compaction, the inner blastomeres differentiate into the ICM. Intercellular contact in the ICM is by gap junctions. Thus, while TE cells are tightly apposed, the cells of the ICM are loosely apposed with only occasional points of contact (Plante and King, 1994).

The cow embryo reaches the early blastocyst stage approximately Day 7 after oestrus. The blastocoel cavity continues to grow resulting in the expansion and thinning of the zona pellucida. Between Days 8 and 10 after oestrus, the blastocyst hatches from the zona pellucida (Betteridge, 1988), possibly via the secretion of plasminogen to partially lyse the zona pellucida (Menino and Williams, 1987). Unlike embryos of rodents and primates which implant shortly after hatching (Pratt, 1987), the ruminant embryo has an extended period of growth and elongation prior to the attachment of the chorion to the uterine epithelium. Attachment in fact occurs around Day 14 in the sheep and Day 19 in the cow (McLaren, 1980; King and Thatcher, 1993; Guillomot, 1995). The largest spherical embryos in the cow are found on Days 11 and 12 of oestrus and are approximately 375 μm in diameter with around 1000 cells (Betteridge, 1988). Elongation of the blastocyst commences on Days 12 to 14 of oestrus (Betteridge, 1988). Attachment of ruminant embryos involves an apposition stage when cell-cell contact is established between trophoblast or TE cells and uterine epithelial cells, followed by an adhesion stage, resulting in the formation of an epithelio-chorial placenta (Guillomot, 1995). Unlike the mouse where trophoblast cells have been shown to displace an epithelial cell monolayer and make contact with the culture vessel, trophoblast cells of cow blastocysts have been shown to form an attachment plate on top of the epithelial monolayer without actually penetrating the monolayer (Linderg *et al.*, 1989). While the rodent embryo is termed the 'pre-implantation' embryo prior to the process of implantation, the ruminant embryo is referred to as the 'pre-attachment' embryo.

1.3 IN VITRO MATURATION

Edwards (1965) was the first to show that primary cow oocytes from non-ovulatory follicles could resume meiosis and complete MI in vitro. Subsequent early research on the in vitro maturation of cow oocytes revealed that immature primary oocytes, retrieved from small antral follicles of slaughterhouse cows of unknown stage of their oestrous cycle, could be matured and fertilized in vitro to produce live offspring (Newcomb *et al.*, 1978; Crister *et al.*, 1986; Sirard *et al.*, 1988). Fertilizability, developmental competence and viability following uterine transfer, however, have been reported to be lower in in vitro matured oocytes than in in vivo matured oocytes (Greve *et al.*, 1987; Leibfried-Rutledge *et al.*, 1987).

With the development of techniques for the maturation of oocytes in vitro, it was discovered that oocyte maturation was in fact a complex process involving maturation of both the nucleus and the cytoplasm. The ability of an oocyte to resume meiosis and progress to MII was found to be not necessarily indicative of either its ability to be fertilized or its developmental competence (Thibault, 1972). That is, nuclear and cytoplasmic maturation are in fact two distinct processes. Cytoplasmic maturation has in fact been shown to involve numerous biochemical and physiological events that may or may not be related to nuclear maturation, but that have a significant effect on subsequent fertilization and embryo development (Hunter and Moor, 1987; Kastrop *et al.*, 1991; Levesque and Sirard, 1994; Eppig *et al.*, 1996). Inadequate cytoplasmic maturation may indeed be the reason for the reduced developmental capacity of in vitro produced mammalian embryos.

Fukui *et al.* (1982) and Bavister *et al.* (1992) demonstrated that a variety of media could be successfully used for maturation of cow oocytes in vitro. Maturation

conditions, however, were shown to affect the developmental competence of resultant embryos (Bavister *et al.*, 1992).

Mammalian oocytes at the GV stage will spontaneously resume meiosis, undergoing GVBD when placed in a variety of media (Eppig, 1991b). In the absence of serum, gonadotrophins such as LH and FSH have been found to be beneficial for the maturation of cow oocytes, cumulus cell expansion and the subsequent fertilizability of oocytes (Zuelke and Brackett, 1990; Saeki *et al.*, 1991; Kobayashi *et al.*, 1994; Armstrong *et al.*, 1996). There have, however, been conflicting reports as to whether gonadotrophins confer any benefits during maturation of cow oocytes when serum is present (Fukui *et al.*, 1982; Fukushima and Fukui, 1985; Sirard *et al.*, 1988; Fukui and Ono, 1989; Keefer *et al.*, 1993; Dominko and First, 1997). Dominko and First (1997) found that with respect to FSH, LH did not affect the proportion of cow oocytes reaching MII after 24 h but increased the rate of nuclear maturation in the presence of serum. Oocytes reaching MII by 16 h maturation had an improved developmental competence to those reaching MII at 20 or 24 h maturation (Dominko and First, 1997).

The successful IVM of cow oocytes is dependent on the presence of intact cumulus cells (Ball *et al.*, 1983; Sirard *et al.*, 1988; Liu *et al.*, 1995). Sirard *et al.* (1988) found that the optimal concentration of COC's was 10 COC's/50 µl maturation medium, in the presence or absence of gonadotrophins. Cumulus cells are believed to supply the oocyte with energy substrates such as amino acids, pyruvate and lactate (Brower and Schultz, 1982; Leese and Barton, 1985; Gardner and Leese, 1990; Gardner *et al.*, 1996a; Cetica *et al.*, 1997), as well as ATP via gap junctions between cumulus cell projections and the oolemma (Eppig, 1991; Downs *et al.*, 1996). It has

also been suggested that cumulus cells prevent hardening of the zona pellucida (De Felici and Siracusa, 1982). Only oocytes with several intact layers of compact cumulus cells are generally selected for IVM, as subsequent embryo development has been correlated with the number of cumulus cell layers surrounding the oocyte (Shiyoa *et al.*, 1984).

Many early studies matured cow oocytes in the presence of granulosa cells to mimic the in vivo environment (Crister *et al.*, 1986; Lu *et al.*, 1987; Lutterbach *et al.*, 1987; Xu *et al.*, 1987). It has been suggested that fertilizability of cow oocytes and subsequent developmental competence are both greater following maturation in the presence of granulosa cells (Crister *et al.*, 1986; Lutterbach *et al.*, 1987; Fukui and Ono, 1989). More recent studies, however, indicate that high levels of morula and blastocyst development can be achieved from maturation in granulosa cell-free cultures (Gardner, 1994; Edwards *et al.*, 1997), equivalent to the 45% reported for in vivo matured oocytes (Leibfried-Rutledge *et al.*, 1987).

The in vitro maturation of cow oocytes has also been shown to be improved by culture with specific growth factors. The addition of EGF to maturation medium for cow oocytes, in the absence of serum or gonadotrophins, stimulated the resumption of meiosis (Lorenzo *et al.*, 1994; Kato and Seidel, 1996) and significantly increased the proportion of embryos reaching MII (Sanbuissho *et al.*, 1990; Kato and Seidel, 1996; Lonergan *et al.*, 1996), cumulus cell expansion (Kobayashi *et al.*, 1994; Lorenzo *et al.*, 1994; Kato and Seidel, 1996; Lonergan *et al.*, 1996), the fertilizability of matured oocytes (Kobayashi *et al.*, 1994; Gandolfi *et al.*, 1996; Kato and Seidel, 1996) and subsequent embryo development (Coskun *et al.*, 1991; Harper and Brackett, 1993; Lonergan *et al.*, 1996). Maturation of cow oocytes with IGF-I also improved the

proportion of oocytes reaching MII but did not affect cumulus expansion (Lorenzo *et al.*, 1994; Armstrong *et al.*, 1996). Lorenzo *et al.* (1994) found a synergistic effect between EGF and IGF-I on both nuclear maturation and cumulus cell expansion. Maturation of cow oocytes with TGF-α was also found to stimulate cumulus cell expansion and the fertilizability of matured oocytes (Kobayashi *et al.*, 1994). The stimulatory effects of EGF and TGF-α were reported to be simular to the effects of serum during oocyte maturation (Kobayashi *et al.*, 1994). Maturation of cow oocytes with GH was also found to increase the fertilizability of cow oocytes (Izadyar *et al.*, 1997) but did not influence the proportion of oocytes reaching MII (Izadyar, 1996). It has, therefore, been suggested that GH specifically affects cytoplasmic maturation (Izadyar *et al.*, 1997). The presence of other growth factors during cow oocyte maturation, specifically insulin, bFGF and TGF-β1, had no effect on cumulus cell expansion or fertilizability (Kobayashi *et al.*, 1994).

The positive effects of growth factors such as EGF and IGF-I during maturation of cow oocytes have been reported to be dependent on the presence of cumulus cells, suggesting that the action of growth factors during oocyte maturation is mediated through the cumulus cells (Sanbuissho *et al.*, 1990; Lorenzo *et al.*, 1994). Lonergan *et al.* (1996), however, found that EGF stimulated polar body formation in denuded cow oocytes and suggested that inconsistencies in reports of the actions of growth factors on denuded oocytes may be due to the methods of denuding and circumstances under which oocytes are devoid of cumulus cells. Interestingly, when combined with gonadotrophins (LH or FSH), EGF had no additional affect on cumulus cell expansion, fertilizability of cow oocytes or subsequent embryo development (Kobayashi *et al.*, 1994). It has been suggested that EGF and gonadotrophins act via

the same stimulatory mechanism, specifically a c-AMP-dependent process (Kobayashi *et al.*, 1994; Downs *et al.*, 1988).

TCM-199 with Earle's salts, supplemented with 10% (v/v) heat-treated FCS, sodium pyruvate (0.2 mM), FSH, LH and oestradiol 17β (1 μg/ml), is widely used for the maturation of cow oocytes in vitro (Rosenkrans *et al.*, 1993; Pinyopummintr and Bavister, 1996a; Dominko and First, 1997; Furnus *et al.*, 1997). Oocytes are typically retrieved from follicles of 2-6 mm (Keskintepe *et al.*, 1995; Liu and Foote, 1995b; Dominko and First, 1997; Edwards *et al.*, 1997) as developmental competence has been shown to be poor in oocytes derived from small follicles of less than 2 mm in diameter (Pavlok *et al.*, 1992). Cow oocytes are generally matured for 22-24 h at 39°C, at 5% CO_2 and air (Chian *et al.*, 1992; Kim *et al.*, 1993a; Pinyopummintr and Bavister, 1996b; Edwards *et al.*, 1997; Morales *et al.*, 1999).

1.4 IN VITRO FERTILIZATION

Early attempts to produce cow embryos in vitro proved that embryos fertilized in vitro could subsequently develop to the blastocyst stage and were viable following uterine transfer (Newcomb *et al.*, 1978; Xu *et al.*, 1987; Sirard *et al.*, 1988). There have, however, been reports of ultrastructural abnormalities in in vitro matured and in vitro fertilized oocytes compared with those matured and fertilized in vivo. Such abnormalities include delayed and asynchronous pronuclear development, pre-activation of cytokenesis, polygyny and polyspermy which is possibly linked to the observed delayed and incomplete release and distribution of cortical granules (Hytell *et al.*, 1988b; Xu and Greve, 1988; Hytell *et al.*, 1989). Furthermore, developmental changes from the zygote to the blastocyst stage have been found to be delayed in

embryos fertilized in vitro compared with those fertilized in vivo (Plante and King, 1994). Research into the events involved in fertilization has enabled the development of improved in vitro fertilization systems.

Tyrodes Albumin Lactate Pyruvate Medium (Fert-TALP) (Bavister and Yanagimachi, 1977; Parrish *et al.*, 1988) is frequently used for the fertilization of cow oocytes in vitro (Gardner *et al.*, 1996b; Lee and Fukui, 1996; Pinyopummintr and Bavister, 1996b; Thompson *et al.*, 1996), however, modifications of media such as SOF (Tervit *et al.*, 1972), CR1aa (Rosenkrans and First, 1994) and Defined Medium (Brackett and Oliphant, 1975) have all been successfully used for the in vitro fertilization of cow oocytes (Choi *et al.*, 1991; Long *et al.*, 1994; Saeki *et al.*, 1994; Tajik *et al.*, 1994; Keskintepe and Brackett, 1996; Earl *et al.*, 1997). Frozen-thawed spermatozoa are routinely used for the insemination of cow oocytes, following the separation of motile sperm from immotile sperm and milk solids, used in the freezing of spermatozoa, on a Percoll density gradient.

Capacitation of ruminant spermatozoa in vitro requires exposure to oviductal components such as GAG's (Parrish *et al.*, 1989b). Heparin, a GAG which was found to capacitate bull spermatozoa in vitro (Parrish *et al.*, 1988) is routinely added to media for the in vitro fertilization of cow oocytes. Heparin has been found to increase the ability of bull spermatozoa to bind to the zona pellucida (Watanabe *et al.*, 1997) and the proportion of oocytes penetrated by spermatozoa (Susko-Parrish *et al.*, 1990). The optimal concentration of heparin for the fertilization of cow oocytes has been reported to be approximately 10 μg/ml (Wales, 1970; Greve *et al.*, 1987; Niwa and Ohgoda, 1988). There is evidence, however, to suggest that a higher concentration of heparin (100 μg/ml) can reduce the time for induction of capacitation in cow

spermatozoa (Fukui *et al.*, 1989; Fukui *et al.*, 1990). It has also been suggested that the optimal heparin concentration for fertilization of cow oocytes may vary with different bulls (Fukui *et al.*, 1990). Caffeine and/or calcium ionophore A23187 have also been used to capacitate bull spermatozoa (Byrd, 1981; Shiyoa *et al.*, 1984).

Glucose has been shown to stimulate a number of processes during fertilization of oocytes from rodents and humans including capacitation, the AR and the proportion of oocytes fertilized (Niwa and Iritani, 1978; Fraser and Quinn, 1981; Perrault and Rogers, 1981). Glucose, however, is detrimental to the fertilization of cow oocytes, inhibiting the AR in cow spermatozoa (Parrish *et al.*, 1985). It was determined that the metabolism of glucose by bull spermatozoa resulted in an acidification of pHi due to the generation of H^+ during glycolysis, delaying capacitation (Parrish *et al.*, 1989a). Capacitation of cow spermatozoa is in fact associated with a slight alkalization of pHi (Parrish *et al.*, 1989a). Consequently, glucose is typically not included in media for fertilization of cow oocytes.

Penicillamine (P), hypotaurine (H) and epinephrine (E) are often added to media for the fertilization of cow oocytes to enhance the motility of spermatozoa. Fertilization with either P, H or E in the presence of heparin was shown to increase the proportion of cow oocytes penetrated by spermatozoa (Susko-Parrish *et al.*, 1990). Long *et al.* (1994), however, found no additional benefit of adding P, H and E to fertilization media and suggested that P, H and E may only enhance fertilization results in systems where penetration of oocytes by spermatozoa is low.

Earl *et al.* (1997) reported that the addition of glutathione to media for the preparation of spermatozoa for insemination of cow oocytes, significantly increased subsequent blastocyst development. It was suggested that glutathione may function to

protect spermatozoa from damage induced by ROS (Earl *et al.*, 1997). Glutathione has in fact been implicated in the treatment of male infertility in humans through its action as an antioxidant (reviewed by Irvine, 1996). At present, however, glutathione is not routinely added to media for the preparation of bull spermatozoa for insemination.

Cow oocytes have been shown to maintain fertilizability for 20 to 24 h after ovulation (Thibault, 1967). Spermatozoa penetrate oocytes in vitro as early as 3 h after insemination (Park *et al.*, 1989; Saeki *et al.*, 1991b) and continue to penetrate oocytes until 24 h after insemination (Chian *et al.*, 1992). The incidence of polyspermy, however, increases with the incubation period (Saeki *et al.*, 1991b; Chian *et al.*, 1992; Long *et al.*, 1994). Cow oocytes are routinely incubated with spermatozoa for approximately 18 to 24 h, at 39°C, in 5% CO_2 and air (Xu *et al.*, 1992; Gardner *et al.*, 1996b; Pinyopummintr and Bavister, 1996b; Thompson *et al.*, 1996; Hill *et al.*, 1997).

Dominko and First (1997) found that the timing of insemination following arrest at MII was critical to the subsequent developmental competence of cow embryos. Oocytes inseminated 8 h after arrest at MII had a higher developmental competence than oocytes inseminated immediately after reaching MII. This suggests that the oocyte's ability to support fertilization and subsequent embryo development is partially acquired after nuclear maturation. This phenomenon has also been reported for human (Trounson *et al.*, 1982), mouse (Kubiak, 1989) and cat (Johnston *et al.*, 1989) oocytes.

The in vitro fertilization of cow oocytes has been reported to be subject to a 'bull effect'. Spermatozoa from different bulls have been found to have different capabilities of fertilizing oocytes (Long *et al.*, 1994; Tajik *et al.*, 1994; Kurtu *et al.*, 1996; Sumantri *et al.*, 1996) and have been shown to affect subsequent embryo

development (Long *et al.*, 1994; Avery and Quetglas, 1996). The reported 'bull effect' could be due to a number of factors such as the age of bulls (Sirard and Lambert, 1985), factors affecting normal testicular function such as mild heat stress (Januskauskas *et al.*, 1995), variations in the contents of seminal plasma (Fukui *et al.*, 1988), poor genetics or differences in the generation of ROS by spermatozoa from different bulls, as fertilization in the presence of glutathione was shown to improve blastocyst production (Earl *et al.*, 1997).

It has been suggested that cumulus cells are important for the induction of the AR in cow spermatozoa (Tajik *et al.*, 1993). Several studies have demonstrated that the presence of cumulus cells was important for the fertilization of cow oocytes (Fukui, 1990; Saeki *et al.*, 1994; Chian *et al.*, 1996). This, however, remains controversial with other studies finding that fertilization of cow oocytes was improved following the removal of cumulus cells prior to insemination (Lu *et al.*, 1987; Xu *et al.*, 1987; Fukui *et al.*, 1989). The requirement for cumulus cells during fertilization has been found to be partly dependent on the presence of protein. When enclosed by cumulus cells, the penetration of oocytes by spermatozoa was found to be equivalent in the presence and absence of BSA (Tajik *et al.*, 1993). Oocyte penetration, however, did not occur when cumulus-free oocytes were inseminated in the absence of BSA (Tajik *et al.*, 1993; Saeki *et al.*, 1994; Tajik *et al.*, 1994). Saeki *et al.* (1994) have in fact reported a synergistic effect between cumulus cells and BSA on oocyte fertilization. These findings suggest that both cumulus cells and BSA contain a factor or factors that enhance sperm penetration in vitro. Fraser (1985) suggested fertilization of mouse oocytes in protein-free medium was possibly due to albumin or

a similar macromolecule from follicular or oviductal fluid trapped by the cumulus complex.

Both FCS and BSA have been shown to support fertilization of cow oocytes in vitro (Leibfried-Rutledge *et al.*, 1986; Tajik *et al.*, 1993), however, there is evidence that the incidence of polyspermy is higher following fertilization in the presence of FCS than in the presence of BSA (Tajik *et al.*, 1993). Saeki *et al.* (1994) found that the macromolecule PVA could replace protein during the fertilization of cumulus enclosed cow oocytes. In the absence of cumulus cells, however, the penetration of oocytes by spermatozoa has been found to be lower in the presence of PVA than in the presence of BSA (Saeki *et al.*, 1994; Tajik *et al.*, 1994). Interestingly, oocyte penetration in the presence of PVA was improved by increasing the concentrations of both sperm and bicarbonate (Tajik *et al.*, 1994). Tajik *et al.* (1994) suggested that proteins have an important role in the capacitation of bull spermatozoa and oocyte penetration.

1.5 IN VITRO CULTURE

With the evolution of techniques for the in vivo and in vitro maturation of mammalian oocytes and subsequent fertilization in vitro, it was discovered that many species of mammalian embryos exhibited a developmental block. Cow and sheep embryos were found to block at the 8- to 16-cell stage (Wright and Bondoli, 1981; Thibault, 1966), some strains of mouse embryos blocked at the 2-cell stage (Whitten, 1956; Goddard and Pratt, 1983), pig embryos blocked at the 4-cell stage (Davis and Day, 1978), while hamster embryos were found to block at both the 2-cell and 4-cell stages (Bavister *et al.*, 1983). Such blocks to development were attributed to in vitro

induced artefacts. The timing of the developmental blocks in each species corresponds to the major onset of embryonic genome activation (Telford *et al.*, 1990). Furthermore, in cow and sheep embryos, the block to development occurs when the embryo moves from the oviduct to the uterus (Bavister, 1988).

The desire to overcome blocks to mammalian embryo development in vitro has generated the need to understand the requirements of mammalian embryos during culture and the varied effects that culture components can have on both the development and viability of embryos. As a result, numerous culture systems have been employed to support the development of mammalian embryos from the zygote to the blastocyst stage. Elongation of ruminant embryos in vitro, however, has never been achieved.

1.5.1 Co-Culture and Conditioned Media

Many of the earliest culture systems which were successful in supporting development of ruminant embryos past the 8- to 16-cell block, involved the co-culture of embryos with somatic cells (Camous *et al.*, 1984; Gandolfi and Moor, 1987; Heyman *et al.*, 1987; Goto *et al.*, 1988; Eyestone and First, 1989). Such co-culture systems were devised in an attempt to mimic the maternal environment. Subsequent studies have revealed that a variety of cell lines from different species are effective in supporting development of ruminant embryos past the 8- to 16-cell stage, including trophoblastic vesicles (Camous *et al.*, 1984; Heyman *et al.*, 1987), cumulus and/or granulosa cells (Goto *et al.*, 1988; Wiemer *et al.*, 1991; Lim and Hansel, 1998), BOEC (Ellington *et al.*, 1990; Wiemer *et al.*, 1991; Xu *et al.*, 1992; Shamsuddin *et*

al., 1993), BRL cells (Hasler *et al.*, 1994; Rehman *et al.*, 1994; Van Inzen *et al.*, 1995) and green monkey kidney epithelial (Vero) cells (Carnegie *et al.*, 1996).

There are several possible explanations for the observed benefits of culturing embryos in the presence of somatic cells. Firstly, somatic cells may secrete embryotrophic factors such as proteins (Heyman *et al.*, 1987) and growth factors. BRL cells have in fact been shown to produce growth factors such as LIF, IGF-II and TGF-β (Massague *et al.*, 1985; Smith and Hooper, 1987; Carr *et al.*, 1989) which have been found to improve development of cow embryos in vitro (see section 1.6.8). Secondly, somatic cells may remove components that are toxic to the embryo such as toxic metal ions, via chelation (see section 1.6.5) or ROS (see section 1.6.3.3). Thirdly, somatic cells may improve development of embryos by modifying the culture medium. Takahashi *et al.* (1995) reported that the utilization of cystine in TCM-199 by 6- to 8-cell cow embryos was largely dependent on the reduction of cystine to cysteine by cumulus-granulosa cells. Edwards *et al.* (1997) found that BRL cells, BOEC and fibroblasts all modified the carbohydrate composition of culture media (TCM-199). Glucose concentration was reduced, especially in the presence of BOEC (5.6 to 2.7 mM), and the concentrations of both lactate and pyruvate increased following co-culture. Interestingly, when the concentrations of carbohydrates in SOFaa (referred to as 20aa in the present study) were adjusted to reflect the concentrations of carbohydrates in TCM-199 following incubation with BOEC, 57% of zygotes developed to the blastocyst stage compared with 11% in conventional TCM-199 (Edwards *et al.*, 1997). This indicates that somatic cells modify the carbohydrate composition of culture media in a fashion consistent with the physiological requirements of the developing embryo.

Eyestone and First (1989) demonstrated that medium conditioned with oviductal tissue supported equivalent rates of cow embryos to the morula and blastocyst stages as a co-culture system with medium containing oviductal tissue. Ellington *et al.* (1990), however, found that culture of cow embryos with medium conditioned with BOEC inferior to co-culture with BOEC. Likewise, Pinyopummintr and Bavister (1991) found no benefit from conditioning medium with oviductal cells for culture of cow embryos. Although Van Inzen *et al.* (1993) reported that development of cow embryos in TCM-199 conditioned with BRL cells improved development of cow embryos to the blastocyst stage, development was extremely low following culture in both conditioned medium (9.4%) and unconditioned medium (5.3%).

The success of co-culture has been shown to be dependent on the media used to culture embryos and somatic cells (Ellington *et al.*, 1990; Xu *et al.*, 1992; Applewhite and Westhusin, 1995). It is important to note that media used in co-culture or conditioned systems have typically been complex tissue culture media with serum, designed to support the requirements of somatic cells rather than those of the embryos. Invariably, culture of embryos in complex tissue culture media without somatic cell support has resulted in extremely poor embryo development, with development of zygotes to the morula and blastocyst stages as low as 3% (Eyestone and First, 1989) and 18% (Heyman *et al.*, 1987) in TCM-199 supplemented with serum. Likewise, conditioning of a simple medium HTF, formulated for the culture of human embryos (Quinn *et al.*, 1985), with either Vero cells or granulosa cells improved development of cow embryos to the blastocyst stage compared with embryos cultured in unconditioned HTF (Maeda *et al.*, 1996). Development of cow embryos in unconditioned HTF, however, was extremely poor with only 3% of

embryos developing to the blastocyst stage. The success of co-culture and conditioned media thus lies in the apparent inadequacy of many media in supporting the development of cow embryos, highlighting the problem of trying to satisfy two distinct cell types.

1.5.2 Somatic Cell-Free, Unconditioned Media

The first embryo culture media were simple media designed for the culture of mouse embryos, many years before the implementation of co-culture systems. Using a CO_2- bicarbonate buffered balanced salt solution (Kreb's Ringer Bicarbonate or KRB) containing glucose and BSA, Whitten (1956) found that 8-cell mouse embryos could be cultured to the blastocyst stage. Importantly, McLaren and Biggers (1958) demonstrated that mouse embryos cultured from the 8-cell stage could result in live offspring after transfer of resultant blastocysts to recipient mice. Whitten (1957) discovered that the early cleavage stage embryo had different requirements to the later stages of development as lactate was required to support development of mouse embryos from the 2-cell stage. It was subsequently found that either pyruvate, phosphoenolpyruvate or oxaloacetate could be substituted for lactate, however, pyruvate and lactate acted synergistically in supporting development of mouse 2-cell embryos to the blastocyst stage (Brinster, 1965a, 1965c).

It was evident that the mouse zygote had different requirements to the 2-cell embryo as culture with lactate and glucose could not support development through the first cleavage division (Whitten, 1957). Although pyruvate and oxaloacetate were shown to facilitate development of zygotes through the first cleavage division, development was subsequently arrested at the 2-cell stage (Biggers *et al.*, 1967). This

phenomenon has since been termed the '2-cell block' (see section 1.5) and has only been overcome following many years of studies analysing the metabolism and physico-chemical and nutritional requirements of the mouse embryo. The '2-cell block' was in fact found to be confined to certain strains of mice. In contrast to zygotes from outbred and random bred strains, zygotes from certain inbred strains of mice and their F1 hybrids could develop from the zygote to the blastocyst stage in medium containing glucose, pyruvate and lactate (Whitten and Biggers, 1968).

Thus, early research pertaining to the culture of mouse embryos in simple media led to several important discoveries that paved the way for the development of culture systems for mammalian embryos. Firstly, the fertilized egg and cleavage stage embryos were quite unlike somatic cells which can utilize glucose as the sole energy substrate. Secondly, the requirements of the embryo changed during development from the zygote to the blastocyst stage. Thirdly, embryos produced in vitro were viable as evidenced by the birth of live offspring following transfer of cultured embryos to recipients. Further, the ability of the embryo to develop in culture varied according to genetic make-up. Early studies on the culture of rabbit embryos also led to a very important discovery. Rabbit zygotes were shown to develop through several cleavage divisions in the absence of any exogenous energy substrates and could in fact develop to the blastocyst stage in the absence of any carbohydrates (Kane, 1972; Kane, 1987). Thus, it become evident that the requirements of embryos are species specific.

Tervit *et al.* (1972) developed the first simple somatic cell-free medium for the successful extended culture of cow and sheep zygotes. The medium termed Synthetic Oviduct Fluid (SOF) was formulated from the ionic and lactate compositions of the

sheep reproductive tract. In SOF, in vivo matured and fertilized oocytes were able to develop past the 8- to 16-cell stage, with sheep embryos reaching the morula (5/6 embryos: 91%) and early blastocyst (1/11 embryos: 9%) stages and cow embryos developing as far as the morula stage (5/10 embryos: 50%) after 5 or 6 days of culture (Tervit *et al.*, 1972). Importantly, Tervit and Rowson (1974) reported the birth of lambs after transfer of embryos to recipient ewes following culture of cleavage stage embryos in SOF for up to 6 days.

The development of simple, somatic cell-free media has been a fundamental aspect of improving the development of large numbers of viable embryos in vitro. Such media have enabled the determination of the effects of various media components on mammalian embryo development and viability. Furthermore, somatic cell-free media have allowed for determinations of embryo metabolism during development.

There have been many somatic cell-free media developed for the culture of cow embryos from the zygote to the blastocyst stage. Some of the media currently used to culture cow embryos are listed in Table 1.1. One of the most successful media for the development of cow embryos from the zygote to the blastocyst stage, SOFaa (Table 1.1; referred to as 20aa in the present study) has been based on SOF with the addition of amino acids (Gardner *et al.*, 1994b). The fact that culture of ruminant embryos in SOFaa without any somatic cell support could result in 64% of in vitro produced cow zygotes (Edwards *et al.*, 1997) and 95% of in vivo matured and in vivo fertilized sheep zygotes (Gardner *et al.*, 1994b) developing to the blastocyst stage, indicates that somatic cell support is not a requirement for the development of ruminant embryos in vitro. In support of this, Gardner *et al.* (1994b) reported a 62% pregnancy rate after

Table 1.1 Composition of somatic cell-free media designed for the culture of cow embryos.

Component (mM)	SOFaa[a]	CR1aa[b]	mHECM-3+NEA[c]	mKSOM[d]
NaCl	99.7	114.7	113.8	95.0
KCl	7.16	3.1	3.0	2.5
KH_2PO_4	1.19	-	-	0.35
$CaCl_2$	1.71	-	1.9	1.71
$MgCl_2$	0.49	-	0.5	-
$MgSO_4$	-	-	-	0.2
$NaHCO_3$	25.1	26.2	25.0	25.0
glucose	1.5	0.2	-	0.2
Na lactate	3.3	-	4.5	10.0
Ca lactate	-	5.0	-	-
Na pyruvate	0.33	0.4	-	0.2
HCl (µl/100 ml)	-	-	100	-
HEPES	-	-	-	2.5
Glutamine	1.0	1.0	0.2	1.0
Non-essential amino acids	yes MEM[†]	yes MEM[†]	yes TCM-199[†]	yes MEM[†] (half concentration)
Essential amino acids	yes MEM[†]	yes BME[†]	no	yes MEM[†]
Taurine	-	-	0.5	0.4
EDTA	-	-	-	0.01
BSA (mg/ml)	8[‡]	3	-	1[‡]
PVA (mg/ml)	#	-	0.1	1[‡]

[a]Edwards *et al.*, 1997, adapted from Gardner *et al.* (1994b).

[b]Rosenkrans and First (1994).

[c]Pinyopummintr and Bavister (1996a). Note that this media was optimized for culture between 18 h and 72 h pi.

[d]Liu and Foote (1995b).

[†]amino acid composition of either Minimum Essential Medium (MEM) modified to contain no glutamine, Basal Medium Eagle (BME) or Tissue Culture Medium-199 (TCM-199).

media has also been used with PVA instead of BSA (Lee and Fukui, 1996).

[‡]either BSA or PVA added to media.

transfer of the cultured sheep blastocysts to recipient ewes, compared with 78% in in vivo developed controls.

The ultimate aim of developing somatic cell-free culture media is to design media based on the physiological, nutritional and metabolic requirements of developing embryos from different species. The study of individual medium components and the physico-chemical parameters of embryo culture have enabled the development and refinement of somatic cell-free culture media.

1.6 COMPONENTS AND PHYSICO-CHEMICAL ASPECTS OF SOMATIC CELL-FREE CULTURE MEDIA

1.6.1 Ions and Osmolytes

Analyses of the ionic composition of mammalian reproductive fluids for the cow (Olds and VanDemark, 1957; Schultz *et al.*, 1971), sheep (Restall and Wales, 1966), mouse (Borland *et al.*, 1977), rabbit (Leese, 1988) and human (Borland *et al.*, 1980) have revealed that fluids within the reproductive tract are not simply serum transudates. Fluids of the reproductive tract vary from serum not only in the concentration of specific ions but also in the ratios of ions present (Carlson *et al.*, 1970; Schultz *et al.*, 1971; Gardner and Sakkas, 1993). The ionic composition of the ruminant reproductive tract can be found in Table 1.2. Attempts to culture embryos in a physico-chemical environment similar to that of the reproductive tract have been varied. Unlike the sheep, mouse and hamster, there has been little attempt to determine the inorganic ionic requirements of the in vitro produced cow embryo.

Table 1.2 Ionic composition of reproductive tract fluids of the cow and sheep.

Component (mM)	Cow oviductal fluid[a]	Cow uterine fluid[a]	Sheep oviductal fluid[b]
Sodium	90.3	95.7	137.0
Potassium	57.0	46.8*	8.2
Calcium	2.9	3.8	2.8
Magnesium	nd[†]	nd	0.75
Phosphate	1.0	0.78	1.1
Chloride	112.7	101.9	121.0
Bicarbonate	nd	nd	19.7

[a]determined by Durward Olds and VanDemark (1957).

[b]determined by Restall and Wales (1966).

*potassium reported to be 15.7 mM in cow uterine fluid by Schultz *et al.* (1971).

[†]nd indicates not determined.

Potassium is found at high concentrations in fluids of the mammalian reproductive tract, however, increasing the concentration of potassium in culture media has not been beneficial to the development of sheep (Walker *et al.*, 1988) or hamster (Bavister and Golden, 1989; McKiernan and Bavister, 1990) embryos. Culture of mouse embryos in medium with a potassium concentration (25 mM) similar to that found in the mouse reproductive tract resulted in an improvement in embryo development, cell number and viability (Roblero and Riffo, 1986). In contrast, Wiley (1986) found that mouse embryo development was increased following culture with a reduced concentration of potassium (1.4 mM). The highest concentration of potassium tested, however, was 6.0 mM and results may have been confounded by the use of different proteins (BSA and cow serum immunoglobulins) and protein concentrations for the different concentrations of potassium tested (Wiley *et al.*, 1986).

While inorganic phosphate (Pi) is present in the cow oviduct and uterus (Olds and VanDemark, 1957; Schultz *et al.*, 1971), Pinyopummintr and Bavister (1991) reported that Pi was not necessary for development of cow embryos in vitro. In contrast, Kim *et al.* (1993b) reported that Pi (0.35 mM) was required for development of cow embryos to the 8-cell stage and blastocyst. Unlike cow embryos (Pinyopummintr and Bavister, 1991; Kim *et al.*, 1993b), development of hamster (Schini and Bavister, 1988; Seshagiri and Bavister, 1989a) and rat (Miyoshi *et al.*, 1994) embryos has been reported to be inhibited by Pi.

Calcium has been shown to be required for the process of compaction within the embryo (Wales, 1970), while both sodium and chloride ions have been shown to be important components for blastocoel expansion (Manejwala *et al.*, 1989). Although

magnesium was not a requirement for embryo development, the presence of magnesium improved mouse blastocyst development (Wales, 1970). Culture of sheep and hamster embryos, however, with a wide range of calcium concentrations did not affect embryo development (Walker *et al.*, 1988; McKiernan and Bavister, 1990). Furthermore, it has been shown that mouse and hamster embryos can develop in media containing a wide range of magnesium and sodium concentrations (Wales, 1970; McKiernan and Bavister, 1990). In addition, altering the ratios of Ca^{2+}:Mg^{2+} and Na^{+}:K^{+} was found to have no effect on development of the hamster embryo (Bavister and Golden, 1989; McKiernan and Bavister, 1990). Recently, however, Lane *et al.*, (1998) reported that an increase in the concentration of magnesium in the culture medium, from 0.5 mM to 2.0 mM, resulted in an increase in intracellular magnesium ion concentrations with a concomitant decrease in intracellular calcium concentrations. The observed change in intracellular ratio of Ca^{2+}:Mg^{2+} was in fact associated with an increase in development to the blastocyst stage, an increase in blastocyst cell number and ICM cell number as well as an increase in fetal potential following transfer of embryos to recipients (Lane *et al.*, 1998).

Lawitts and Biggers (1991a) used a process called simplex optimization to formulate culture media for mouse embryos, by modifying several components simultaneously. This resulted in the development of a medium SOM, which supported the development of embryos from the outbred CF1 strain of mice through the 2-cell block (Lawitts and Biggers, 1992). Subsequently, an increase in the concentration of potassium in SOM to 2.5 mM (KSOM) improved the rate of compaction, blastocyst development and trophoblast cell number in mouse embryos. KSOM was also shown to support the development of cow embryos to the blastocyst stage (Yang *et al.*, 1994)

and has subsequently been modified with the addition of amino acids (Table 1.1) for the culture of cow embryos (Liu and Foote, 1995b; Liu and Foote, 1997).

Unfortunately, with the exception of a few studies, the majority of studies have used only embryo development as the end-point for assessing the effects of altering the concentrations of specific ions in culture media. In order to truly understand the effects of varying ion concentrations on the embryo, parameters such as cell number, the differentiation of cells in the blastocyst, metabolism and viability following transfer must be examined.

Reports of culture of mouse and human embryos in medium with an ionic composition similar to that of oviductal fluid have been conflicting (Quinn *et al.*, 1985; Van Winkle *et al.*, 1990; Cummins *et al.*, 1999). Gardner and Sakkas (1993) formulated a culture medium (KK) based on both the carbohydrate and ionic composition of the mouse reproductive tract. KK inhibited development of the mouse embryo, however, the inhibitory effects were alleviated upon the removal of phosphate and the addition of amino acids, vitamins, transferrin and insulin (Gardner and Sakkas, 1993). Similarly, Tervit *et al.* (1972) formulated SOF, based on the ionic composition of sheep oviductal fluid (Restall and Wales, 1966; Table 1.2), for the culture of sheep and cow embryos. Subsequently, SOF has been widely used as the base medium for culture of cow embryos and, with the addition of amino acids (Table 1.1), can support the highest reported levels of cow blastocyst development (45-64%) when compared to co-culture and culture in other somatic cell-free media such as CR1aa, KSOM and mHECM-3 (reviewed by Gardner, 1998a).

Determinations of the osmolarity of uterine and oviductal fluids using electron probe x-ray microanalysis revealed that the fluids were hyperosmotic, with an

osmolarity of approximately 350 to 360 mOsm (Olds and Van Demark, 1957; Borland *et al.*, 1980). Mouse, hamster and sheep embryos, however, have been found to develop well in vitro at a lower osmolarity, in the range of 250 to 300 mOsm (Brinster, 1965b; Whitten and Biggers, 1968; McKiernan and Bavister, 1990; Walker *et al.*, 1992). Mouse embryos have even been shown to develop well in media with an osmolarity as low as 229 mOsm (Lawitts and Biggers, 1992). There is an increasing amount of evidence revealing that culture of mammalian embryos in media over 300 mOsm, in the absence of organic osmolytes, is detrimental to embryo development (Maurer *et al.*, 1970; McKiernan and Bavister, 1990; Van Winkle *et al.*, 1990; Miyoshi *et al.*, 1994; Li and Foote, 1996; Liu and Foote, 1996; Dawson and Baltz, 1997). Recently, Collins and Baltz (1999) used two novel, independent methods involving volumetric changes of zygotes and embryos under different ionic conditions, to estimate the tonicity of mouse oviductal fluid. Collins and Baltz (1999) determined the actual osmolality to be in the range of 290 to 300 mOs/kg, which equates to a range of osmolarities that have been determined to support embryo development in vitro.

It has been suggested that the observed detrimental effects of culturing embryos in media with an osmolarity of over 300 mOsm are not due to the increase in osmotic pressure, but rather due to the increase in the concentration of NaCl (Beckmann and Day, 1993; Li and Foote, 1996). It is believed that an increase in NaCl concentration could affect a number of intracellular processes, including ATP production (Beckmann and Day, 1993), glucose-6-phosphate isomerase function (Lawitts and Biggers, 1991b), mRNA synthesis (Ho *et al.*, 1994) and protein synthesis (Anbari and Schultz, 1993). Dawson and Baltz (1997), however, showed that the inhibitory effects

of an increased concentration of NaCl on mouse embryo development were identical to the inhibitory effects of increasing the osmotic pressure of culture media with raffinose. Interestingly, the inhibitory effects of increasing osmotic pressure to 310 mOsm with either NaCl or raffinose were counteracted by the addition of the organic osmolytes glutamine, betaine, proline, glycine or alanine (Dawson and Baltz, 1997). This indicates that osmotic pressure is an important component of embryo culture systems and that developmental effects are not simply due to changes in NaCl concentration.

Organic osmolytes function in the regulation of cell volume to protect protein structure and intracellular biochemical processes that would normally be disrupted by changes in the concentration of intracellular inorganic ions (Yancey *et al.*, 1982). Thus, it would seem appropriate that osmolytes be included as components of embryo culture media. Like Dawson and Baltz (1997), Biggers *et al.* (1993) found that both glutamine and betaine were able to protect the early cleavage stage mouse embryo from detrimental increases in NaCl concentration. In addition, an observed decrease in protein synthesis by mouse embryos cultured at increased concentrations of NaCl, was counteracted by culturing embryos with betaine (Anbari and Schultz, 1993). It has been suggested that taurine may function in mouse and human oocytes and embryos as an inorganic osmolyte (Dumoulin *et al.*, 1997). In contrast to the mouse, Liu and Foote (1996) found that neither betaine nor glutamine was able to protect the developing cow embryo from an increase in NaCl (95-122 mM). In the study by Liu and Foote (1996), however, the potential effect of organic osmolytes was studied only from the 4 cell stage onwards. There has in fact been very little research into the role of osmolytes during the production of cow embryos in vitro.

1.6.2 Carbohydrates

Pinyopummintr and Bavister (1996b) were the first to show that very few cow zygotes underwent the first cleavage division in the absence of exogenous energy substrates. Numerous studies on the effects of carbohydrates during culture of the cow zygote to the blastocyst stage have led to the formulation of media based on the energy requirements of the developing cow embryo.

1.6.2.1 Glucose

As was found for mouse embryos (Whitten, 1957), glucose as the sole energy source was unable to support development of cow embryos (Kim *et al.*, 1993a, 1993b; Pinyopummintr and Bavister, 1996a). Pinyopummintr and Bavister (1996b) found that increasing the concentration of glucose from 0.2 to 2.0 mM increased the proportion of cow embryos that underwent two cleavage divisions, compared with cleavage in the absence of energy substrates. Few embryos, however, were capable of development beyond the second cleavage division.

The presence of glucose in culture media has been found to be detrimental to development of cow (Ellington *et al.*, 1990; Kim *et al.*, 1993a, 1993b; Rosenkrans *et al.*, 1993), sheep (Thompson *et al.*, 1992b), hamster (Schini and Bavister, 1988; Seshagiri and Bavister, 1989b; Barnett and Bavister, 1996a) and mouse (Chatot *et al.*, 1989; Lawitts and Biggers, 1991b) embryos. The inhibitory effect of glucose on the development of hamster embryos was in fact found to be dependent on the presence of Pi in the culture media (Seshagiri and Bavister, 1989a; Schini and Bavister, 1988). Pinyopummintr and Bavister (1991) reported a similar inhibitory interaction between glucose and Pi during culture of cow embryos in HECM, however, such an interaction was not supported in a subsequent study by Kim *et al.* (1993b), following culture in

mTLP-PVA medium. While both media contained amino acids, pyruvate was not present in HECM. There is strong evidence to suggest that the inhibitory affects of glucose are somewhat dependent on the composition of the culture media. Glucose, in the presence of Pi, was reported to be detrimental to the development of mouse embryos in CZB medium (Chatot *et al.*, 1989) and Whittingham's M16 medium (Lawitts and Biggers, 1991b) but not in SOM (Lawitts and Biggers, 1992) or KSOM with amino acids, even at a concentration of 5.56 mM (Biggers *et al.*, 1997). Interestingly, in the study by Pinyopummintr and Bavister (1991), blastocyst development was equivalent in HECM which lacked glucose and Pi and in TCM-199 which contained both glucose and Pi. Thus, culture media composition is an important consideration when assessing the effects of specific media components.

The observed inhibitory effects of glucose during culture of ruminant embryos appear to be concentration dependent. Glucose concentrations of greater than 3 mM were inhibitory to development of sheep (Thompson *et al.*, 1992b) and cow embryos both in the presence (Takahashi and First, 1992; Kim *et al.*, 1993b; Furnus *et al.*, 1997) and absence (Matsuyama *et al.*, 1993) of amino acids. Furthermore, the inhibitory effects of glucose during culture of cow embryos have been found to be stage specific. The addition of glucose to medium for culture of cow embryos after Day 4 pi significantly improved development to the blastocyst stage both in the presence (Kim *et al.*, 1993b) and absence of amino acids (Matsuyama *et al.*, 1993) and increased blastocyst cell number (Matsuyama *et al.*, 1993). Kim *et al.* (1993b) reported that 2.78 to 5.56 mM glucose was optimal for development of cow embryos from Day 5 pi, however, Matsuyama *et al.* (1993) found that 1.5 mM glucose was optimal for development from Day 4 pi, with development significantly reduced at 5

mM glucose. The fact that cow embryos appear to be more sensitive to higher concentrations of glucose on Day 4 pi than from Day 5 pi onwards is supported by the study of Kim *et al.* (1993a) which reported that development of cow embryos was inhibited by the addition of 5.56 mM glucose on Day 4 pi but was improved by the addition of glucose on Day 5 pi.

Early cleavage stage cow embryos have in fact been found to be more sensitive to the concentration of glucose in culture media than the later stages of development (Kim *et al.*, 1993a; Matsuyama *et al.*, 1993). Although the early cleavage stages showed a requirement for glucose, development was largely inhibited at concentrations of 3 mM and 5 mM glucose (Matsuyama *et al.*, 1993). The optimal concentration of glucose for development from Day 0 to Day 3 pi was in fact reported to be 0.188 mM (Matsuyama *et al.*, 1993). Ellington *et al.* (1990) found that culture of cow zygotes for only the first 48 h in the absence of glucose increased subsequent blastocyst development. Similarly, Furnus *et al.* (1997) reported that culture of cow zygotes in the absence of glucose for the first 24 h improved development to the blastocyst stage. Thus, it is evident that early cleavage stage cow embryos are quite sensitive to glucose, especially during the first two cleavage divisions.

The concentration of glucose in oviduct fluid of humans (Gardner *et al.*, 1996a) and pigs (Nichol *et al.*, 1992) has been reported to be low, particularly around the time of ovulation when glucose is reduced to approximately 0.5 mM. Furthermore, the concentration of glucose in mouse oviduct fluid was reduced in the presence of cumulus cells which surround the oocyte and early embryo, possibly through the use of glucose by cumulus cells as an energy source (Gardner and Leese, 1990). In addition, the concentration of glucose in uterine fluid has been reported to be higher

than that found in the oviduct (Gardner *et al.*, 1996a), indicating that the embryo is in fact exposed to a higher glucose concentration beyond the 8- to 16-cell stage than during the early cleavage stages of development.

The complete removal of glucose for the optimization of culture media for development of cow embryos from the zygote to the 8- to 16-cell stage (Pinyopummintr and Bavister, 1996a) does not appear to be warranted and could in fact be detrimental to embryo development (Matsuyama *et al.*, 1993). Glucose is present in the cow oviduct at a concentration of up to 0.2 mM (0.06 to 0.09 mM reported by Carlson *et al.*, 1970; 0.1 to 0.2 mM reported by Parrish *et al.*, 1989a). Furthermore, cow zygotes have developed to the morula and blastocyst stages in the oviducts of sheep (Eyestone *et al.*, 1987) and rabbits (Hamner, 1973; Leese, 1988) where glucose is present. Together, these would suggest that glucose is required by early cleavage stage cow embryos, albeit at very low levels. As suggested by Gardner (1998b), the role of glucose during development of the cleavage stage embryo is likely to be biosynthetic. Studies in the cow (Javed and Wright, 1991), mouse (O'Fallon and Wright, 1986) and pig (Flood and Wiebold, 1988) have revealed that a significant proportion of glucose is metabolized through the PPP in early cleavage stage embryos (see section 1.8.2). The metabolism of glucose via the PPP results in the generation of ribose moieties for the synthesis of nucleic acids as well as NADPH which is used as a reductant during the synthesis of fatty acids. In addition, glucose would be required for the formation of glycoproteins and the synthesis of triacylglycerols.

1.6.2.2 Pyruvate and lactate

Determinations of the concentration of lactate in mammalian oviductal fluids have varied among species with approximately 3.5 mM found in the cow (Restall and Wales, 1966), sheep (Restall and Wales, 1966) and rabbit (Leese, 1988), 4.79 mM in the mouse (Gardner and Leese, 1990), 6.48 mM in the pig (Nichol *et al.*, 1992) and 10.5 mM in the human (Gardner *et al.*, 1996a). Levels of pyruvate in mammalian oviductal fluids have been found to be more uniform across species with pyruvate reported to be at approximately 0.3 mM in oviductal fluids of mice, rabbits, pigs and humans (Leese, 1988; Gardner and Leese, 1990; Nichol *et al.*, 1992; Gardner *et al.*, 1996a). The concentration of pyruvate in fluids of the cow reproductive tract, however, has not been reported.

Pinyopummintr and Bavister (1996b) demonstrated that, as the sole energy substrate, lactate (4.5 mM) was able to support 37.2% of inseminated cow oocytes through the first three cleavage divisions, equivalent to development in TCM-199. Similarly, Kim *et al.* (1993b) reported that lactate (10 mM), as the sole carbohydrate, supported 34% of inseminated ova to the morula stage by 144 h pi and 10% to the blastocyst stage by 192 h pi. Although the culture medium contained amino acids, no zygotes underwent the first cleavage division with amino acids as the only energy source (Kim *et al.*, 1993b). Cow zygotes are thus different to mouse zygotes which have an absolute requirement for pyruvate (or oxaloacetate) to undergo the first cleavage division (Whitten, 1957). Lactate was also able to support the first cleavage division of hamster embryos (McKiernan *et al.*, 1991).

Pyruvate (0.25 mM) was able to support development of cow embryos through the first three cleavage divisions to a rate that was similar to that observed for lactate

(Pinyopummintr and Bavister, 1996b). Likewise, culture of cow zygotes with pyruvate or lactate at 0.5 mM and 10 mM, respectively (Kim *et al.*, 1993b) and pyruvate or lactate at 0.3 mM and 3.3 mM, respectively (Takahashi and First, 1992) as the sole carbohydrate, resulted in equivalent development to the morula (Takahashi and First, 1992) and blastocyst stages (Kim *et al.*, 1993b). Culture of cow embryos with pyruvate as the sole energy source at a concentration of 1 mM, however, inhibited development of embryos to the blastocyst stage (Rosenkrans *et al.*, 1993). This is in contrast to mouse embryos in which development was found to be unaffected (Cross and Brinster, 1973) or improved (Kouridakis and Gardner, 1995) by increasing pyruvate to 1 mM.

The concentrations of both pyruvate and lactate in culture media will ultimately affect the intracellular concentrations of each substrate, influencing the intracellular levels of NADH and NAD^+, respectively. The ratio of NADH:NAD^+ represents the oxidation/reduction potential or redox state within the embryo, possibly affecting embryo metabolism. As discussed in Section 1.8.3, it is not known whether embryos have the appropriate or active NADH/NAD^+ shuttles to maintain an appropriate redox equilibrium between cytoplasmic and mitochondrial compartments. If this were the case, then extracellular concentrations of pyruvate and lactate would be critical to the intracellular ratio of NADH:NAD^+. Cross and Brinster (1973) found that the ratio of lactate:pyruvate was in fact a critical parameter for development of mouse embryos, with optimal development at a ratio of approximately 10:1. Similarly, a lactate:pyruvate ratio of 10:1 was reported to be optimal for sheep embryo development (Thompson *et al.*, 1993). For the culture of cow embryos, Edwards *et al.* (1997) found that the optimal concentration of lactate in SOFaa ranged from 3.3 to

13.2 mM, with the lactate:pyruvate ratio ranging from 10:1 to 40:1. Conversely, Thompson *et al.* (1993) found that a concentration of lactate greater than 10 mM and a lactate:pyruvate ratio of 30:1 impaired development of sheep embryos (Thompson *et al.*, 1993). This contrast may be due to differences in the amino acid composition of SOF medium between the two studies, with sheep embryos cultured in the presence of Eagle's non-essential amino acids (Thompson *et al.*, 1993) and cow embryos cultured in the presence of both Eagle's non-essential and essential amino acids (Edwards *et al.*, 1997). Thompson *et al.* (1993) in fact showed that the presence of different energy substrates, including amino acids, affected the rate of [1-^{14}C] pyruvate utilization. Alternatively, it has been suggested that the lactate:pyruvate ratio during culture of cow embryos could be less important than the total concentrations of lactate and pyruvate (Edwards *et al.*, 1997; Rosenkrans *et al.*, 1993).

1.6.3 Gas Phase and Antioxidants

1.6.3.1 Carbon Dioxide

Together with bicarbonate ions, CO_2 is routinely used to buffer embryo culture media for the development of embryos in vitro. Typically 5% CO_2 is used in conjunction with 25 mM bicarbonate to maintain an extracellular pH (pHo) of 7.3 to 7.4. Carney and Bavister (1987b) found that doubling CO_2 from 5% to 10% significantly increased development of 8-cell hamster embryos to the blastocyst stage. The observed stimulatory effect on embryo development was not due to changes in pHo, as adjusting bicarbonate to maintain a pHo of 7.4 while increasing CO_2 to 10%, had an equivalent effect in stimulating embryo development. Carney and Bavister

(1987b) hypothesised that the stimulatory effects of an increased concentration of CO_2 were due to CO_2 acting as a weak acid, facilitating the regulation of intracellular pH (pHi) in the embryo. This was supported by the fact that culture at 5% CO_2 with the non-metabolizable weak acid DMO improved embryo development to rates equivalent for culture at 10% CO_2 in the absence of DMO (Carney and Bavister, 1987b). DMO has recently been shown to decrease the pHi of the early cleavage stage mouse embryo (Edwards *et al.*, 1998a, 1998b).

Hallden *et al.* (1992) found that increasing CO_2 from 5% to 10% was beneficial for development of the rabbit embryo. In contrast, Vajta *et al.* (1997) reported that increasing CO_2 from 5% to 7.5% significantly decreased development of cow embryos to the blastocyst stage. In the study by Vajta *et al.* (1997), however, embryos were cultured in a co-culture system with serum increased from 5% to 10% on Day 4 pi. The effects of an increased concentration of CO_2 were likely to have been affected by the presence of somatic cells and serum which may alter pH. Thus, the role of CO_2 during culture of the cow embryos in a somatic cell-free system remains undetermined.

1.6.3.2 Oxygen

The oxygen tension of mammalian reproductive tracts has been found to be substantially lower than atmospheric O_2 (21%). Oxygen tensions ranging from 25 - 60 mm Hg (3.5 - 8.5% O_2) were found in the reproductive tracts of the rabbit and hamster, while O_2 tension was found to fall below 2% O_2 in the reproductive tract of the rhesus monkey (Fischer and Bavister, 1993). Culture of embryos at 5% to 10% O_2, compared with culture at atmospheric concentrations of O_2 significantly improved development of rabbit (Li and Foote, 1993; Lindenau and Fischer, 1994), hamster

(McKiernan and Bavister, 1990), mouse (Whitten, 1971; Gardner and Lane, 1996), rat (Kishi *et al.*, 1991), cow (Nakao and Nakatsuji, 1990; Thompson *et al.*, 1990; Fukui *et al.*, 1991; Liu and Foote, 1995a; Fujitani *et al.*, 1996; Lonergan *et al.*, 1999b), goat (Batt *et al.*, 1991), sheep (Tervit *et al.*, 1972; Thompson *et al.*, 1990; Bernardi *et al.*, 1996) and human (Gardner *et al.*, 1999a) embryos. Furthermore, culture at a reduced oxygen concentration increased embryo cell number in the rabbit (Li and Foote, 1993), mouse (Gardner and Lane, 1996), cow (Thompson *et al.*, 1990; Lonergan *et al.*, 1999b), goat (Batt *et al.*, 1991) and sheep (Thompson *et al.*, 1990). The optimal O_2 concentration for the development of cow embryos from Day 5 to Day 7 pi was in fact reported to be as low as 2% O_2 (Thompson *et al.*, 2000). In contrast, 20% O_2 was required for embryos produced in co-culture systems using complex culture media (Fukui *et al.*, 1991; Voelkel and Hu, 1992; Watson *et al.*, 1994). This indicates that the influence of oxygen concentration on embryo development is in fact affected by culture conditions. Furthermore, reducing oxygen concentration from 20% during either IVM or IVF of cow oocytes has been shown to be detrimental to the maturation and fertilization processes (Pinyopummintr and Bavister, 1995).

One possible mechanism for the effect of oxygen concentration on embryo development is the evident effect of oxygen concentration on embryo metabolism. In mice, a reduction in oxygen concentration from 20% O_2 increased the catabolic utilization of glucose by morulae and blastocysts, as evidenced by the increased production of CO_2 and lactate (Khurana and Wales, 1989). Culture of sheep embryos at 5% O_2 on Days 3 and 6 pi reduced the amount of [U-^{14}C] glucose converted to lactate, compared with culture at 20% O_2 (Du and Wales, 1993a). Another possible cause of the observed effects of atmospheric concentrations of oxygen during culture

of mammalian embryos is the toxic accumulation of ROS. While cells require oxygen, oxidative metabolism results in the formation of ROS such as superoxide anion and hydrogen peroxide. ROS are toxic to cells, resulting in damage to DNA, alterations in protein structure and function and peroxidation of lipid membranes (Fridovich, 1976; Freeman and Crapo, 1982). ROS can also affect pHi (Shibanuma *et al.*, 1988; Ikebuchi *et al.*, 1991). Interestingly, hydrogen peroxide was recently shown to inhibit development of in vitro produced cow embryos (Morales *et al.*, 1999). ROS have in fact been found to increase during development of embryos in vitro (Nasr-Esfahani *et al.*, 1990a; Manes, 1992; Goto *et al.*, 1993) to levels higher than those found in vivo (Nasr-Esfahani *et al.*, 1990a; Johnson and Nasr-Esfahani, 1994).

1.6.3.3 Antioxidants

Aerobic organisms have developed intracellular mechanisms to counteract the toxic effects of ROS. These mechanisms involve antioxidants such as SOD, catalase, glutathione and taurine. While both mouse and cow embryos express the genes for several antioxidant enzymes including catalase, glutathione peroxidase (Harvey *et al.*, 1995) and SOD (Harvey *et al.*, 1995; Wrenzycki *et al.*, 2000), the environments of the oviduct and uterus likely play a significant role in the provision of antioxidants for the developing embryo (Harvey *et al.*, 1995). Reports of the effects of supplementation of culture media with antioxidants on embryo development are variable. The addition of SOD to culture media has been reported to alleviate the inhibitory effects of culture at atmospheric concentrations of oxygen on the development of mouse embryos (Noda *et al.*, 1991; Nonogaki *et al.*, 1991). Other studies, however, have found that SOD did not have a beneficial effect during culture of mouse embryos (Legge and Sellens, 1991; Payne *et al.*, 1992). As suggested by Payne *et al.* (1992), conflicting reports as

to the effects of SOD during culture are likely due to differences in the sensitivities of various strains of mice to ROS. Similarly, conflicting reports of the effects of SOD during culture of cow embryos are possibly due to the media in which embryos were cultured. Fujitani *et al.* (1996) found a beneficial effect of SOD during culture of cow embryos in TCM-199, whereas studies determining the effects of SOD in media designed for embryo culture, specifically KSOM and SOF with non-essential amino acids and citrate, found no beneficial effects of SOD (Liu and Foote, 1995a; Luvoni *et al.*, 1996, respectively). Similarly, taurine and its precursor hypotaurine improved development of cow embryos cultured at 20% O_2 to levels observed following culture at reduced concentrations of oxygen in TCM-199 (Fujitani *et al.*, 1996) and KSOM (Liu and Foote, 1995a). A recent study by Lonergan *et al.* (1999b), however, found no benefit from culturing cow embryos in SOF with taurine at a concentration within the range previously reported to have a beneficial effect (Fujitani *et al.*, 1996). Thus, culture conditions may well be a significant factor when determining the effects of antioxidants during embryo culture.

In contrast to SOD and taurine, there have been no reports to date of a beneficial effect of catalase during embryo culture (Legge and Sellens, 1991; Payne *et al.*, 1992). The reduced form of glutathione, GSH, however, was shown to reduce levels of hydrogen peroxide in mouse embryos (Nasr-Esfahani *et al.*, 1990a). In addition, the inhibition of GSH synthesis in cow embryos resulted in an increase in intracellular hydrogen peroxide and damage to DNA (Takahashi *et al.*, 1997). Supplementing culture media with GSH improved development of both mouse (Legge and Sellens, 1991; Gardiner and Reed, 1994) and cow embryos (Luvoni *et al.*, 1996). Furthermore, the addition of β-mercaptoethanol to culture medium, which increases intracellular

glutathione, significantly improved development of cow embryos to the blastocyst stage following culture at 20% O_2 but not at 5% O_2 (Caamano *et al.*, 1998). Pyruvate may also have a role as an antioxidant for mammalian embryos. Pyruvate has been shown to function as an antioxidant in somatic cells through its direct reaction with hydrogen peroxide (Andrae *et al.*, 1985). Interestingly, pyruvate has been reported to reduce levels of hydrogen peroxide in both cow (Morales *et al.*, 1999) and mouse embryos (Kouridakis and Gardner, 1995; O'Fallon and Wright, 1995). Morales *et al.* (1999) found that, in the absence of pyruvate (0.33 mM), cow embryos were sensitive to increased levels of hydrogen peroxide, particularly up to the 8-cell stage and at the blastocyst stage. Although not as effective as pyruvate, non-essential and essential amino acids were also found to reduce levels of hydrogen peroxide in culture media (Morales *et al.*, 1999).

Thus, while antioxidants appear to be an important component of media for the optimal culture of mammalian embryos, the effectiveness of antioxidants is likely linked to other factors regulating embryo development such as species, strain and culture conditions.

1.6.4 Protein Sources and Macromolecules

A variety of protein sources have been used in the culture of mammalian embryos. Different fractions of serum and serum albumin from a variety of species are routinely added to culture media, making it difficult to compare results between studies. As well as acting as surfactants to facilitate the manipulation of embryos during culture, serum and serum albumin preparations are likely to contain growth factors, vitamins, fatty acids and amino acids (Maurer, 1992; Walker *et al.*, 1996) and

may act as chelating agents for toxic contaminants such as metal ions (Van Winkle and Campione, 1982).

Albumin is the most abundant protein in the mammalian reproductive tract and has thus been considered by many to be a suitable additive to media for the culture of mammalian embryos. Culture with BSA at a reduced concentration of oxygen (7%), in SOF containing amino acids, has resulted in comparatively high percentages of blastocyst development in both the cow (Edwards *et al.*, 1997; Gardner *et al.*, 2000a) and sheep (Gardner *et al.*, 1994b; Walker *et al.*, 1996). Krisher *et al.* (1999) recently reported that cow embryos showed a requirement for BSA during the final stages of culture (from 72 h to 168 h culture) but not during the first 72 h culture from the zygote stage. The reason for this has not been established but may be reflective of the beneficial effects of energy sources attached to the BSA such as citrate (Gray *et al.*, 1992) at a time when energy production has been shown to increase in the cow embryo (Thompson *et al.*, 1996).

A number of studies have reported that the addition of serum to culture media, either protein-free media or media containing serum albumin, improved development of ruminant embryos compared with culture with serum albumin alone (Thompson *et al.*, 1992a; Lim *et al.*, 1994; Salamone *et al.*, 1995; Jones and Westhusin, 1996; Van Langendonckt *et al.*, 1997; Thompson *et al.*, 1998a). Serum appears to have a biphasic effect on the cow embryo, inhibiting the first cleavage division (Pinyopummintr and Bavister, 1994) but stimulating development when added just prior to the 8- to 16-cell stage at 42 h pi (Pinyopummintr and Bavister, 1994; Van Langendonckt *et al.*, 1997). Despite reports of the beneficial effects of culturing ruminant embryos with serum compared with culturing with BSA, there have also been reports of no beneficial

effect of serum (Yamashita *et al.*, 1996) or an inhibitory effect of culture with serum (Milovanov and Herradon, 1996). Discrepancies among studies are likely due to a number of factors. Firstly, the effects of serum during embryo culture have been shown to vary with both the source of the serum (Fukui and Ono, 1989; Thompson *et al.*, 1992; Pinyopummintr and Bavister, 1994; Sinclair *et al.*, 1998) and with batches of serum (Price and Gregory, 1982; Pinyopummintr and Bavister, 1994). Furthermore, batch and fraction variations have been found with BSA (Batt *et al.*, 1991; McKiernan and Bavister, 1992). In addition, a number of studies that have shown a beneficial effect of culture of cow embryos with serum have reported surprisingly low rates of embryo development in control groups, with less than 10% of embryos developing to the morula and blastocyst stages (Salamone *et al.*, 1995; Jones and Westhusin, 1996; Van Langendonckt *et al.*, 1997) or less than 17% of embryos developing to the blastocyst stage (Pinyopummintr and Bavister, 1994). This indicates that the beneficial effects of serum reported in these studies could be due to sub-optimal culture conditions possibly resulting from culture of the embryos at atmospheric concentrations of oxygen (see section 1.6.3).

A number of concerns have been raised regarding the culture of embryos with serum (Gardner, 1994; Bavister, 1995; Gardner and Lane, 1997a). In vivo, embryos are not exposed to serum and while it is likely that serum contains a number of embryotrophic components, it may also contain components that are toxic to the embryo (Maurer, 1992). Furthermore, there is a growing amount of evidence to suggest that culture of embryos with serum results in morphological and metabolic abnormalities (Gardner, 1994; Gardner *et al.*, 1994b). Several studies have found that culture with serum leads to precocious blastocyst formation (Walker *et al.*, 1992;

Pinyopummintr and Bavister, 1994; Thompson *et al.*, 1995; Van Langendonckt *et al.*, 1997). Gardner *et al.* (1994b) documented both metabolic and morphological differences in sheep blastocysts following culture with serum versus media containing BSA. Blastocysts cultured in the presence of serum contained lipid-like vesicles and there was no relationship between the uptake of glucose and the production of lactate as was observed for embryos cultured with BSA, inferring that metabolism had been altered (Gardner *et al.*, 1994b). Thompson *et al.* (1995) reported that culture of sheep embryos with serum resulted in a number of perturbations including an increase in cytoplasmic lipid inclusions within the embryo, an increase in the length of gestation and an increased birth weight. Other studies have also confirmed an effect of serum on lamb birth weight (Walker *et al.*, 1992; Sinclair *et al.*, 1998). Furthermore, it has been demonstrated that cow morulae and blastocysts produced in vitro in a medium containing serum, had a larger number of lipid droplets than in vivo produced morulae and blastocysts (Plante and King, 1994; Abe *et al.*, 1999; Ferguson and Leese, 1999) and blastocysts produced in vitro in the absence of serum (Ferguson and Leese, 1999). Recently, Abe *et al.* (1999b) found that cow morulae and blastocysts cultured in the presence of serum had a larger number of lipid droplets, particularly in trophoblast cells, compared with embryos cultured in the absence of serum. Furthermore, culture of both sheep and cow embryos with serum resulted in abnormal mitochondrial ultrastructure (Dorland *et al.*, 1994; Thompson *et al.*, 1995; Abe *et al.*, 1999b) and perturbations in energy metabolism (Gardner, 1994). Interestingly, Menke and McLaren (1970) reported that serum increased oxidative metabolism in mouse embryos cultured to the blastocyst stage but not in in vivo produced blastocysts. The observed increase in oxidative metabolism could have resulted from an uncoupling of

oxidative phosphorylation in the cultured embryos because of mitochondrial damage, increasing the consumption of energy substrates without a corresponding increase in ATP production. Thompson *et al.* (1998a) reported that although culture of cow embryos with serum from Day 5 of development increased blastocyst development at Day 7 compared with culture with serum albumin, embryo viability was impaired, as evidenced by post-transfer development. Interestingly, treatment of serum with charcoal to remove fatty acids prior to embryo culture, increased blastocyst development (from 21.6% in BSA to 39.4% in serum) and resulted in equivalent embryo survival rates to those observed following culture with serum albumin (Thompson *et al.*, 1998a).

It has been suggested that both serum and serum albumin be removed from culture media and replaced with a synthetic polymer, PVA to act as a surfactant (Bavister, 1981; Liu and Foote, 1995b; Jones and Westhusin, 1996; Milovanov and Herradon, 1996). Of concern has been the fact that the effects of both BSA and serum on embryo development can vary with different batches and fractions (Price and Gregory, 1982; Batt *et al.*, 1991; McKiernan and Bavister, 1992; Pinyopummintr and Bavister, 1994). Culture with a lipid-stripped BSA or serum, however, has been shown to reduce variations in development (Bavister, 1995; Thompson *et al.*, 1998a). A further concern has been that BSA could mask the effects of media components such as amino acids in studies determining embryo requirements. BSA has been shown to be taken up by the embryo via endocytosis, thus potentially acting as a source of substrates such as amino acids and citrate (Pemble and Kaye, 1986; Gray *et al.*, 1992; Dunglison and Kaye, 1993; Thompson *et al.*, 1998b). Many previous studies, however, have reported similar developmental rates of ruminant embryos

cultured with BSA and PVA (Keskintepe *et al.*, 1995; Liu and Foote, 1995b; Jones and Westhusin, 1996; Milovanov and Herradon, 1996) indicating that embryo development was not significantly affected by amino acids contributed by BSA. Furthermore, Walker *et al.* (1996) reported that concentrations of individual amino acids were equivalent in media in the presence and absence of BSA.

There is evidence to suggest that culture of mouse embryos with PVA may result in a higher incidence of fetal loss after implantation (Gardner *et al.*, 1999b). Furthermore, Thompson *et al.* (1998b) have suggested that culture of cow embryos in protein-free media could result in metabolic perturbations within the embryo. Cow embryos cultured with PVA had a lower protein content at the blastocyst stage than embryos cultured with BSA or serum (Thompson *et al.*, 1998b). In fact, embryos cultured in the presence of protein had a similar protein content to in vivo produced embryos. The observed differences in protein content, however, were not due to different levels of protein synthesis, but rather due to the fact that embryos cultured in the presence of protein, as is the case in vivo, took up exogenous protein via endocytosis (Pemble and Kaye, 1986; Dunglison and Kaye, 1993; Thompson *et al.*, 1998). In addition, pyruvate uptake by cow embryos cultured in a protein-free medium (SOF with PVA), was found to be significantly higher than for embryos cultured in the SOF with BSA (Eckert *et al.*, 1998). Thus, care must be taken when interpreting the results of studies determining the requirements and metabolism of cow embryos cultured in protein-free systems.

In light of the documented concerns of culturing embryos in protein-free media, the recent report of a 50% (5/10) live calf rate following culture of embryos in a protein-free medium is most interesting (Holm *et al.*, 1999). Holm *et al.* (1999)

showed that, providing citrate and myo-inositol were present in SOFaa, cow blastocyst development was equivalent in medium containing PVA, BSA and serum and that embryos were in fact viable under such protein-free conditions.

A physiological alternative to serum and serum albumin is GAGS. GAGS are abundant in mammalian follicular, oviductal and uterine fluids (Ax and Ryan, 1979; Yanagishita *et al.*, 1979; Grimek *et al.*, 1984; Lee and Ax, 1984; Sato *et al.*, 1987; Sato *et al.*, 1990). GAGS such as HA have been found to promote viability of pig (Sato *et al.*, 1990) and mouse (Sato *et al.*, 1987) oocytes and reduce degeneration of pig embryos during culture (Kano *et al.*, 1998). Further, HA increased blastocyst development in pig embryos (Miyano *et al.*, 1994; Edwards *et al.*, 1998). Gardner *et al.* (1999b) found that transfer of mouse embryos with HA increased implantation rates and fetal development compared with transfer of embryos with BSA or no macromolecule. Interestingly, both BSA and HA were required during culture to support high rates of proliferation and hatching, while only HA was required in the transfer medium to support optimal rates of implantation and fetal development.

HA is primarily located on cell surfaces and is involved in cell adhesion, proliferation and differentiation (Lindahl and Hook, 1978; Hook *et al.*, 1984; Wright *et al.*, 1991). Thus HA may play a significant role in influencing cell organization and regulation and cell-cell interaction during development of the embryo and fetus. It has also been suggested that HA may bind or interact with specific growth factors, stimulating cell proliferation (Sato *et al.*, 1991). Furthermore, HA has been shown to stimulate differentiation of extra-embryonic tissue in the mouse (Hamashima, 1982) as well as having a role in embryonic morphogenesis and organogenesis through its interaction with the membrane protein CD44 (Underhill, 1992). In addition, HA was

found to stimulate protein kinase activity in intact cells (Turley, 1989). HA was also found to induce local changes in cell membrane properties by influencing the distribution of lipids or ions (Kujawa *et al.*, 1986). Further research is required to elucidate the precise mechanisms by which HA exerts its effects on oocytes and embryos during the pre- peri- and post-implantation periods. Furthermore, the potential use of HA as an alternative or an additional macromolecule in culture media for the production of cow embryos in vitro needs to be determined.

1.6.5 Chelators

Culture media and oil for overlaying culture media can contain cationic metals which may be toxic or inhibitory to embryo development (Van Winkle and Campione, 1982; Nasr-Esfahani *et al.*, 1990; Erbach *et al.*, 1995). The addition of various metal ion chelators to culture media has been found to have a beneficial effect on embryo development. Culture of mouse embryos with EDTA, a chelator of cationic metals, significantly improved development (Abramczuk *et al.*, 1977; Loutradis *et al.*, 1987; Fissore *et al.*, 1989; Mehta and Kiessling, 1990; Nasr-Esfahani *et al.*, 1992) and could in fact replace BSA (Fissore *et al.*, 1989), supporting the hypothesis that BSA acts as a chelating agent. In addition, EDTA was found to act in synergy with glutamine and Eagle's non-essential amino acids in helping mouse embryos through the 2-cell block (Gardner and Lane, 1996). Transferrin is a chelator of iron which is inhibitory to mouse embryo development (Nasr-Esfahani *et al.*, 1990b), possibly through its role in the production of hydrogen peroxide (see section 1.6.3.2). Culture of mouse embryos with transferrin alleviated the inhibitory effects of iron (Nasr-Esfahani *et al.*, 1990b; Nasr-Esfahani and Johnson, 1992), however, in the presence of amino acids and

EDTA, transferrin had no additional beneficial effects on development (Gardner and Lane, 1996). Amino acids have also been shown to act as chelating agents (Lindenbaum, 1973).

With the exception of the study by Gardner *et al.* (2000a), previous reports of the effects of EDTA during culture of cow embryos have cultured embryos with EDTA up to the blastocyst stage (Rosenkrans *et al.*, 1993; Keskintepe *et al.*, 1998). ETDA, however, exerts its beneficial effects during the first few cleavage divisions and is in fact inhibitory to development of mouse and cow embryos post-compaction (Gardner and Lane, 1996, from Gardner and Lane, 1997a). Culture of the cow embryo with EDTA for just 72 h from the zygote stage, significantly increased subsequent blastocyst cell number and the proportion of cells in the inner cell mass (Gardner *et al.*, 2000a).

It was subsequently determined that EDTA reduced glycolytic activity through inhibition of phosphoglycerate kinase, via the chelation of Mg^{2+}. Thus, the beneficial effects of EDTA during culture of the early cleavage stage embryo are perhaps due to the alleviation of a Crabtree-like effect (see section 1.8.4). EDTA, however, was inhibitory post-compaction (Gardner and Lane, 1996; Gardner *et al.*, 2000a) when glucose metabolism increases in the embryo with increased energy demands (Gardner and Leese, 1986; Rieger *et al*, 1992a, 1992b; Houghton *et al.*, 1996; Thompson *et al.*, 1996).

1.6.6 Amino Acids

The significance of the addition of amino acids to culture media was overlooked for many years. This was due to the fact that mouse embryos, cultured in balanced salt

solutions containing carbohydrates and BSA, developed well from the 1-cell and 2-cell stages to the blastocyst and could be transferred back to mice to produce live young (Whitten, 1957; McLaren and Biggers, 1958; Brinster, 1963; Brinster, 1965c; Whitten and Biggers, 1968). Thus, it was believed that amino acids were not required for development of the mammalian embryo in vitro.

There were, however, some early clues that amino acids were required by the mammalian embryo during development. Significant concentrations of amino acids were found in reproductive tract fluids of the cow (Fahning *et al.*, 1967), rabbit (Miller and Schultz, 1987), sheep (Perkins and Goode, 1967; Menezo, 1972; Menezo and Winterberger-Torres, 1976) and human (Casslen, 1987). The concentrations of amino acids found to be present in the ruminant reproductive tract are listed in Table 1.3. Of the amino acids documented in fluids of the reproductive tract, glutamate, aspartate, taurine, alanine, serine, threonine and glycine were found to be present at the highest concentrations, with glycine comprising up to 50% of the total amino acid pool (Miller and Schultz, 1987). Substantial endogenous pools of amino acids were also found in oocytes and embryos (Miller and Schultz, 1987; Schultz *et al.*, 1981). The size of endogenous amino acid pools actually increased with embryo development (Schultz *et al.*, 1981; Miller and Schultz, 1987). Further, specific transport mechanisms for amino acids were found in oocytes and embryos (Keefer and Tasca, 1984; Van Winkle, 1988; Van Winkle *et al.*, 1988). Amino acids have been reported to be secreted by perfused rabbit oviducts (Leese *et al.*, 1979). Amino acids were also found to stimulate hatching and outgrowth of the pre-implantation mouse embryo cultured from the blastocyst stage (Spindle and Pedersen, 1972) and

Table 1.3 Composition of amino acids in cow and sheep reproductive tract fluids and in Eagle's non-essential and essential amino acid groups.

Amino acid (mM)	Cow uterine fluid[a]	Sheep oviductal fluid[b]	Non-essential[c]	Essential[c]
Alanine	0.98	0.5	0.10	-
Arginine	not determined	0.1	-	0.60
Asparagine	0.07*	0.02	0.10	-
Aspartic acid	0.37	0.02	0.10	-
Citrulline	not determined	0.15	-	-
Cystine	trace	not determined	-	0.10
Glutamic acid	1.38	0.05	0.10	-
Glutamine	0.07*	0.21		2.00[†]
Glycine	3.27	1.50	0.10	-
Histidine	not determined	0.05	-	0.20
Isoleucine	0.15	0.10	-	0.40
Leucine	0.24	-	-	0.40
Lysine	0.06	2.20	-	0.40
Methionine	0.08	0.05	-	0.10
Ornithine	not determined	0.02	-	-
Phenylalanine	0.10	0.10	-	0.20
Proline	0.20	0.05	0.10	-
Serine	0.16	0.01	0.10	-
Taurine	1.69		-	-
Threonine	0.04	0.01	-	0.40
Tryptophan		-	-	0.05
Tyrosine	0.12	0.01	-	0.20
Valine	0.30	0.27	-	0.40

[a]determined by Fahning *et al.* (1967) on Day 5 to Day 7 of oestrus.

[b]determined by Nancarrow *et al.* (1992).

[c]classification of non-essential and essential amino acids by Eagle (1959).

*asparagine and glutamine measured together.

[†]glutamine is typically excluded from this group for the determination of the role of essential amino acids during embryo culture.

were required by the rabbit blastocyst from the morula stage onwards for blastocyst formation (Kane and Foote, 1970).

1.6.6.1 Development

The first extensive studies of the amino acid requirements of the embryo were in the hamster. Initial studies revealed that the amino acids glutamine, isoleucine, methionine and phenylalanine stimulated nuclear maturation of the hamster oocyte (Gwatkin and Haidri, 1973). Subsequently, the addition of these four amino acids to a simple medium containing lactate, pyruvate and BSA supported development of the hamster embryo through the first cleavage division, but could not overcome the 2-cell block to development (Juetten and Bavister, 1983). These four amino acids were also found to have a positive effect on development beyond the 8-cell stage, with 36% of in vivo produced 8-cell embryos developing to the blastocyst stage, compared with 2% in the absence of amino acids (Bavister *et al.*, 1983). The amino acid responsible for the stimulatory effect was in fact glutamine (Carney and Bavister, 1987a). Glutamine was not stimulatory at a concentration of 0.1 mM, but was required at 1.0 mM. Glutamine (0.2 mM), as the sole amino acid, was in fact able to support development of the hamster embryo from the 1-cell stage to the blastocyst, in HECM-3 containing salts, lactate and no protein (McKiernan *et al.*, 1991). It was subsequently determined that the hamster embryo had a greater requirement for amino acids. McKiernan *et al.* (1995) found that the combination of glutamine with glycine and taurine, two amino acids present at high concentrations in fluid of the reproductive tract, increased development of the 1-cell hamster embryo to the blastocyst stage. Further improvement in development required the addition of a group of eight amino acids to glutamine, glycine and taurine (McKiernan *et al.*, 1995).

The eight amino acids included asparagine, aspartate, serine, glutamate, histidine, lysine, proline and cysteine.

The study by Carney and Bavister (1987a) was the first indication that certain amino acids may in fact inhibit embryo development. In a subsequent study, McKiernan *et al.* (1995) found that, at a concentration of 0.5 mM, leucine, tyrosine, valine, methionine, phenylalanine, arginine, cysteine and isoleucine all inhibited development of 1-cell hamster embryos to the blastocyst stage. Isoleucine, phenylalanine and tryptophan were inhibitory even at a concentration of 0.05 mM. Three of these amino acids, methionine, phenylalanine and isoleucine, were not in fact inhibitory beyond the 8-cell stage (Bavister *et al.*, 1983) suggesting that the hamster embryo changes its requirements for amino acids with development.

The studies on the effects of amino acids during culture of the hamster embryo raised interest in the potential effects of amino acids in the culture of embryos from other mammalian species. Zhang and Armstrong (1990) found that culture of the rat embryo from the 8-cell stage with 13 amino acids and insulin, not only improved embryo development, but significantly increased fetal development following embryo transfer. The amino acids found to stimulate development and viability of the rat embryo were those found in the essential group of Eagle's amino acids. Eagle's essential and non-essential amino acid groups (Table 1.3) were identified by Eagle (Eagle, 1959) as being required (essential) or not required (non-essential) in culture medium for the maintenance and growth of somatic cells. The majority of amino acids found at the highest concentrations in mammalian reproductive tract fluids, with the exception of taurine and threonine, are components of Eagle's non-essential amino acid group (Table 1.3). The concentration of many of the amino acids present in

Eagle's essential group are much higher than concentrations found in the reproductive tract (Table 1.3). Interestingly, the amino acids found to inhibit development of the hamster embryo from the 1-cell stage (isoleucine, cysteine and phenylalanine) were all components of Eagle's essential amino acid group (Table 1.3).

Based on a physiological approach, Gardner and Lane (1993a) studied the effects of Eagle's non-essential and Eagle's essential amino acid groups, in the presence and absence of glutamine, on development of the mouse embryo in culture. Due to reports of the stimulatory effect of glutamine as the sole amino acid during embryo culture, glutamine is often not included in Eagle's essential amino acid group, but rather tested as a separate media component. Gardner and Lane (1993a) found that culture of F1 mouse embryos from the 1-cell stage with all twenty amino acids present in Eagle's amino acid groups (including glutamine), significantly increased blastocyst development, cell number and hatching. The positive effects of culture with Eagle's amino acid groups were found to be due to the stimulatory effects of the non-essential amino acids during the first 72 h culture (Gardner and Lane, 1993a). Interestingly, in the absence of glutamine, culture with the essential amino acids decreased blastocyst cell number. It was subsequently determined that the combination of glutamine and the non-essential amino acids stimulated development of the early mouse embryo by decreasing the time of the first two cleavage divisions (Lane and Gardner, 1997b).

Mehta and Kiessling (1990) reported that culture of the mouse embryo to the 4-cell stage with Eagle's amino acids improved post-implantation development only in the presence of 100 μM EDTA. Gardner and Sakkas (1993) found that culture of the mouse embryo for three days with the non-essential and essential amino acids, glutamine, MEM vitamins, insulin, transferrin and EGF significantly increased fetal

development following transfer. Lane and Gardner (1994) subsequently determined that culture of the mouse embryo with glutamine and Eagle's non-essential amino acids for the first 48 h, significantly increased post-implantation development without requiring the presence of vitamins or growth factors. Interestingly, culture with all of Eagle's amino acids during the first 48 h significantly decreased fetal development following transfer, compared with culture with just glutamine and Eagle's non-essential amino acids. Thus, as found with culture of the hamster embryo, either some or all of the components of Eagle's essential amino acids were inhibitory to the mouse embryo during the first 48 h culture.

Like the hamster, the mouse embryo also changes its requirements for amino acids with development. Lane and Gardner (1997a) determined the effect of culturing mouse embryos with different amino acid groups during both the first and the second 48 h culture. It was established that optimal blastocyst viability was obtained following culture for the first 48 h with glutamine and Eagle's non-essential amino acids, followed by culture for the second 48 h with all twenty of Eagle's amino acids. In addition, each amino acid group was found to have quite specific functions in the development of the mouse embryo. During culture from the 8-cell stage to the blastocyst, glutamine and the non-essential amino acids stimulated blastocyst formation and hatching while the essential amino acids (no glutamine present) increased blastocyst cell number and allocation of cells to the ICM. This study highlighted the importance of using a two-step culture system to evaluate the requirements of the developing embryo.

The in vitro produced ruminant embryo has also been shown to have a requirement for amino acids during development. Culture of both sheep and cow

embryos with amino acids significantly increased the development of embryos to the morula and/or blastocyst stages, when compared to culture in the absence of amino acids (Takahashi and First, 1992; Kim *et al.*, 1993b; Gardner *et al.*, 1994b; Rosenkrans and First, 1994; Liu and Foote, 1995b; Keskintepe *et al.*, 1995; Pinyopummintr and Bavister, 1996a). Rosenkrans and First (1994) found that culture with either the non-essential or the essential amino acids improved development of cow embryos from the 1-cell stage to the blastocyst. Liu and Foote (1995b), however, found that development of the cow embryo to the blastocyst was greatest in the presence of both the non-essential and the essential amino acids (no glutamine present), with respect to culture with individual groups. Embryos were cultured, however, from only the 4 -cell stage and thus any influence of amino acids during the first two cell divisions was not determined. Lee and Fukui (1996) found that, in the presence of the non-essential and essential amino acids, glutamine was not required for optimal cow blastocyst development. Again, development was determined only from the 2- to 4-cell stages.

Moore and Bondioli (1993) reported that two amino acids present at high concentrations in fluids of the mammalian reproductive tract, alanine and glycine, independently stimulated development of cow embryos from the 1-cell stage to the blastocyst. This was later confirmed by Lee and Fukui (1996). The concentrations of alanine (1 and 5 mM) and glycine (10 mM) found to be stimulatory, however, were approximately ten times the concentrations of these amino acids found in the ruminant reproductive tract and ten to fifty times and one hundred times the concentrations of alanine and glycine found in Eagle's non-essential amino acids (Table1.3), respectively (Moore and Bondioli, 1993; Lee and Fukui, 1996). Interestingly, BOEC

were found to produce significant amounts of glycine, alanine and proline over a 48 h period (Moore and Bondioli, 1993), with concentrations similar to those found in Eagle's non-essential amino acids. Culture of cow embryos with concentrations of amino acids present in sheep oviductal fluid (Table 1.3) resulted in a higher blastocyst development than culture with Eagle's amino acid groups (Hill *et al.*, 1997). The Eagle's amino acid solution, however, did not contain glutamine (unlike the oviductal fluid solution) and blastocyst cell numbers were very low, with less than 60 cells per blastocyst following culture with either amino acid solution. Interestingly, there was no difference in embryo viability following culture with oviductal amino acids and Eagle's amino acids (Hill *et al.*, 1997).

As already mentioned, some recent studies have limited the culture period with amino acids to after the first two or three cleavage divisions, rendering the determination of amino acid requirements of the cow embryo incomplete. Furthermore, previous studies to date have failed to address the fact that amino acid requirements potentially change with development of the cow embryo. Pinyopummintr and Bavister (1996a) reported the effects of different amino acid groups during the first 54 h culture, however, subsequent development to the blastocyst stage was in a complex medium (TCM-199) with serum. Interestingly, culture of the cow embryo for 54 h from the zygote, with glutamine or the non-essential amino acids and glutamine, stimulated cleavage to the 8-cell stage and subsequent blastocyst development. The presence of the essential amino acids actually negated the stimulatory effects of glutamine and the non-essential group. Importantly, Partridge and Leese (1996) found that the rate of depletion of individual amino acids from culture medium changed with development of the cow embryo. Thus, there is a

need to determine the amino acid requirements of both the early cleavage stage embryo and the later stages of development.

Despite the fact that amino acids are required by the mammalian embryo, culture with amino acids can result in a deleterious effect. Both the metabolism of amino acids by the embryo and the spontaneous breakdown of amino acids in culture media at 37°C, result in a significant build-up of ammonium in the culture medium (Gardner and Lane, 1993a). The resultant ammonium has been shown to inhibit development and viability of the mouse embryo (Gardner and Lane, 1993a; Lane and Gardner, 1994). Culture of mouse embryos in the presence of ammonium was also associated with an increase in fetal exencephaly (Lane and Gardner, 1994). Lane and Gardner (1994) found that the toxic effects of ammonium could be alleviated by renewing culture media containing amino acids every 48 h. Ammonium has in fact been shown to inhibit the oxidative metabolism of acetyl-CoA (Maddaiah and Kumbar, 1994) which could result in a decrease in ATP production in the embryo.

There has been some question as to whether ruminant embryos are as susceptible to ammonium toxicity as mouse embryos. Gardner and Lane (1993) reported that culture of sheep embryos in medium containing amino acids for six days from the 1-cell stage, without renewal of the medium, resulted in significantly more embryos arresting at the 8- to 16-cell stages. Studies determining the effects of ammonium during culture of cow embryos, however, have been conflicting. Fukui *et al.* (1996) and Ikeda (2000) found no evidence of an inhibitory effect of an increase in ammonium during culture of cow embryos with amino acids. Hammon *et al.* (2000) reported that culture of cow embryos with ammonium increased the number of degenerative ova and hence decreased the proportion of ova developing to the

blastocyst stage. In the study by Fukui *et al.* (1996), the effects of ammonium during culture of cow embryos were assessed from the 2- to 4-cell stages. Thus, cow embryos may be sensitive to ammonium during the first cleavage division. Interestingly, the addition of ammonium to medium during in vitro fertilization of cow oocytes significantly increased blastocyst development (Hammon *et al.*, 2000). Furthermore, the addition of ammonium to medium during in vitro maturation, fertilization and culture significantly improved development to the morula stage, suggesting that the effects of ammonium during culture of cow embryos are in fact dependent on the developmental stage at which cow oocytes/embryos are exposed to ammonium.

1.6.6.2 Role of Amino Acids

There have been numerous suggestions as to the role of amino acids in sustaining growth and development and maintaining viability during culture of mammalian embryos (Table 1.4).

1.6.6.2.1 Biosynthesis

Amino acids are the basis of proteins and undoubtedly have a significant anabolic function in the synthesis of new proteins, particularly when protein synthesis increases during embryo development (Frei *et al.*, 1989).

1.6.6.2.2 Energy substrates

Amino acids may be used catabolically by the embryo as energy substrates, through their conversion into major metabolic intermediates, such as fatty acids and ketone bodies. Glutamine has been found to be an important medium component for

Table 1.4 Possible functions of amino acids during culture of in vitro produced mammalian embryos.

Function	References
Biosynthesis	Crosby *et al.* (1988); Thompson *et al.* (1998); Chandolia *et al.* (1999).
Energy Substrates	Petters *et al.* (1990); Rieger *et al.* (1992a, 1992b).
Regulation of Metabolism	Gardner and Lane (1993b); Vella *et al.* (1997).
Regulation of pHi	Edwards *et al.* (1998b).
Osmolytes	Van Winkle *et al.* (1990); Lawitts and Biggers (1992); Biggers *et al.* (1993); Dawson and Baltz (1997); Dumoulin *et al.* (1997).
Antioxidants	Liu and Foote (1995b); Fujitani *et al.* (1996); Guyader-Joly *et al.* (1997); Morales *et al.* (1999).
Chelators	Lindenbaum (1973).

maturation of rabbit oocytes (Bae and Foote, 1975), culture of mouse (Chatot *et al.*, 1989; Gardner and Lane, 1996), hamster (McKiernan *et al.*, 1991) and pig embryos (Petters *et al.*, 1990) through developmental blocks and stimulation of the early cleavage stage cow embryo (Pinyopummintr and Bavister, 1996a). Glutamine has been shown to be used as a major energy source for some somatic cells and can even replace glucose as an energy source (Zielke *et al.*, 1978; Reitzer *et al.*, 1979). Petters *et al.* (1990) found that glutamine could in fact replace glucose as an energy substrate for the developing pig embryo. Significant amounts of glutamine are in fact taken up by the cow embryo (Rieger *et al.*, 1992b; Partridge and Leese, 1996), with the majority metabolized through the TCA cycle (Rieger *et al.*, 1992b) (see section 1.8.1). Thus glutamine, as well as other amino acids, appears to be an important energy source for the developing cow embryo.

1.6.6.2.3 Regulators of metabolism

As well as stimulating metabolism, there is evidence to suggest that amino acids could function in the regulation of cellular metabolic activity in the embryo. Alanine has been shown to be an inhibitor of pyruvate kinase which is a rate limiting inhibitor of glycolysis (Feliu and Sols, 1976; Giles *et al.*, 1977; Gosalvez *et al.*, 1978). Interestingly, the amino acids phenylalanine, alanine and cysteine were found to increase respiration and decrease glycolytic activity in ascites tumour cell suspensions, via the inhibition of pyruvate kinase (Gosalvez *et al.*, 1975). An increase in cellular respiration results in an increase in the production of ATP, a known inhibitory effector of pyruvate kinase (Giles *et al.*, 1977; Gosalvez *et al.*, 1978; Torres *et al.*, 1984) and PFK (Gosalvez *et al.*, 1978; Torres *et al.*, 1984). An increase in the production of ATP by ascites tumour cells, in the presence of glutamine and

asparagine, was in fact found to inhibit glycolysis via the allosteric inhibition of PFK (Gonzales-Mateos *et al.*, 1993).

Gardner and Lane (1993b) found that Eagle's non-essential amino acids and glutamine reduced glycolytic activity and increased pyruvate oxidation in CF1 mouse embryos at the 2-cell stage. The observed regulation of metabolism in the presence of amino acids was associated with an improved development through the 2-cell block. Thus, there is evidence to suggest that specific amino acids may function in the alleviation of a potentially inhibitory Crabtree-like effect (see section 1.8.4). In further support of this theory, Barnett and Bavister (1996a) found that the presence of amino acids alleviated the observed inhibitory effects of Pi and glucose on development of the early cleavage stage hamster embryo. It has also been shown that Eagle's non-essential amino acids and glutamine affected glycolytic activity of F1 mouse 4- to 8-cell embryos by decreasing both glucose flux and the Km of glycolysis (Vella *et al.*, 1997). It is currently not known whether amino acids play a role in the regulation of metabolism in the cow embryo.

1.6.6.2.4 Regulators of intracellular pH

There is recent evidence that amino acids have a function in the regulation of pHi in the embryo. It has been suggested that the oviductal environment plays an important role in the regulation of pHi in the mammalian embryo and that culture of embryos in unfavourable conditions may result in a detrimental increase in pHi (Bavister, 1995). It has been hypothesised that culture of embryos in the absence of amino acids could lead to a mass efflux of amino acids and protons from the embryo, resulting in an increase in pHi (Bavister and McKiernan, 1993; Bavister, 1995). To

date, however, there have been no reports of the effects of extended culture of the mammalian embryo under different conditions, on pHi.

The pHi of early cleavage stage mouse and hamster embryos is affected by the presence of weak acids in culture media (Carney and Bavister, 1987b; McKiernan and Bavister, 1990; Edwards *et al.*, 1998a, 1998b). Prior to compaction, the mouse embryo is unable to recover from an acid load (Edwards *et al.*, 1998a, 1998b). Early studies indicated that the cleavage stage mouse embryo lacked the Na^+/Cl^- antiporter which is one of the mechanisms by which somatic cells remove excess protons (Baltz *et al.*, 1990; Baltz *et al.*, 1991). There is recent evidence, however, to show that the antiporter is in fact present in human, hamster and cow cleavage stage embryos (Lane *et al.*, 1998; Lane and Bavister, 1999; Lane *et al.*, 1999; Phillips *et al.*, 2000). Bavister and McKeirnan (1993) suggested that amino acids could act as intracellular buffers of pHi due to their zwitterionic nature. Edwards *et al.* (1998b) have recently shown that amino acids can function as buffers of pHi for in vivo produced, early cleavage stage mouse embryos in response to an increasing acid load. Although the HCO_3^-/Cl^- exchanger, used for the regulation of pHi in the alkaline range, is present in the early cleavage stage mouse embryo (Phillips and Baltz, 1999), amino acids were also found to act as buffers in response to an increasing alkaline load (Edwards *et al.*, 1998b). The HCO_3^-/Cl^- exchanger was also found to be present in the cleavage stage cow embryo, however, cow embryos were unable to recover from a slight increase in pHi, suggesting that a prolonged culture period may have reduced the ability of the exchanger to function properly (Lane and Bavister, 1999). Thus, the potential role of amino acids in the regulation of pHi of the in vitro produced cow embryo needs to be examined.

1.6.6.2.5 Osmolytes

A further role for amino acids in embryo culture media is in the capacity of osmolytes, to help alleviate fluctuations in osmotic pressure. The regulation of osmotic pressure is critical for the maintenance of cell function. Changes in the osmotic pressure of culture media can have profound effects on embryo development (see section 1.6.1). Amino acids, specifically taurine glutamine, glycine, proline and alanine, have been found to act as osmolytes in mouse and human oocytes and embryos in response to detrimental increases in the osmolarity of culture media (Van Winkle *et al.*, 1990; Lawitts and Biggers, 1992; Biggers *et al.*, 1993; Dawson and Baltz, 1997; Dumoulin *et al.*, 1997). Taurine also functions as an osmolyte for somatic cells (Burg, 1995). Liu and Foote (1996) found that the presence of glutamine in culture medium could not in fact protect the cow embryo from detrimental increases in osmolarity. Embryos were cultured, however, from the 4-cell stage, possibly overlooking a protective action of glutamine between the 1 and 4-cell stages.

Although culture of cow embryos in medium with an osmolarity of around 270 mOsm can support comparatively high levels of embryo development, it cannot be assumed that the embryo is not under some degree of osmotic stress. Recent evidence indicates that the osmolality of the reproductive tract is 290 to 300 mOs/kg (Collins and Baltz, 1999), however, in the reproductive tract, embryos are in the presence of potential organic osmolytes such as amino acids (Fahning *et al.*, 1967).

1.6.6.2.6 Antioxidants

Amino acids may also function as antioxidants to protect embryos from oxidative stress during culture. Culture of many species of mammalian embryos at atmospheric concentrations of oxygen has been found to suppress embryo

development, possibly through the accumulation of ROS (see section 1.6.3.2). Taurine supported development of the cow embryo to the blastocyst stage at a concentration of oxygen (20%) that was shown to inhibit development (Liu and Foote, 1995a). Similarly, hypotaurine improved blastocyst development in cow embryos cultured at an oxygen tension of 20% (Fujitani *et al.*, 1996; Guyader-Joly *et al.*, 1997). Morales *et al.* (1999) recently demonstrated that the non-essential or essential amino acid groups reduced the amount of hydrogen peroxide (10^{-6} mM) in cow embryos, from levels found to be detrimental to embryo development.

1.6.6.2.7 Chelators

Amino acids may function as chelators of free metal ions and heavy metal contaminants (Lindenbaum, 1973) which have been found to affect embryo development (Van Winkle and Campione, 1982; Nasr-Esfahani *et al.*, 1990b) (see section 1.6.5). In such a way, amino acids may regulate metabolic activity via the chelation of free metal ion cofactors in the same way that EDTA was found to reduce glycolytic activity of the mouse embryo via the chelation of Mg^{2+} (Lane and Gardner, 1997c). In addition, the chelation of metal ions could limit the formation of toxic hydroxy radicals (Johnson and Nasr-Esfahani, 1994) and could, thus, reduce the potential effects of ROS on embryo development.

It is most likely that amino acids have more than one function during embryo culture and that such functions may change with development from the 1-cell stage to the blastocyst. Further, the potential functions of amino acids in the embryo are not necessarily mutually exclusive. The function/s of amino acids during culture of the cow embryo remain largely undetermined.

1.6.7 Vitamins

Despite the fact that vitamins are key components of cellular metabolism, the role of vitamins in the culture of mammalian embryos has received little attention. This has largely been due to the fact that culture with water soluble B-complex vitamins did not increase the proportion of embryos developing to the blastocyst stage in hamsters (Bavister *et al.*, 1983; Kane and Bavister, 1988b), rabbits (Kane and Foote, 1970), cows (Takahashi and First, 1992; Rosenkrans and First, 1994), sheep (Gardner *et al.*, 1994b) or mice (Gardner and Sakkas, 1993). The requirements of mammalian embryos, however, for vitamins appears to be at the blastocyst stage. Water soluble vitamins were found to be required for expansion of the rabbit blastocyst (Daniel, 1967; Kane and Foote, 1970). Inositol was found to be the greatest stimulator of blastocyst expansion, however pyridoxine, riboflavin and nicotinamide also stimulated blastocyst expansion (Kane, 1988). Kane and Bavister (1988b) reported that several of the vitamins found in Ham's F-10 (Table 1.5), inositol, pantothenate and choline, stimulated hatching of hamster blastocysts. Holm *et al.* (1999) reported that the addition of myo-inositol to SOFaa containing citrate, overcame the negative effect, on cow embryo development and cell number, of the removal of protein from the culture medium. Interestingly, culture of in vivo produced mouse and rat blastocysts for just 6 h in the absence of amino acids and vitamins decreased embryo viability following transfer (Lane and Gardner, 1998).

Water soluble vitamins have also been found to stimulate development of mammalian embryos post-implantation. Rat embryos, explanted on Day 9 of gestation, required pantothenic acid, riboflavin, inositol, folic acid and nicotinamide

Table 1.5 Vitamin composition of MEM vitamins, Ham's F-10 medium and human serum.

Vitamin	MEM[a]	Ham's F-10[b]	Serum[c]
	μM	μM	μM
B_{12}	-	1.0	0.000006
Biotin	-	0.1	0.000708
Choline	7.5	5.0	-
Folic acid	2.3	3.0	-
Inositol	11.0	3.0	-
Nicotinamide	8.2	5.0	0.69
Pantothenate	4.2	3.0	0.013
Pyridoxine	4.9	1.0	0.0054
Riboflavin	0.27	1.0	0.0018
Thiamine	3.0	3.0	0.00039

[a]vitamins present in Minimum Essential Medium (MEM).

[b]vitamins present in Ham's F-10 medium.

[c]vitamins present in human serum, from Tsai and Gardner (1994).

for normal development (Cockroft, 1988). In addition, pantothenic acid, riboflavin and folic acid significantly improved development of rat embryos cultured at the head-fold stage (Cockroft, 1979). Recently, McKiernan and Bavister (2000) reported that culture of hamster embryos with pantothenate not only stimulated blastocyst development, but significantly improved fetal development following transfer of embryos to recipients.

Studies have indicated that vitamins affect the metabolism of the blastocyst. Gardner *et al.* (1994b) reported that the addition of MEM vitamins to SOF containing amino acids, significantly increased the uptake of glucose and the production of lactate by sheep blastocysts on a per cell basis. Concurrent with the observed beneficial effects on embryo viability, Lane and Gardner (1998) found a synergistic effect between MEM vitamins (Table 1.5) and amino acids, in reducing the proportion of glucose converted to lactate in mouse blastocysts.

There have been several reports of inhibitory effects of vitamins on embryo development and viability. In medium lacking amino acids, culture of the mouse embryo with Ham's F-10 vitamins significantly decreased blastocyst formation, while culture with either Ham's F-10 vitamins or MEM vitamins significantly decreased blastocyst cell number (Tsai and Gardner, 1994). Culture of the mouse embryo with MEM vitamins in the presence of Eagle's amino acids did not depress blastocyst cell number, but did reduce blastocyst hatching (Gardner and Sakkas, 1993). Shirley (1989) reported that riboflavin, nicotinamide, and biotin all inhibited development of the mouse embryo to the blastocyst stage, when present at one thousand times the concentration of the individual vitamins in human serum (Table 1.5). Tsai and Gardner (1994) subsequently determined that riboflavin and biotin were not inhibitory

to mouse embryo development at concentrations found in either human serum or Ham's F-10 (Table 1.5). In fact, culture of mice embryos with 1 μM riboflavin significantly increased blastocyst cell number and fetal development following transfer to recipient females (Tsai and Gardner, 1994). Nicotinamide, however, inhibited development of the mouse embryo at concentrations found in both serum and Ham's F-10 and significantly reduced embryo viability following transfer (Tsai and Gardner, 1994). Vitamin B-12, present in Ham's F-10 but not in MEM vitamins, was found to inhibit expansion of the rabbit blastocyst (Kane, 1988) and development of the mouse embryo to the blastocyst stage (Tsai and Gardner, 1994).

To date, the role of water soluble vitamins in the expansion and metabolism of the cow blastocyst has not been determined. Since the cow blastocyst undergoes a significant period of growth and expansion prior to attaching to the uterine wall, it is likely that vitamins play a significant role in both expansion and energy metabolism.

1.6.8 Growth Factors

Embryos and tissues of the maternal reproductive tract express genes for growth factor ligands and receptors. Thus, growth factors could potentially play an important role in development of the mammalian embryo in vivo. The majority of research on the effect of growth factors during development of embryos in vitro has been mainly focused on the mouse. Many growth factors, including IGF-1, IGF-II, EGF, LIF, insulin, TGF-α, PAF, CSF-1 and TGF-β, have all been reported to stimulate development of mouse embryos during culture (Paria and Dey, 1990; Gardner and Kaye, 1991; Pampfer *et al.*, 1991; Harvey and Kaye, 1992a, 1992b; Lim *et al.*, 1993; Roberts *et al.*, 1993; Lavranos *et al.*, 1995). The presence of genes for specific

maternal and embryonic ligands and receptors, however, differs between mammalian species (reviewed by Kane *et al.*, 1997). The action of many growth factors during embryo development is thus likely to be species specific.

Cow embryos have been found to have genes for receptors for insulin, IGF-I, IGF-II, PDGF and TGF-α (Watson *et al.*, 1992). With the exception of IGF-II, receptor transcripts for these growth factors have also been found in somatic cells of the cow reproductive tract (Watson *et al.*, 1992). While research into the role of growth factors during culture of the cow embryo has not been extensive, it is evident that specific growth factors or combinations of growth factors can affect embryo development. EGF was found to stimulate cow blastocyst development (Yang *et al.*, 1993; Lee and Fukui, 1995; Lonergan *et al.*, 1996), however, there are conflicting reports of the effect of EGF on blastocyst cell number (Lee and Fukui, 1995; Lonergan *et al.*, 1996). There is also evidence to suggest that EGF acts in synergy with TGF-β to increase hatching of cow blastocysts (Keefer, 1992). TGF-β1 has also been found to act synergistically with bFGF in reducing the period of the fourth cell cycle in cow embryos (Larson *et al.*, 1992). Evidence for the stimulatory effects of TGF-β1, when present as the sole growth factor, however, are conflicting (Yang *et al.*, 1993; Lee and Fukui, 1995). Culture of cow embryos with PDGF increased development beyond the 8-cell stage (Thibodeaux *et al.*, 1993) by reducing the period of the 4th cell cycle (Larson *et al.*, 1992). LIF was shown to increase hatching of both cow (Han *et al.*, 1995) and sheep (Fry *et al.*, 1992) blastocysts, however, reports of the beneficial effect of LIF on morula and blastocyst development are conflicting (Fry *et al.*, 1992; Fukui and Matsuyama, 1994; Han *et al.*, 1995).

The reported beneficial effects of culturing embryos in groups or in reduced volumes of media is believed to be largely due to the autocrine and paracrine effects of growth factors. Culture of cow (Keefer *et al.*, 1994; Ahern and Gardner, 1998), sheep (Gardner *et al.*, 1994b) and mouse embryos (Wiley *et al.*, 1986; Paria and Dey, 1990; Lane and Gardner, 1992) in groups or in reduced volumes significantly increased embryo development. Importantly, mouse embryo viability was improved following culture in groups or in reduced volumes (Lane and Gardner, 1992), likely reflecting the observed increase in total cell number and size of the inner cell mass in both mouse (Gardner *et al.*, 1997) and cow (Ahern and Gardner, 1998) blastocysts. Thus the stimulatory effect of culturing embryos in groups or reduced volumes was not due to a general mitogen, rather a mitogen specific to the ICM. Thibodeaux *et al.* (1995) provided evidence that the beneficial effects of culturing cow embryos in groups was partly due to the production of PDGF. Further, Paria and Dey (1990) found that culture with EGF, TGF-α or TGF-β1 mimicked the beneficial effects of culturing mouse embryos in groups.

While the majority of studies have used single or paired growth factors to determine the effects of specific growth factors during culture of mammalian embryos, it is likely that, in vivo, the embryo will be exposed to several growth factors simultaneously. Therefore, the effects of growth factors on the embryo when presented individually, may differ markedly to the effect of a specific growth factor when in combination with a number of growth factors. Research into the role of growth factors during culture of the cow embryo has not been extensive and thus growth factors are not routinely added to culture media. Further research is required to

determine the effects of combinations of growth factors on cow embryo development and viability because of the potential to produce aberrant fetal growth.

1.7 IN VITRO PRODUCTION OF EMBRYOS FROM PRE-PUBERTAL ANIMALS

While techniques such as co-culture and culture with conditioned and somatic cell-free, non-conditioned media have provided reliable methods for producing embryos from adult cows in vitro, the production of embryos from pre-pubertal cows has proven to be somewhat more difficult. During the past decade there has been a considerable amount of interest in the use of pre-pubertal cows as oocyte donors. The ovaries of the pre-pubertal cow contain a greater number of oocytes than the ovaries of the adult cow, thus providing a larger number of oocytes per animal (Erickson, 1966b). Furthermore, the use of oocytes from pre-pubertal cows for assisted reproductive technologies offers the potential to reduce the generation interval between cows, thus increasing the rate of genetic gain in a given population (Lohuis, 1995; Duby *et al.*, 1996).

While it has been possible to obtain pregnancies and live births following transfer of blastocysts derived from oocytes of pre-pubertal cows (Kajihara *et al.*, 1991; Armstrong *et al.*, 1992; Revel *et al.*, 1995) developmental competence is often impaired compared with embryos derived from adult cows (Onuma and Foote, 1969; Kajihara *et al.*, 1991; Palma *et al.*, 1993; Levesque and Sirard, 1994; Revel *et al.*, 1995). Poor development has also been demonstrated in oocytes from pre-pubertal sheep (O'Brien *et al.*, 1996; O'Brien *et al.*, 1997) and pigs (Pinkert *et al.*, 1989). It has been suggested that the success of the production of embryos from pre-pubertal

animals is dependent on the age of the donor, with poor development in oocytes from cows less than eight months old (Looney *et al.*, 1995). There have been several reports, however, of equivalent developmental competence of oocytes from calves of six to nine weeks of age and adult cows (Armstrong *et al.*, 1992; Irvine *et al.*, 1993; Armstrong *et al.*, 1994).

While the precise reasons for the often observed poor developmental competence of oocytes from pre-pubertal animals remain unknown, there is an increasing amount of evidence that there are morphological, physiological and biochemical differences between oocytes from pre-pubertal and adult cows. Oocytes from pre-pubertal cows were reported to be smaller than oocytes from adult cows (Duby *et al.*, 1996; Gandolfi *et al.*, 1998), however, this was not found in sheep (O'Brien *et al.*, 1996). Duby *et al* (1996) reported that the pattern of inositol 1,4,5 triphosphate induced intracellular calcium release was different in pre-pubertal and adult cows and that the overall amount of calcium released was in fact lower in pre-pubertal cows. In addition, protein profiles have been shown to be markedly different in oocytes from pre-pubertal and adult cows (Levesque and Sirard, 1994; Gandolfi *et al.*, 1998), with protein profiles in pre-pubertal oocytes similar to those found in developmentally incompetent oocytes from adult cows (Levesque and Sirard, 1994). Further, Gandolfi *et al.* (1998) found a significant decrease in protein synthesis in pre-pubertal oocytes after 9 h maturation that was not evident in oocytes from adult cows until 24 h maturation. Damiani *et al.* (1989) found that the activities of histone H1 kinase and mitogen activated protein (MAP) kinase were 50% lower in pre-pubertal oocytes, even after 24 h maturation. Recently, Gandolfi *et al.* (1998) found that the metabolism of glutamine and pyruvate was lower in oocytes from pre-pubertal cows

during the first 3 h maturation, indicating that metabolic pathways may not be appropriately activated in oocytes from pre-pubertal calves. Perturbations in energy metabolism have also been reported in pre-pubertal sheep oocytes (O'Brien *et al.*, 1996).

Oocyte size, intracellular calcium release, protein synthesis, histone H1 kinase and MAP kinase activity are all important factors in the initiation and/or completion of meiosis (Schultz and Wassarman, 1977; Fair *et al.*, 1995; Dekel, 1996; Whitaker, 1996; Otoi *et al.*, 1997). The present findings suggest that cytoplasmic maturation and potentially nuclear maturation are perturbed in oocytes from pre-pubertal animals. Palma *et al.* (1993) in fact found that fewer oocytes from pre-pubertal cows were at MII after 24 h maturation than oocytes from adult cows. Fertilization, however, has been found to be equivalent in oocytes from pre-pubertal and adult cows (Duby *et al.*, 1996). Although few, reports of equivalent rates of blastocyst development between pre-pubertal and adult cows gives promise to the use of pre-pubertal cows as oocyte donors (Irvine *et al.*, 1993; Armstrong *et al.*, 1992; Armstrong *et al.*, 1994). Further research is thus necessary to understand the differences between oocytes/embryos from pre-pubertal cows and adult cows and to relate such differences to developmental competence.

1.8 METABOLISM OF THE EMBRYO

The processes involved in the development of the mammalian oocyte and embryo from the initiation and completion of maturation, fertilization and development from the zygote to the blastocyst are energetically consuming. Within a week the mammalian embryo must switch from maternal to embryonic genome

regulation and undergo rapid blastomere proliferation, an increase in protein synthesis, blastomere compaction involving the formation of junctions, formation of a large blastocoel, cell differentiation and, in the case of ruminant embryos, extensive growth of the blastocyst. In order to meet such demands, both energy production and the generation of ribose moieties for biosynthesis must be efficient in the developing embryo. Cellular energy is produced in the form of ATP via the metabolism of various energy substrates. ATP cannot be stored thus the appropriate metabolic and biosynthetic pathways must be functioning accurately at the appropriate times to meet the specific energy demands of each and every process.

Thompson *et al.* (1996) calculated ATP production in cow embryos from measurements of oxygen consumption and the proportion of exogenous glucose metabolized to lactate and found that ATP production increased during the development of embryos from the zygote to the blastocyst stage. The production of ATP by early cleavage stage embryos was low (54 - 60 pmoles/embryo/h for 1-cell to 8-cell stage embryos) and increased exponentially with development to the 16-cell stage and morula (85 pmoles/embryo/h), compaction at the morula stage (124 pmoles/embryo/h) and blastocyst formation (221 pmoles/embryo/h).

A high level of ATP in a cell indicates that ATP is not being used and thus ATP production is low. Conversely, a low level of ATP reflects high usage of ATP and thus high production. Studies of the ATP and ADP contents of mouse and sheep embryos have revealed that ATP levels decreased during development, resulting in a high ATP:ADP ratio in the early cleavage stages, which decreased with development from the 8- to 16-cell stage to the blastocyst (Rozell *et al.*, 1992; Leese *et al.*, 1984). The ATP content of cow embryos has also been found to decrease with embryo

development (Rieger *et al.*, 1997). Changes in the production of ATP and the ATP content of embryos during development suggest that there are significant changes in the activities of energy generating pathways during development from the zygote to the blastocyst stage. Recently, Thompson *et al.* (2000) reported that ATP production (from oxidative phosphorylation) was in fact critical for development of cow embryos.

1.8.1 Energy Generating Pathways

ATP is produced through only two metabolic mechanisms, glycolysis and oxidative phosphorylation. Glycolysis involves the conversion of one molecule of glucose to two molecules of pyruvate via the EMP (Figure 1.1) in the cytoplasm of the cell. Glycolysis utilizes two molecules of ATP and produces four molecules of ATP, resulting in a net of two molecules of ATP. In the presence of oxygen, pyruvate enters mitochondria and is metabolized through the TCA cycle (Figure 1.2), resulting in the production of CO_2, H_2O and thirty eight molecules of ATP. In order to operate, the TCA cycle requires supplies of NAD^+ and FAD which are generated through the process of oxidative phosphorylation. NADH and $FADH_2$ transfer electrons to oxygen through the electron transport chain, resulting in the formation of NAD^+, FAD and ATP. Thus, the rate of activity of the TCA cycle is governed by the cell's requirements for ATP. The complete metabolism of glucose to CO_2 and H_2O via the EMP and TCA cycle yields a net of thirty six molecules of ATP. Under anaerobic conditions, pyruvate cannot be oxidized via the TCA cycle but is converted to lactate by LDH (EC 1.1.1.27) (Figure 1.1). This is termed anaerobic glycolysis and is typical of muscle cells during exercise. The generation of NAD^+, resulting from the

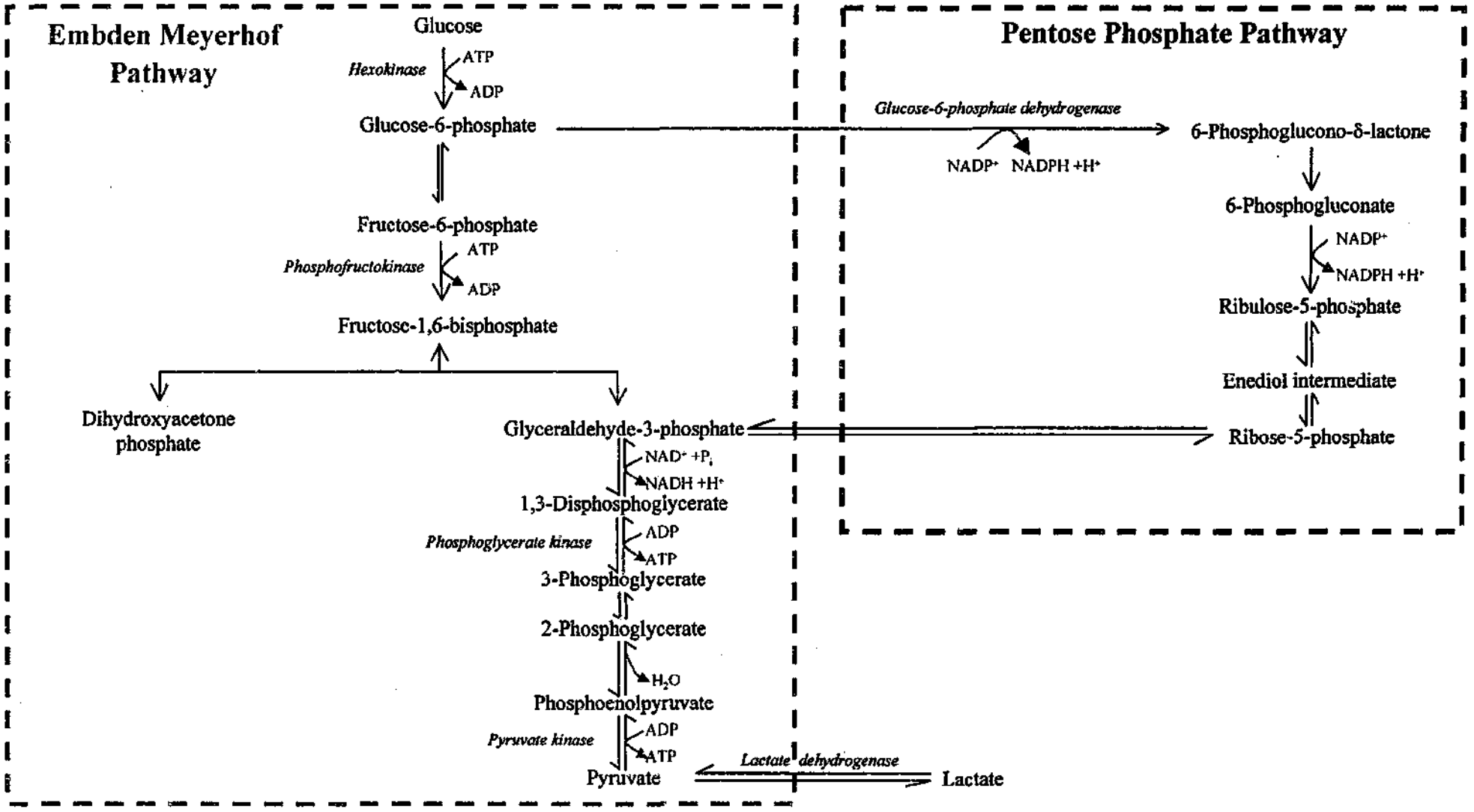

Figure 1.1 The Embden Meyerhof Pathway (EMP) and the Pentose Phosphate Pathway (PPP).

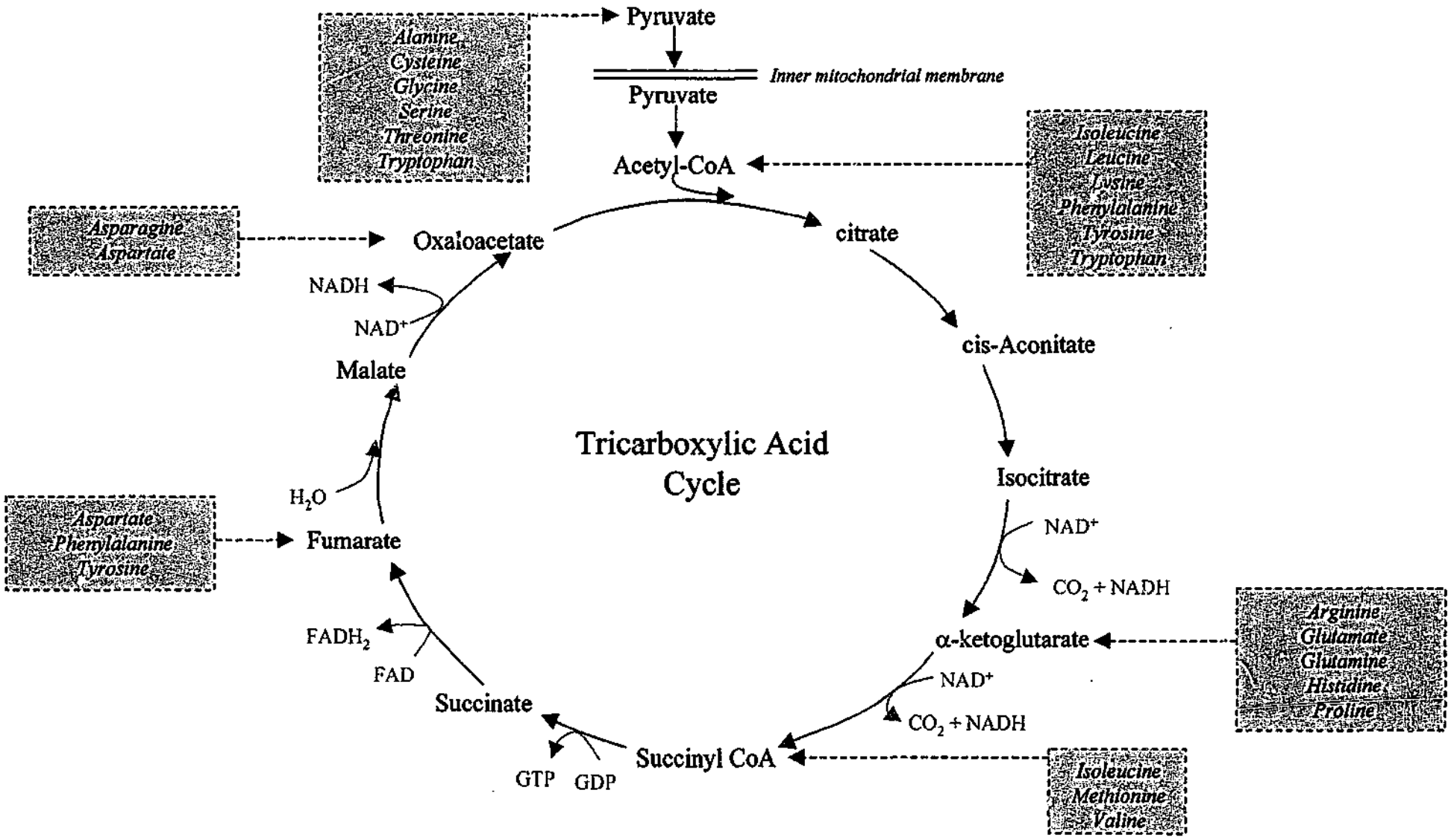

Figure 1.2 The Tricarboxylic Acid (TCA) cycle showing entry points for amino acids.

conversion of pyruvate to lactate, ensures the continuation of glycolysis. The conversion of one molecule of glucose to two molecules of lactate results in the net production of two molecules of ATP. Thus, the TCA cycle is a far more efficient means of generating energy than anaerobic glycolysis.

Glucose can also be metabolized through the PPP to produce another type of energy critical to cellular function, reducing power (Figure 1.1). The metabolism of glucose through the PPP leads to the production of NADPH for reductive biosyntheses as well as ribose moieties for the formation of nucleotides such as RNA and DNA.

Glucose is not the only exogenous substrate that can be metabolized to produce energy. Pyruvate can be taken up by the cell and metabolized through the TCA cycle (net production of thirty two molecules of ATP). Furthermore, exogenous lactate can be converted to pyruvate by LDH and metabolized through the TCA cycle. Various EMP and TCA cycle intermediates can also be taken up by cells and metabolized for the generation of energy. In addition, various amino acids can be transaminated to TCA cycle intermediates (Figure 1.2).

1.8.2 The Metabolism of Radiolabelled Energy Substrates

The metabolism of radiolabelled substrates has been used to measure the activities of specific metabolic pathways in mammalian embryos. Energy substrates such as glucose, pyruvate, lactate and glutamine are typically labelled with tritium or ^{14}C, resulting in the liberation of $^{3}H_2O$ and $^{14}CO_2$, respectively, at a given point in a metabolic pathway. Radiolabelled substrates have also been used to determine rates of

nutrient uptake by embryos. Such techniques have enabled the determination of the changing metabolic requirements of developing embryos from a number of species.

Early studies were only able to determine mean metabolic rates from a group of embryos. The determination of the utilization of ^{14}C-labelled glucose, a measure of the oxidation of glucose through the PPP and TCA cycle, revealed that glucose utilization was low in the early cleavage stages of the mouse embryo, increasing with development to the morula and blastocyst stages (Brinster, 1967b). The oxidation of pyruvate and lactate by early cleavage stage mouse embryos was found to be greater than that of glucose and increased with development to the blastocyst stage (Brinster, 1967a).

The development of a more sensitive technique by O'Fallon and Wright (1986) enabled the metabolism of radiolabelled substrates by individual mouse embryos to be determined. The proportion of glucose metabolized through the PPP with respect to the EMP was found to vary with embryo development, peaking at the 2-cell and compacted morula stages and lowest at the blastocyst stage (O'Fallon and Wright, 1986). Javed and Wright (1991) found that the proportion of glucose metabolized through the PPP in in vivo produced cow embryos was highest during the early cleavage stages of development, decreasing by four to seven fold at the morula and blastocyst stages. Conversely, the proportion of glucose metabolized via the EMP was lowest in cleavage stage embryos, increasing to over 90% at the morula and blastocyst stages (Javed and Wright, 1991). A similar pattern of glucose metabolism was found in the developing pig embryo (Flood and Wiebold, 1988). Thus, production of NADPH and ribose moieties from glucose appears to be very important during

development of the cleavage stage embryo, whereas the metabolism of glucose via the EMP becomes more important with compaction of morulae and blastocyst formation.

Although significant amounts of glucose are metabolized through the PPP and EMP, very little glucose was found to be oxidized through the TCA cycle in cow embryos from the zygote to the blastocyst stage (Rieger and Guay, 1988; Rieger *et al.*, 1992a, 1992b). The utilization of glucose through the TCA cycle was also found to be minimal in sheep blastocysts (Thompson *et al.*, 1991). Significant amounts of pyruvate (Rieger and Guay, 1988; Rieger *et al.*, 1992b), lactate (Waugh and Wales, 1993), glutamine (Rieger and Guay, 1988; Tiffin *et al.*, 1991; Rieger *et al.*, 1992a, 1992b) and acetate (Waugh and Wales, 1993) have been found to be metabolized by cow embryos through the TCA cycle, however, indicating that the TCA cycle is in fact active in cow embryos. Rieger and Guay (1988) demonstrated that DNP, an uncoupler of oxidative phosphorylation, significantly increased the activity of the TCA cycle with respect to the utilization of pyruvate and glutamine, but had no effect on glucose oxidation. Thus, it was hypothesised that the cow embryo had a block to glycolysis due to a lack or inhibition of pyruvate kinase (Rieger and Guay, 1988). Recent evidence indicates that there is not a block to glycolysis in the sheep embryo. Thompson *et al.* (1991) found that the oxidation of glucose by sheep blastocysts increased in the presence of DNP when both lactate and pyruvate were removed from the culture medium. Thus, it was suggested that the metabolism of glucose through the TCA cycle was low due to the successful competition of exogenous substrates for entry into the TCA cycle. Further, the fact that significant amounts of lactate were produced by sheep blastocysts when glucose was the only exogenous energy source (see section 1.8.3 with respect to aerobic glycolysis) revealed that glycolysis was not

in fact blocked in the sheep embryo (Gardner *et al.*, 1993). Thompson *et al.* (1996) found that lactate production by cow embryos was significant and could account for 100% of the glucose taken up at the blastocyst stage. Pyruvate, however, was also present in the medium so the source of the lactate could not be determined conclusively. In order to establish whether there is in fact a block at pyruvate kinase in the cow embryo, lactate production needs to be determined with glucose present as the only energy substrate.

Significant amounts of glucose carbon have been found to be incorporated into the storage molecule glycogen in mouse embryos (Brinster 1969, Pike and Wales, 1982). There is, however, very little incorporation of glucose into glycogen pools in either cow (Waugh and Wales, 1993) or sheep embryos (Pike and Wales, 1979; Wales *et al.*, 1989). Thus, glycogen storage does not appear to be a significant fate of glucose in ruminant embryos.

Like glucose, the metabolism of pyruvate and glutamine changes during embryo development. Rieger *et al.* (1992b) found pyruvate metabolism through the TCA cycle to be high in cow embryos at the 2- to 4-cell stages, low at 16-cell and morula stages, increasing again with blastocyst formation. Similarly, glutamine metabolism was high at the 2- to 4-cell stages, decreased at the 8-cell stage, then increased significantly with blastocyst development (Rieger *et al.*, 1992a, 1992b). This is in contrast to the mouse embryo, as glutamine utilization was reported to be lowest in the early cleavage stages, increasing with development to the blastocyst stage (Chatot *et al.*, 1990; Du and Wales, 1993b). The metabolism of pyruvate and glutamine by cow embryos is, therefore, different to that of glucose which was found to be lowest during the early cleavage stages (Rogers *et al.*, 1979; Javed and Wright, 1991; Rieger

et al 1992a, 1992b). Although the metabolism of glutamine and pyruvate are both measures of the activity of the TCA cycle, the metabolism of the two substrates was not found to be entirely parallel. Rieger *et al.* (1992b) reported that glutamine metabolism by cow embryos increased markedly with blastocyst expansion and hatching, while pyruvate metabolism peaked at the blastocyst stage and remained constant during the expanding and hatching stages. Thus, it is evident that in cow embryos metabolic activity differs for different sections of the TCA cycle.

1.8.3 The Uptake of Nutrients by Embryos

While the use of radiolabelled energy substrates has provided valuable information about the activities of various metabolic pathways during embryo development, the exposure of embryos to radioisotopes is invasive and has in fact been found to affect the development of mouse and pig embryos (Wiebold and Anderson, 1985; Flood and Wiebold, 1988) and the viability of mouse embryos following transfer of embryos to recipients (Wiebold and Anderson, 1985).

Non-invasive microfluorescent techniques, based on the generation of or decrease in the fluorescence of reduced forms of pyridine nucleotides when exposed to UV light (detailed in section 2.9), have been used to determine the uptake of nutrients by embryos from a variety of species. Studies in the cow (Thompson *et al.*, 1996), sheep (Gardner *et al.*, 1993), human (Gott *et al.*, 1990), goat (Gardner *et al.*, 1994a) and mouse (Leese and Barton, 1984; Gardner and Leese, 1986) have revealed that glucose uptake is low during the early cleavage stages of mammalian embryo development, increasing exponentially after the 8-cell stage with compaction and blastocyst development. This supports findings that activity of the EMP is low during

development of the early cleavage stages (see section 1.8.2). Further, the fact that culture of embryos with glucose at high concentrations is inhibitory to development (see section 1.6.2.1) indicates that a high utilization of glucose is not favoured by the early cleavage stage embryo.

The reason for the low utilization of glucose by early cleavage stage embryos is not fully understood. Interestingly, the marked increase in glucose uptake and metabolism occurs around the time of the major onset of activation of the embryonic genome (Telford *et al.*, 1990). The low utilization of glucose by the early cleavage stage mouse embryo is believed to be due to a blockade in glycolysis, resulting from inhibition of PFK (Barbehenn *et al.*, 1974; Barbehenn *et al.*, 1978) by a high ratio of ATP:ADP (Leese *et al.*, 1984; Gardner and Lane, 1997). With the decrease in the ratio of ATP:ADP with development from the 8- to 16-cell stage to the blastocyst (Rozell *et al.*, 1992; Leese *et al.*, 1984) PFK would no longer be inhibited and glucose flux would thus increase. It has also been suggested that low glucose uptake and metabolism by early cleavage stage cow embryos could be due to the observed low level of GLUT-1 mRNA after maturation and fertilization of cow oocytes (Lequarre *et al.*, 1997). GLUT proteins were shown to mediate the uptake of glucose by mouse embryos (Gardner and Leese, 1988) with GLUT-1 transcripts present during development of the early cleavage stages (Hogan *et al.*, 1991; Lequarre *et al.*, 1997). The increase in glucose uptake and metabolism after the 8- to 16-cell stage in cow embryos could be due to the reported increase in hexokinase mRNAs at the morula stage (Lequarre *et al.*, 1997).

Not surprisingly, pyruvate uptake has been reported to be greater than that of glucose during development of the early cleavage stages in cows (Thompson *et al.*,

1996), sheep (Gardner *et al.*, 1993), humans (Hardy *et al.*, 1989b), mice (Leese and Barton, 1984; Gardner and Leese, 1986) and goats (Gardner *et al.*, 1994a). While pyruvate uptake was found to increase at the blastocyst stage in the cow (Thompson *et al.*, 1996), sheep (Gardner *et al.*, 1993) and goat (Gardner *et al.*, 1994a) pyruvate uptake by human embryos actually decreased with blastocyst development (Hardy *et al.*, 1989b) and was reduced to almost zero uptake with development of the mouse blastocyst (Leese and Barton, 1984; Gardner and Leese, 1986) and the rat blastocyst (Brison and Leese, 1991). Pyruvate uptake by human, rat and mouse embryos, however, was determined in the presence of lactate. Lane and Gardner (2000a) showed that, when lactate was removed from the culture medium, pyruvate uptake increased in mouse blastocysts. Furthermore, the presence of lactate, in combination with glucose and amino acids, was found to decrease the rate of utilization of pyruvate by sheep blastocysts (Thompson *et al.*, 1993). Similarly, Dorland *et al.* (1991) demonstrated that, in the presence of lactate (31.3 mM), pyruvate uptake by cow blastocysts was zero. In the absence of lactate, however, pyruvate uptake was 8.84 pmoles/embryo/h. Interestingly, in the presence of 10 mM lactate, Thompson *et al.* (1996) reported a significant increase in pyruvate uptake by cow embryos from the morula stage (9.9 pmoles/embryo/h) to the blastocyst stage (20.5 pmoles/embryo/h). Thus, careful consideration must be given to media components and their concentrations when determining and interpreting the uptake and utilization of different energy substrates by embryos.

Microfluorescent techniques have also enabled the determination of the metabolic fate of specific energy substrates. Gardner and Leese (1988) found that considerable amounts of glucose taken up by the mouse embryo were converted to

lactate, with around 25% of glucose converted to lactate at the 2-cell stage, increasing to 40% at the blastocyst stage. The proportion of glucose converted to lactate by mouse blastocysts has in fact been shown to be as high as 91% (Gardner and Leese, 1990). Similarly, Gardner *et al.* (1993) reported that almost 100% of the glucose taken up by sheep blastocysts was converted to lactate. Although other energy substrates were present in the culture media, nearly 100% of the glucose taken up by rat and cow embryos could be accounted for by lactate production from the 8-cell stage to the blastocyst (Brison and Leese, 1991; Thompson *et al.*, 1996). Conversely, only about 40% of the glucose taken up by the cow zygote was converted to lactate (Thompson *et al.*, 1996). Thus aerobic glycolysis appears to be a major metabolic process in mammalian embryos, particularly in the later stages of development.

Gardner *et al.* (1993) found that around 65% of exogenous pyruvate taken up by the sheep blastocyst was converted to lactate, indicating that only 35% of pyruvate was potentially oxidized through the TCA cycle. Likewise, using radiolabelled substrates, Rieger *et al.* (1992b) found that only 14% of pyruvate taken up by cow blastocysts was oxidized. Conversely, on the basis of pyruvate uptake and oxygen consumption, Thompson *et al.* (1996) suggested that the majority of pyruvate was oxidized through the TCA cycle in the cow embryo. The production of lactate from exogenous pyruvate has not previously been determined in cow embryos. Again, it must be considered that determinations of pyruvate metabolism in the above studies, were carried out in very different culture media, including media lacking amino acids (Thompson *et al.*, 1996), media lacking amino acids and lactate (Gardner *et al.*, 1993) and complex tissue culture media (Rieger *et al.*,1992b). As detailed in section 1.8.4,

culture media components can have a significant effect on the uptake and metabolism of substrates.

The reasons for the prevalence of aerobic glycolysis in mammalian embryos is unknown. Aerobic glycolysis is not as efficient as the TCA cycle with respect to the production of ATP. It has been suggested that aerobic glycolysis may in fact be a culture induced artefact as the proportion of glucose converted to lactate by mouse embryos increased with the culture period (Gardner and Leese, 1988). This is supported by the fact that, in the mouse, this phenomenon can be corrected with appropriate culture conditions (Gardner and Leese, 1990; Gardner and Sakkas, 1993; Lane and Gardner, 1998). Furthermore, the proportion of glucose taken up by mouse embryos that was converted to lactate was as high as 91% for embryos cultured from the 8-cell stage compared with 44% for fresh blastocysts (Gardner and Leese, 1990). Conversely, the proportion of glucose accounted for by lactate production was found to be approximately 100% in in vivo produced sheep blastocysts (Gardner *et al.*, 1993). Thompson *et al.* (1991) in fact found no difference in the glycolytic activity of sheep blastocysts derived in vivo and in vitro. Similarly, the conversion of glucose to lactate by rat blastocysts was almost 100% for both in vitro and in vivo derived blastocysts (Brison and Leese, 1991). Furthermore, glucose oxidation through the TCA cycle in in vivo produced cow blastocysts was less than 1% of the total amount of glucose metabolized through the EMP (Rieger and Guay, 1988). Measurements of the metabolic activity of in vivo produced ruminant and rat embryos, however, have been made in medium lacking amino acids (Brison and Leese, 1991; Thompson *et al.*, 1991), medium lacking amino acids and lactate (Gardner *et al.*, 1993) and in complex tissue culture medium (Rieger and Guay, 1988). As media components can have a

significant effect on substrate metabolism (discussed in section 1.8.4) it is difficult to determine to what degree the observations of glucose metabolism in in vivo produced embryos are in vitro induced artefacts.

Significantly high levels of aerobic glycolysis are also a characteristic of rapidly dividing tumour cells (Stubbs *et al.*, 1995). It is has been suggested that embryos may lack either the glycerol phosphate shuttle or the malate-aspartate shuttle to transport NADH from the cytoplasm into mitochondria and conversely, NAD^+ from the mitochondria to the cytoplasm (Thompson *et al.*, 1993; Gardner, 1998a). If this were the case, then the conversion of pyruvate to lactate would be the only means by which the embryo could generate the NAD^+ required for glycolysis to proceed. In addition, the generation of NAD^+ via the production of lactate could be critical to the ratio of NADH:NAD^+, or redox state of the embryo, which has been found to be an important factor in the development of embryos from some species (see section 1.6.2.2). The transfer of reducing equivalents generated by glycolysis has in fact been shown to be impaired in Morris hepatoma cells (Lanoue *et al.*, 1974). It has been hypothesised that the ratio of NAD^+:NADH drives the conversion of pyruvate to lactate (Stubbs *et al.*, 1995). Recently, Lane and Gardner (2000b) reported that the malate-aspartate shuttle was in fact present and active in in vivo derived mouse embryos at the 2-cell stage and the blastocyst stage. It was suggested that the high rate of aerobic glycolysis observed in cultured blastocysts was due to inactivity of the malate-aspartate shuttle, proposing that culture impaired activity of the shuttle.

It has not yet been established whether reducing-equivalent shuttles are present in ruminant embryos. The observed high rates of aerobic glycolysis in ruminant blastocysts derived both in vitro and in vivo could be reflective of the absence or

inactivity of reducing-equivalent shuttles. The absence or inactivity of such shuttles could possibly be an in vitro induced artefact, as the metabolism of in vivo derived blastocysts has been determined in vitro. Alternatively, this could be an adaptation of the embryo to survive in a relatively anoxic environment. It has previously been suggested that a high rate of aerobic glycolysis in rodent embryos could be an adaptation of the embryo to survive in a potentially anoxic environment within the uterus (Ellington, 1987; Gardner, 1987, PhD thesis; Leese, 1989). The endometrium has in fact been shown to become avascular around the time of implantation of the rat blastocyst (Rogers *et al.*, 1982a, 1982b; 1993). Implantation or rather attachment does not occur in ruminant species, however, for a further one to two weeks after the blastocyst hatches from the zona pellucida. Interestingly, Du and Wales (1993a) reported that a reduction in oxygen concentration during culture of structures of the advanced sheep conceptus from Days 13 to 19 of pregnancy, significantly reduced the oxidation of glucose but increased lactate production. The reported significantly high levels of glycolysis at the blastocyst stage in ruminant embryos may, thus, be an adaptation to prepare the conceptus for survival in a relatively anoxic uterine environment.

1.8.4 The Effects of Culture on Embryo Metabolism

The culture of embryos in vitro can have profound effects on embryo metabolism. Menke and McLaren (1970) revealed that pyruvate and lactate oxidation were significantly reduced in in vitro produced mouse blastocysts compared with in vivo produced blastocysts. Furthermore, both glutamine and glucose oxidation were shown to be reduced in in vitro developed mouse embryos (Du and Wales, 1993b).

Brison and Leese (1991) reported that the overall level of glycolytic activity in rat blastocysts was significantly reduced by culture compared with in vivo derived embryos. Furthermore, Gardner and Leese (1990) showed that culture of mouse embryos from the 8-cell stage increased the proportion of glucose converted to lactate by blastocysts, compared with in vivo derived blastocysts. Although the glycolytic activity of sheep blastocysts was not affected by culture in vitro, glucose oxidation was significantly reduced compared with in vivo produced blastocysts (Thompson *et al.*, 1991). Javed and Wright (1991) found that PPP increased three fold in cow embryos after 24 h culture. Culture of in vivo produced mouse embryos for just 6 h in a simple medium lacking amino acids and vitamins reduced pyruvate oxidation and increased the proportion of glucose converted to lactate (Lane and Gardner, 1998).

Embryo metabolism is further influenced by the particular system in which embryos are cultured. The presence of energy substrates has been found to affect both the uptake and utilization of other substrates. Pyruvate uptake and utilization have been found to be affected by the presence of glucose and lactate (Gardner and Leese, 1988; Dorland *et al.*, 1991; Thompson *et al.*, 1993; Lane and Gardner, 2000a). Further, the utilization and uptake of glutamine and glucose by mouse embryos has been found to be reduced in the presence of the other substrate (Gardner *et al.*, 1989; Du and Wales, 1993). Culture with HEPES has also been shown to reduce the uptake of glucose by mouse embryos (Butler *et al.*, 1988). Furthermore, amino acids have been found to affect the uptake and metabolism of nutrients by mouse embryos (Lane and Gardner, 1997a). As a result, embryo metabolism and nutrient uptakes have been found to vary when embryos are cultured in different types of media (Gardner and

Sakkas, 1993; Rieger *et al.*, 1995; Barnett and Bavister, 1996; Leppens *et al.*, 1996; Krisher *et al.*, 1999).

Increasing the concentration of glucose in culture media can increase the activity of glycolytic enzymes (Edwards and Gardner, 1995) and stimulate glycolytic flux (Vella *et al.*, 1997). Culturing embryos at a high concentration of glucose may, therefore, upset the metabolic activity of the embryo by inducing a Crabtree effect, manifested by an increase in glycolytic activity with a concomitant decrease in oxidation. (Crabtree, 1998). It has been suggested that, in some cell types, the phosphorylation of glucose with ATP by hexokinase results in the generation of ADP, which is subsequently rephosphorylated to ATP through the respiratory chain, leading to the depletion of mitochondrial pools of Pi (Koobs, 1972). Pi is required for energy production through oxidative phosphorylation. If the isoform of hexokinase present is insensitive to inhibition by the build-up of glucose-6-phosphate, then glycolysis will continue, resulting in the depletion of cytosolic Pi and the accumulation of ADP. Increasing Pi should alleviate a Crabtree effect (Koobs, 1972), however, Pi is actually required for the inhibitory effect of glucose on hamster embryo development (Seshagiri and Bavister, 1989a; Seshagiri and Bavister, 1991). Thus, it was proposed that early cleavage stage embryos are subject to a Crabtree-like effect.

Despite the fact that individual culture systems have been found to affect both the metabolism and development of mammalian embryos, very few studies have attempted to correlate changes in the activities of metabolic pathways with developmental competence. The majority of studies have addressed either the developmental capacity of embryos in a particular culture system or the metabolism of embryos at various stages of development. Few studies, however, have attempted to

link the observed changes in embryo development with alterations in embryo metabolism imposed by the prevailing culture system. Further, embryo metabolism has often not been determined under conditions that support optimal embryo development, making it difficult to correlate the two parameters. An understanding of how metabolism affects development will facilitate the optimization of culture systems for mammalian embryos. For example, Gardner and Lane (1993b) found that the 2-cell block in mouse embryos was associated with an increase in glycolytic activity and a decrease in activity of the TCA cycle (a potential Crabtree-like effect). Culture under conditions that reduced glycolytic activity alleviated the 2-cell block (Gardner and Lane, 1993b; Lane and Gardner, 1997c). In addition, culture conditions that have been found to alter either glycolytic activity and/or pyruvate oxidation have been associated with changes in embryo development (Gardner and Leese, 1990; Gardner and Sakkas, 1993; Gardner *et al.*, 1994b; Hewitson *et al.*, 1996; Leppens *et al.*, 1996; Leppens-Luisier and Sakkas, 1997; Krisher *et al.*, 1999), blastocyst cell number (Leppens-Luisier and Sakkas, 1997) and embryo viability (Leppens *et al.*, 1996; Lane and Gardner, 1997a).

Thus, careful consideration must be taken when determining both the metabolism and requirements of embryos during culture. The embryo exhibits a considerable degree of plasticity with respect to the utilization of nutrients, possibly at the expense of it's own viability. Thus, where possible, determinations of embryo requirements and embryo metabolism should be performed under the same conditions that embryos are routinely cultured. Deviations from a particular culture system, for example the determination of embryo metabolism in a HEPES-buffered medium at atmospheric concentrations of oxygen, may render metabolic determinations non-

reflective of embryos in the standard culture system, where embryos are exposed to a bicarbonate buffered medium at a low concentration of oxygen. In addition, the changing requirements of the embryo during development must be considered when optimizing culture conditions for mammalian embryos.

1.9 MARKERS OF EMBRYO VIABILITY

The use of markers of embryo viability with assisted reproduction techniques offers a number of advantages. Firstly, the application of viability markers has the potential to increase pregnancy rates by enabling the selection of the most viable embryos for transfer to recipients. Further, the use of a variety of viability markers provides a means of assessing the suitability of various culture systems and media components for the culture of mammalian embryos. The true test of embryo viability is the transfer of embryos to recipient animals, however, the large number of recipients required for such a trial and the cost of animals mean that very few studies dealing with the requirements of cow embryos during culture are able to follow up clinical trials with transfer data and pregnancy outcomes. Thus, viability markers should be an important component of any cow embryo in vitro production system.

1.9.1 Morphology

Morphology alone is often used as the sole criterion for determining embryo viability or the appropriateness of various culture systems and media components for cow embryos (Kim *et al.*, 1993b; Moore and Bondioli, 1993; Liu and Foote, 1995b; Van Inzen *et al.*, 1995; Pinyopummintr and Bavister, 1996a, 1996b; Lim *et al.*, 1997). The reliability of morphology as a marker of mammalian embryo viability, however,

has been challenged (Bowman and McLaren, 1970; Gandolfi and Moor, 1987; Enders *et al.*, 1989; Hardy *et al.*, 1989a). Lane and Gardner (1996) demonstrated the immense variation in the viability of morphologically identical expanded mouse blastocysts. Furthermore, Van Soom *et al.* (1997a) showed that the morphology of cow blastocysts was not indicative of either the number or proportion of cells in ICM, which has been found to be associated with embryo viability (see section 1.9.2). Gardner *et al.* (2000b), however, have reported that the viability of human embryos could be quantified using a three point scoring system based on assessment of the degree of blastocyst expansion and hatching, the compactness and size of the ICM and the cohesiveness and number of cells in the TE.

Cow embryo culture systems are often assessed in terms of the proportion of embryos developing to the blastocyst stage by Day 8 or Day 9 pi (Fukui and Ono, 1989; Kim *et al.*, 1993b; Matsuyama *et al.*, 1993; Keskintepe *et al.*, 1995; Liu and Foote, 1995b; Pinyopummintr and Bavister, 1996b). The timing of blastocyst formation, however, has been shown to be reflective of embryo viability (Mehta and Kiessling, 1990). Cow embryos reaching the blastocyst stage by Day 7 pi, when we would expect to see embryos reaching the blastocyst stage in vivo, have been shown to be more viable than embryos reaching the blastocyst stage on Day 8 pi, following transfer to recipients (Hasler *et al.*, 1995). In addition, embryos reaching the blastocyst stage by Day 7 pi were better equipped to survive freezing and thawing processes than those reaching the blastocyst stage on Day 8 pi (Hasler *et al.*, 1995). Thus, determinations of the proportion of embryos reaching the blastocyst stage after Day 7 pi may not be a reliable indicator of the appropriateness of specific culture systems for cow embryos. Although hatching is often used as an indicator of

blastocyst viability, its reliability is also uncertain (Gonzales and Bavister, 1995; Lane and Gardner, 1997a).

An increased rate of embryo development has been positively associated with viability following transfer in the mouse (Lane and Gardner, 1994) and hamster (McKiernan and Bavister, 1994). Further, the rate of development of early cleavage stage embryos has been found to be predictive of subsequent developmental potential for morula and blastocyst formation in hamster (Gonzales *et al.*, 1995) and cow (Van Soom *et al.*, 1992; Grisart *et al.*, 1994; Holm *et al.*, 1997; Van Soom *et al.*, 1997b) embryos. A number of studies have associated timing of the first cleavage division of cow embryos with developmental competence (Van Soom *et al.*, 1992; Yadav *et al.*, 1993; Holm *et al.*, 1997; Lonergan *et al.*, 1999a). Interestingly, timing of the first cleavage division was not associated with viability of blastocysts on Day 7 pi following transfer to recipients, however, control and test blastocysts were separated by only a 6 h difference in cleavage time (Lonergan *et al.*, 1999a).

1.9.2 Cell Number

In vitro produced cow embryos have been shown to have a lower blastocyst total cell number (Iwasaki *et al.*, 1990) as well as a reduced number and proportion of cells in the ICM, than in vivo produced embryos (Marquant-Le Guienne *et al.*, 1989; Iwasaki *et al.*, 1990; Du *et al.*, 1996). Gardner and Lane (1997a) found that mouse blastocyst cell number, which has also been reported to be lower in in vitro produced embryos (Papaioannou and Ebert, 1986), and the number of cells in the ICM were positively correlated with fetal development following transfer. The morphology of cow blastocysts was reported to not be an accurate indicator of either the number or

proportion of cells in the ICM (Van Soom *et al.*, 1997a). Development of ruminant blastocysts on Day 7 versus Day 8 pi , which, as discussed above, has been associated with viability of cow embryos (Hasler *et al.*, 1995), has been linked with an increase in total blastocyst cell number (Hill *et al.*, 1997; Van Soom *et al.*, 1997a) and the number of cells in the ICM (Van Soom *et al.*, 1997b). Lonergan *et al.* (1999a) reported that the timing of blastocyst formation and blastocyst cell number were not linked with the timing of the first cleavage division, however, development and cell numbers were assessed on Day 8 pi, when many poorer quality embryos may have had time to reach the blastocyst stage.

1.9.3 Nutrient Uptake and Metabolism

Renard *et al.* (1978) revealed that a high glucose uptake by Day 10 cow blastocysts (measured in groups of three) was retrospectively related to the rate of embryo development in culture and survival following transfer to recipients. This was supported by a further study in which glucose uptake by individual blastocysts (Day 10 and 11 pi) was retrospectively correlated with viability following transfer (Renard *et al.*, 1980). Similarly, Gardner and Leese (1987) showed that the linear rate of glucose uptake by individual, Day 4 mouse blastocysts was positively correlated with embryo viability following transfer. Glucose uptake was also found to be significantly lower in Day 5 human blastocysts with a high degree of fragmentation, compared with unfragmented blastocysts (Hardy *et al.*, 1989b). Gardner *et al.* (1996b) found that the uptake of glucose and the production of lactate by frozen-thawed cow blastocysts could be used to predict, within the first 5 h of thawing, which blastocysts would re-expand and survive the freeze-thawing procedure. Interestingly, there was no overlap

in the distribution of glucose uptake between embryos that survived and those that did not survive the freezing and thawing.

Rieger *et al.* (1994) proposed that embryo metabolism could be used as a marker of embryo viability. The metabolism of [1-^{14}C] glucose by cow blastocysts was found to be significantly lower in degenerate than in normal blastocysts (Rieger, 1994). In the first documented prospective trial, Lane and Gardner (1996) demonstrated that the proportion of exogenous glucose converted to lactate could be successfully used to non-invasively predict the viability of morphologically identical mouse blastocysts, with excessive lactate production associated with non-viable embryos. Further research is needed to determine appropriate metabolic markers of viability for ruminant embryos.

1.10 THESIS AIMS

Despite the large number of studies analysing systems for the production of cow embryos in vitro, very little improvement can be seen in the overall development of cow embryos. The suitability of a particular culture system for embryo production has mostly been assessed with respect to the proportion of embryos developing to the blastocyst stage, with embryos often given several days beyond the time blastocysts would normally be seen in vivo, to develop to the blastocyst stage. Thus, the effects of various culture systems on other aspects of cow embryo physiology such as cell number, cell allocation to the TE and ICM, pHi and metabolic activity are mostly undetermined. All of these factors can potentially influence embryo viability.

Many studies have not had the physiology of the cow embryo in mind when designing experiments for the optimization of culture systems for cow embryos.

Determination of the requirements of the embryo for a particular component have often been made in media lacking many of the other components known to be beneficial to embryo development. The relevance of such findings to the requirements of embryos cultured under more optimal conditions must, therefore, be questioned. In addition, the uptake of glucose and pyruvate by cow embryos at each stage of development from the zygote to the blastocyst stage has not previously been determined under the same conditions of embryo culture. Further, the physiology of the embryo changes markedly as it develops from the zygote to the blastocyst stage. Thus, the effects of various culture components on the embryo and the requirements of the embryo will undoubtedly change with embryo development. Despite this, the majority of studies have cultured embryos in the same medium from the zygote to the blastocyst stage.

The main aim of the present research was to improve our understanding of the requirements of the cow embryo as it develops from the zygote to the blastocyst stage. The research focused on the embryo's requirements for carbohydrates, amino acids and vitamins and their potential roles in the culture system, including their effects on embryo development, cell number, cell allocation to the TE and ICM, blastocyst expansion, pHi and activities of the EMP and the TCA cycle. Determinations of nutrient uptakes and metabolism were made under similar conditions in which embryos were cultured so that uptakes and metabolism could be correlated with observed changes in embryo development. In addition, the studies were designed to identify changes in the requirements of the cow embryo as it develops from the zygote to the blastocyst stage.

Oocytes and embryos from pre-pubertal cows were also studied with respect to energy substrate metabolism or nutrient uptakes in an attempt to identify reasons for the often observed poor developmental competence compared with oocytes and embryos from adult cows.

It is hoped that a better understanding of factors affecting the physiology and metabolism of the cow embryo will assist in improvements to embryo culture systems, ultimately increasing the proportion and viability of embryos developing to the blastocyst stage.

CHAPTER 2

GENERAL EXPERIMENTAL PROCEDURES

2.1 SOURCE OF OOCYTES

Oocytes were obtained from several sources for experimental procedures.

2.1.1 Pubertal Cows

Cow ovaries were obtained from a local abattoir. Ovaries were collected from cows of unknown breed and age, but greater then twelve months old. Immediately after being dissected from the dead animal, ovaries were placed in a thermos flask containing 0.9% saline at 39°C and transported to the laboratory. Once at the laboratory, ovaries were rinsed free of blood with approximately 750 ml sterile saline solution, at 39°C. Ovaries were then placed in fresh saline solution in a beaker in a water bath, at 39°C.

Within five hours of collection, COC's were aspirated from ovarian follicles. The aspiration apparatus was a rubber-stoppered glass conical flask (250 ml) with an inlet hose connected to a suction valve from the hospital's main suction system. An outlet hose was attached to an 18-gauge needle that fed into a rubber-stoppered 10 ml test tube (Falcon, Becton Dickinson, North Ryde, NSW, Australia). The stopper of the test tube had an 18-gauge inlet needle which was attached to the tubing of a 19-gauge butterfly needle. During the aspiration process, the hospital suction was adjusted to ensure a slow and constant flow of follicular fluid through the aspiration tubing (approximately 75 mmHg). The butterfly needle was inserted into surface follicles of 2 - 5 mm in diameter and the needle was then scraped around the inside wall of each follicle to ensure recovery of COC's.

2.1.2 Pre-Pubertal Cows

Pre-pubertal oocytes were obtained from two sources: 1) unstimulated two to three month old Angus x Hereford calves[a] slaughtered at an abattoir, with COC's retrieved as for pubertal cows (Section 2.1.1) and 2) stimulated five to seven month old Friesian calves[b]. The Friesian heifers were housed indoors at VIAS[b] and fed a ration of barley concentrate (16% protein) and hay ad libitum. Each calf had a progestagen ear implant (Crestar, All Hank Trading, South Melbourne, Victoria, Australia) inserted for four days prior to receiving four injections (2,2,1,1 ml i.m.) of FSH (either 133 mg Folltropin V (Vetrepharm, South Melbourne, Victoria, Australia) or 6 mg Ovagen (Pacific Vet, Middle Park, Victoria, Australia) over the two days prior to transvaginal oocyte recovery[c] (TVR) (Fry *et al.*, 1994). At each TVR session, follicle number and diameter were recorded and all follicles greater than 3 mm diameter were aspirated. The aspiration equipment was identical to that described in section 2.1.1, however, suction was provided from an electric pump (75 mmHg).

2.2 PREPARATION OF MEDIA

2.2.1 Glassware

Glassware purchased for the preparation of culture media was thoroughly cleaned before use. Glassware was soaked for 24 h in 3% HCl (BDH, Poole, Dorset,

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[a] Supplied by Genetics Australia Co-op Ltd

[b] Supplied by the Victorian Institute of Animal Science, Werribee, Victoria, Australia (VIAS).

[c] Hormonal stimulation of calves and TVR performed by staff at VIAS.

UK) followed by a further 24 h in 7X detergent (ICN Biomedical, Seven Hills, NSW, Australia). Glassware was rinsed with warm tap water and any stickers or markings removed with acetone. Glassware was then rinsed six times with water from a reverse osmosis system (RO water) and left to soak overnight in RO water in a covered bucket. Glassware was then rinsed six times with fresh Milli-Q water (18 meg Ω) and placed in a sonicator for a minimum of 4 h. Following sonication, glassware was rinsed six times with Milli-Q water and placed upside down in an oven (120°C) to dry for 24 h. Glassware was then covered with foil and left in the oven for a further 24 h before use. Immediately after using glassware for making culture media, glassware was cleaned as above, commencing with the rinsing of glassware with RO water.

2.2.2 Reagents and Consumables

All salts used in the preparation of culture media were Analar grade (BDH). Glucose, sodium pyruvate, sodium lactate, phenol red, HEPES, betaine, D-pantothenic acid, niacinamide, folic acid, d-biotin, riboflavin, myoinositol, L-glutamine, L-alanine, L-asparagine, L-aspartic acid, glycine, L-proline and L-serine and TCM-199 (with Earle's salts and L-glutamine and without sodium bicarbonate) were purchased from Sigma Chemical Company (St Louis, MO, USA). Sodium pyruvate and sodium lactate were Sigma embryo tested products. Penicillin, Streptomycin, MEM vitamins solution and Eagle's essential amino acids solution (without glutamine) were obtained from ICN. BSA was essentially lipid-stripped and was purchased from Gibco BRL (No. PSB10036IM; Life Technologies, Grand Island, NY, USA). FCS was also purchased from Gibco BRL and was heat inactivated in a water bath at 56°C for one hour before use.

All media were stored in either 10 or 50 ml polystyrene tubes (Falcon), or 50 ml polystyrene flasks (Falcon). All media were filtered with 0.2 µm filters (Ministart, non-pyrogenic, Sartorius AG, Göttingen, Germany) attached to a 10, 20 or 50 ml plastic syringe (non-pyrogenic, Becton Dickinson Medical, Singapore, Malaysia). All culture dishes (organ culture dishes, 35 mm: primaria, 60 mm: non-pyrogenic and 110 mm dishes) were purchased from Falcon. Five and 10 ml plastic pipettes (Falcon) were used to measure volumes greater than 1 ml. A range of Gilson pipettes (Pipetman, Gilson Medical Electronics, France) with plastic tips (Quality Scientific Plastics, USA) were used to measure volumes of 1 ml or less. The plastic pipette tips were autoclaved and then oven dried (120°C for 24 h) before use. Light mineral oil (embryo tested) used in embryo culture was purchased from Sigma. Glass pasteur pipettes for manipulating embryos were 230 mm Volac disposable pipettes (John Poulten Ltd., Essex, England).

2.2.3 Quality Control

All reagents, dishes, tubes, oil, syringes and filters used in the preparation of culture media were tested for embryo toxicity prior to use. Items were tested in a routine laboratory media QC bioassay[d] (Gardner and Lane, 1993c). Each component was tested against established 'embryo safe' components (controls) in a mouse embryo culture system. Zygotes from F1 hybrid females were cultured in groups of 10

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[d] QC mouse bioassay performed by staff of the Embryo Physiology Laboratory, The Institute of Reproduction and Development, Monash University, Melbourne,Victoria, Australia.

in 20 µl drops of mMTF (Gardner and Lane, 1993a) with at least 30 embryos for the 'embryo safe' control and for each of the test components. Embryos were cultured for the first 24 h in mMTF containing BSA and were then transferred at the 2-cell stage to mMTF without BSA. Embryos were then cultured for a further 66 h to the blastocyst stage. Test components were classed as 'embryo safe' if at least 80% of zygotes developed to the blastocyst stage. Culture for the first 24 h was in the presence of BSA to facilitate development through the first cleavage division. Embryos were cultured in the absence of BSA beyond the 2-cell stage as BSA can act as a chelator and thus has the potential to mask the effect of toxic culture components (Baltz *et al.*, 1990).

Each test component classified as 'embryo safe' was further tested in a standard cow embryo in vitro production system (see sections 2.3, 2.4 and 2.5) to ensure that each component supported at least 70% cleavage of zygotes to the 2-cell stage and at least 40% of 2-cell embryos developing to the blastocyst stage, following culture in cow embryo culture media (see section 2.2.6).

2.2.4 Maturation Media

2.2.4.1 Composition

Medium used for the maturation of cow oocytes was powdered TCM-199 supplemented with sodium hydrogen carbonate, pyruvate, Penicillin, Streptomycin, FSH, LH and FCS (Table 2.1). Handling medium for collection and washing of cow oocytes was powdered TCM-199 supplemented with sodium hydrogen carbonate, HEPES, pyruvate, Penicillin, Streptomycin and BSA (H-199) (Table 2.1).

Table 2.1 Composition of maturation medium and maturation handling medium.

Compound	Maturation medium	Maturation handling medium (H-199)
TCM-199 powder†	Contents of bottle for 1 litre medium	Contents of bottle for 1 litre medium
$NaHCO_3$	25.00 mM	5.00 mM
Pyruvate	0.20 mM	0.33 mM
Penicillin	0.06 g/l	0.06 g/l
Streptomycin	0.05 g/l	0.05 g/l
FCS	10%	-
FSH	10 U/l	-
LH	10 U/l	-
HEPES	-	20.00 mM
BSA	-	4.00 g/l

†Purchased from Sigma.

2.2.4.2 Preparation

Hormones for maturation medium (LH and FSH) were prepared as stock solutions (Appendix 1) and stored in 50 µl aliquots at -80°C. A base medium of TCM-199 and stock solutions of maturation medium and H-199 were made every two weeks (Appendix 1) and then discarded. The pH of the stock solution of H-199 was adjusted to 7.4 with pellets of NaOH dissolved in H-199 stock solution (approximately pH 9). After addition of BSA to H-199, the final pH of the medium was 7.2 to 7.3.

Media were prepared in a laminar flow and filtered (0.2 µm filter) immediately after preparation. The osmolarity of the stock solutions of maturation and H-199 were checked with an osmometer after the production of each batch of medium. If the osmolarity of each stock medium was not equal to 295 ± 10 mOsm, the medium was discarded. Maturation medium and H-199 were made weekly from stock solutions (Appendix 2).

2.2.5 Fertilization Media

2.2.5.1 Composition

Medium for the preparation of bull sperm for fertilization was sperm-TALP containing salts, sodium hydrogen carbonate, sodium lactate, sodium pyruvate, HEPES and BSA (Table 2.2). Sperm-TALP was used as the base medium for Percoll gradients for purifying sperm (Appendix 3). Medium for the fertilization of oocytes was fert-TALP containing salts, sodium hydrogen carbonate, sodium lactate, sodium pyruvate and BSA (Table 2.2).

Table 2.2 Composition of Fert-TALP and Sperm-TALP media.

Compound	Fert-TALP (mM)	Sperm-TALP (mM)
NaCl	114.00	100.00
KCl	3.20	3.10
$NaHCO_3$	25.00	25.00
$NaH_2PO_4.H_2O$	0.34	0.290
Na Lactate	10.00	21.30
HEPES	-	10.00
Na Pyruvate	0.25	1.00
Phenol Red	0.01 g/l	0.01 g/l
$CaCl_2$	2.00	2.00
$MgCl_2.6H_2O$	0.50	1.50
BSA	6.00 g/l	6.00 g/l

2.2.5.2 Preparation

Sperm-TALP was prepared as a Sperm-TALP stock solution at ten times the required concentration (Appendix 4). This was stored at 4°C for a maximum of three months. Sperm-TALP was made every two weeks from the Sperm-TALP stock solution by the addition of BSA (Appendix 3). A Percoll stock solution was also made every two weeks by diluting neat Percoll with sperm-TALP stock solution (Appendix 3). Percoil gradients of 45%, 70% and 90% Percoll were made with the Percoll stock solution and Sperm-TALP (Appendix 3).

Fert-TALP was made every two weeks from stock solutions (Appendix 5). Stock A was stored at 4°C for a maximum of three months. Stock B and stock C were stored at 4°C for a maximum of two weeks and Stock D was stored at 4°C for a maximum of one month. The osmolarity of sperm-TALP and fert-TALP were checked with an osmometer after the production of each batch of medium. If the osmolarity of each stock medium was outside the required range (sperm-TALP: 300 ± 10 mOsm; fert-TALP: 290 ± 10 mOsm) the medium was discarded. Media were prepared in a laminar flow and filtered (0.2 μm filter) immediately after preparation.

2.2.6 Embryo Culture Media

2.2.6.1 *Composition*

All media for the culture of cow embryos were based on a modification of Synthetic Oviduct Fluid (SOF) (Tervit *et al.*, 1972). SOF is a bicarbonate-buffered balanced salt solution containing lactate, pyruvate, glucose and antibiotics (Table 2.3).

Table 2.3 Base media for the culture and handling of cow embryos.

Stock	SOF (mM)	H-SOF (mM)
NaCl	107.61	107.61
KCl	7.16	7.16
KH_2PO_4	1.19	1.19
$MgCl_2.6H_2O$	0.49	0.49
$NaHCO_3$	25.07	5.00
$CaCl_2.2H_2O$	1.71	1.71
Phenol red	10 mg/l	10 mg/l
Lactate (D/L-isomers)	3.30	3.30
Pyruvate	0.33	0.33
Glucose	1.50	1.50
Penicillin	60 mg/l	60 mg/l
Streptomycin	50 mg/l	50 mg/l
HEPES	-	20.00
BSA	8 mg/ml	4 mg/ml

Various components were added to SOF to determine their effects on cow embryo development. The composition of all culture media used in the study can be found in Appendices 6 and 7. The concentrations of individual amino acids and vitamins added to SOF are listed in Table 2.4. An HEPES-buffered version of SOF (H-SOF) was used to maintain embryos at a pH of 7.4 when out of the incubator for extended periods of time (Table 2.3). When modifications were made to the composition of SOF, HEPES-buffered versions of the media were used (Appendix 8).

2.2.6.2 *Preparation*

All culture media (Appendices 6 and 7) and HEPES-buffered media (Appendix 8) were prepared from stock solutions (Appendix 9). Each batch of medium was used for two weeks and then discarded. After the addition of all medium components, the pH of the bicarbonate-buffered medium was adjusted to 7.8 to 8.0. To adjust pH, a pellet of NaOH was added to 500 µl of the specific culture medium . The NaOH solution (approximately pH 9) was then added drop by drop to the culture medium with a fine pulled glass pipette. Media were prepared in a laminar flow and filtered (0.2 µm filter) immediately after preparation.

2.3 IN VITRO MATURATION

Follicular fluid containing COC's was aspirated directly into a 10 ml tube, standing in a water bath at 39°C (for aspiration procedure see section 2.1.1). Each tube was left standing for at least 15 min to allow COC's to settle and form a pellet in the bottom of the tube. Grids with lines 1 cm apart were scratched onto the lid and base of

Table 2.4 Concentration of amino acids and vitamins added to SOF.

Amino Acids	(mM)	Vitamins	(μM)
Glutamine	1.0	Biotin	4.1
Eagle's non-essential‡		**MEM**	
Alanine	0.1	Pantothenate	4.2
Asparagine	0.1	Choline chloride	7.5
Aspartate	0.1	Inositol	11.0
Glutamate	0.1	Niacinamide	8.2
Glycine	0.1	Pyridoxine HCl	4.9
Proline	0.1	Riboflavin	0.3
Serine	0.1	Thiamine HCl	3.0
		Folic acid	2.3
Eagle's essential‡			
Arginine	0.6		
Cystine	0.1		
Histidine	0.2		
Isoleucine	0.4		
Leucine	0.4		
Lysine	0.4		
Methionine	0.1		
Phenylalanine	0.2		
Threonine	0.4		
Tryptophan	0.05		
Tyrosine	0.2		
Valine	0.4		

‡Amino acids as classified by Eagle (1959): Minimum Essential Medium (MEM) non-essential amino acids and MEM essential amino acids. Note the Eagle's essential amino acids do not contain glutamine.

a 10 cm culture dish, with a razor blade. The lid and base were then warmed to 39°C on a microscope warming stage. Follicular fluid from a 10 ml tube was gently poured into the base of the dish, leaving the COC pellet in the tube in approximately 2 ml of follicular fluid. The pellet was then poured into the lid of the 10 cm dish and the tube was rinsed into the dish with two washes of approximately 4 ml H-199. With the aid of the grid, each dish (lid and base) was searched three times for COC's. Searching was performed under a stereomicroscope (10x - 100x magnification), on a heated microscope stage (39°C). COC's were collected with a 20 µl pipette and transferred to a 35 mm culture dish containing 2.5 ml H-199, at 39°C. COC's were then graded. COC's with a compact cumulus of at least five layers and an even cytoplasm, were then washed two times in 2.5 ml H-199. Groups of fifty COC's were then transferred to 800 µl maturation medium, pre-equilibrated at 5% CO_2 and air, at 39°C. Maturation medium was in the inside well of an organ culture dish with 3 ml maturation stock medium in the outer well to prevent evaporation. COC's were matured for 22 to 24 h, at 5% CO_2 and air, at 39°C.

2.4 IN VITRO FERTILIZATION

On the day of fertilization, 10 µg heparin ml^{-1} (Sigma) was added to a 10 ml tube of pre-equilibrated fert-TALP (5% CO_2 and air, at 39°C). The 10 ml fert-TALP was then divided as follows: 5 ml into a 15 ml conical tube for washing Percoll from sperm; 2.5 ml in a 35 mm culture dish and overlayed with 1.5 ml mineral oil (pre-equilibrated to 5% CO_2 and air, at 39°C) for washing COC's; 1 ml in a 10 cm tube for adjusting sperm concentration; and 15 µl drops in a 60 mm culture dish (one drop of

medium for every ten COC's) containing 9.0 ml of pre-equilibrated mineral oil (5% CO_2 and air, at 39°C). All dishes and tubes containing fert-TALP were then returned to the incubator until use.

2.4.1 Preparation of Eggs

Following 22 to 24 h maturation, COC's were removed from maturation medium and washed three times in 2.5 ml H-199, at 39°C. COC's were then transferred in groups of ten to the equilibrated wash dish containing fert-TALP. Each group of ten was picked up in 15 µl fert-TALP and added to a 15 µl drop of fert-TALP in the 60 mm culture dish. The dish containing COC's was returned to the incubator until the completion of sperm preparation. The preparation of sperm was timed so that the eggs were fertilized within 15 min of leaving the maturation medium.

2.4.2 Preparation of Sperm

Percoll gradients were gently layered into a 15 ml conical tube (1 ml of each of 90%, 70% and 45% Percoll). The Percoll gradients were then warmed to 39°C in a water bath. A straw of frozen spermatozoa from a bull of proven fertility[e] was thawed at room temperature for 10 seconds and then in a water bath set at 35°C for a further

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[e] Straws of frozen semen provided by Genetics Australia Co-op. (Bacchus Marsh, Victoria, Australia). Semen was from the bull Wisdom.

20 seconds. The ends of the straw were cut and the sperm were gently layered on top of the Percoll gradient before being centrifuged at 600 G (1800 rpm) for 20 min. The pellet of motile sperm was then removed from the bottom of the tube in approximately 400 µl and added to the 15 ml tube containing 5 ml fert-TALP. This was done in order to wash Percoll from the sperm. The tube was centrifuged at 300 G (1300 rpm) for 5 min. Fert-TALP was then carefully removed from the tube with a 5 ml pipette leaving the undisturbed pellet of sperm in approximately 700 µl fert-TALP. The pellet of sperm was then resuspended in the remaining fert-TALP.

Sperm concentration was then determined by placing 10 µl of the sperm/fert-TALP solution into a Makler Counting Chamber (EL-OF, Israel) and counting the number of forwardly motile sperm in ten squares (100x magnification). The number of motile sperm in ten squares (n) equalled n x 10^6 sperm/ml. The desired final concentration of sperm for fertilization was $2x10^6$/ml motile sperm[f]. The volume of sperm/fert-TALP to add to drops containing COC's in order to fertilize eggs in a volume of 50 µl was calculated according to the following equation:

$$\text{Volume of sperm/fert-TALP} = (2x10^6 / n \times 10^6) \times 0.050 \times 1000.$$

The calculated volume of Sperm/fert-TALP was then added to drops of fertilization medium containing COC's. Equilibrated fert-TALP was then added to drops to make a final volume of 50 µl. Fertilization drops were then returned to the incubator for 18 to 22 h, at 5% CO_2 and air, at 39°C.

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[f] Optimal sperm and heparin concentrations were pre-determined for the bull Wisdom based on the number of pronuclei at 22 h pi, the number of cleaved embryos versus eggs and subsequent development to the blastocyst stage.

2.5 EMBRYO CULTURE

2.5.1 Handling of Embryos

Fine pulled glass pipettes were used to transfer presumptive zygotes and embryos between different media. The glass pipettes were heat sterilized in an oven for 24 h at 120°C. Just prior to use, the narrow neck of a pipette was heated over a Bunsen burner in order to soften the glass. The pipette was then removed from the flame and each end of the pipette was pulled horizontally. The inside diameter of each pipette was thus reduced to a size just larger than the diameter of the embryo (130 to 160 μm depending on the size of the embryo). The pulled glass was then severed and the tip was briefly placed in the yellow flame of the Bunsen in order to produce a smooth tip with no jagged edges. The large end of the pipette was subsequently attached to tubing with a 0.2 μm filter and a mouth piece. Prior to handling embryos, the capillary action of the pipette was broken by allowing small amounts of media and air to alternately move up the pipette. Embryos could then be collected and dispensed in minimal volumes of media.

2.5.2 Denuding Presumptive Zygotes

Presumptive zygotes were removed from fertilization drops (18 to 22 h pi) and transferred with a fine pulled glass pipette into 1 ml H-SOF in a 15 ml conical tube. The tube was then vortexed for 2 min (high setting) to remove cumulus and corona cells from the presumptive zygotes. Media containing presumptive zygotes was then poured into a 3.5 ml culture dish. The conical tube was rinsed four times with approximately 2 ml H-SOF and the contents distributed among several 3.5 ml dishes.

Denuded presumptive zygotes were then retrieved from the dishes and washed twice in 3 ml H-SOF.

2.5.3 Culture Conditions

Conditions for the culture of cow embryos were based on the study by Gardner *et al.* (1994b). Following denuding, presumptive zygotes were immediately transferred to culture media (see section 2.2.6). Embryos were cultured in 30 µl drops of media in a 5 ml culture dish containing 7.5 ml mineral oil. Five embryos were cultured per 30 µl drop medium. Dishes containing media and oil were set up at least 12 h before the addition of embryos and equilibrated in a modular incubation chamber (MIC: Billups-Rothenberg, Delmar, CA, USA) at 5% CO_2, 7% O_2 and 88% N_2, at 39^{0}C. The MIC was gassed by purging the chamber with 5% CO_2, 7% O_2 and 88% N_2 for 3 min and then sealed. Approximately 100 ml of water were added to the bottom of the MIC to humidify the chamber and prevent evaporation. Embryos were cultured for a total of six days (144 h) with a change into fresh pre-equilibrated culture medium drops at 72 h culture. During the move into fresh medium, the MIC remained in the incubator at 39^{0}C and was continuously gassed at 5% CO_2, 7% O_2 and 88% N_2.

2.6 ASSESSMENT OF OOCYTE MATURATION

2.6.1 Morphology

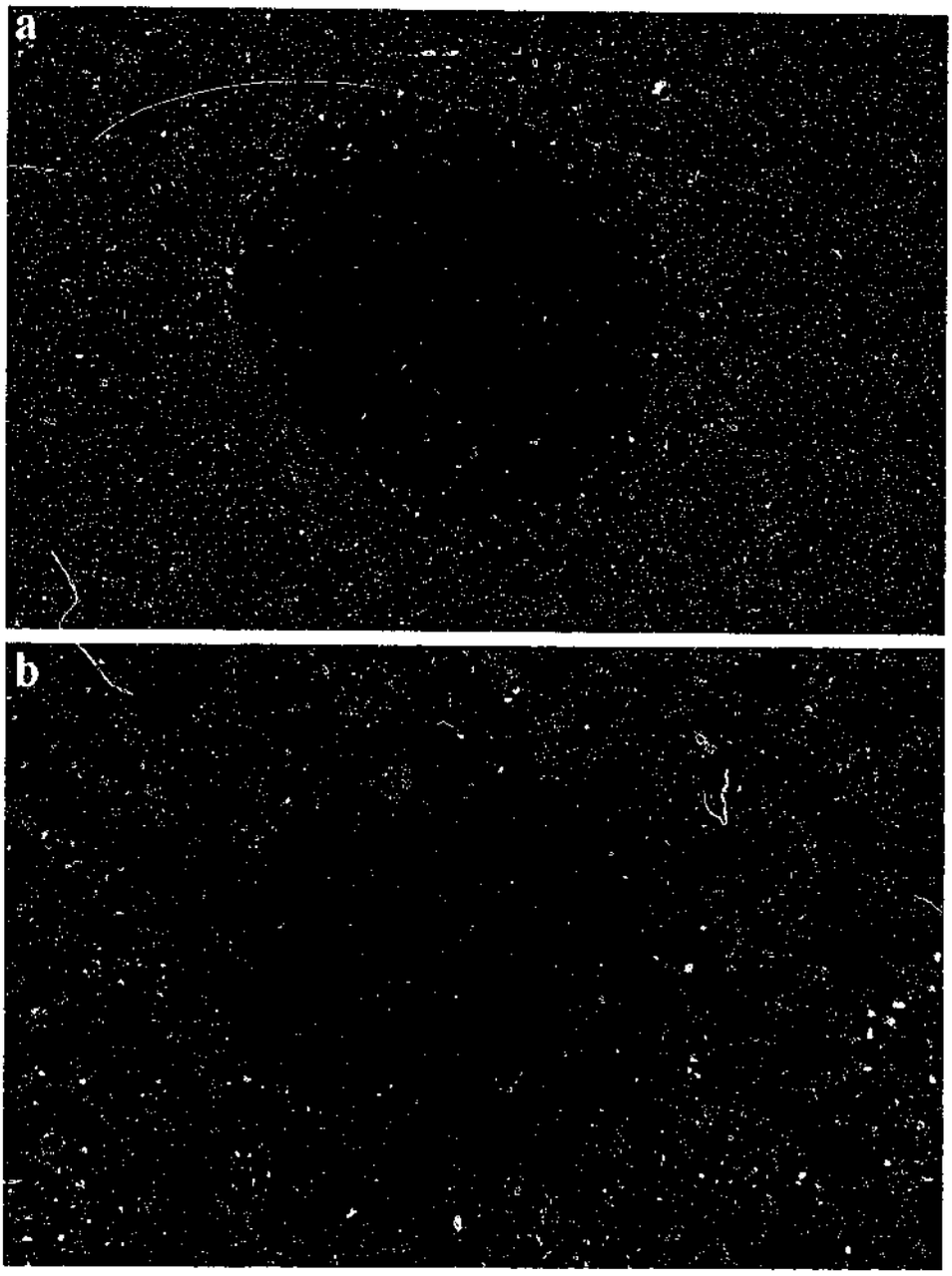
Oocytes retrieved from the follicles of ovaries were classified as immature (Figure 2.1a). The cumulus and corona cells of immature oocytes were tight and compact. Following culture in maturation medium for 22 to 24 h, oocytes were morphologically classified as mature if cumulus and corona cells were expanded and fluffy in appearance (Figure 2.1b).

2.6.2 Determination of Nuclear Maturation

For the determination of GV, GVBD and MII stage oocytes, COC's were transferred to a 15 ml conical tube containing 1 ml of H-199 with 1 mg hyaluronidase ml^{-1} (Sigma), at 39^0C, for 30 seconds. COC's were then vortexed to remove all cumulus and corona cells. The contents of the tube were poured into a 35 mm dish containing 3 ml H-199 and the tube was rinsed three times with H-199. Any remaining corona cells were removed from the oocytes by pipetting up and down with a fine pulled glass pipette. Denuded oocytes were then washed again in H-199.

For the determination of oocytes at GV and GVBD stages, oocytes were transferred to a microscope slide in a minimal volume of medium (approximately 2 µl), with up to ten oocytes per drop. A mixture of paraffin wax and vaseline (1:1) was placed on each corner of a cover-slip and the cover-slip was then placed over the drop containing the oocytes and pushed down gently. The paraffin wax and vaseline mixture allowed for a small space between the oocytes and the cover-slip. Each slide

Figure 2.1 Micrographs of (a) an immature and (b) a mature oocyte.


a
b

was then placed in a jar of ethanol:acetic acid (3:1) for 24 h to fix the oocytes. Once removed from the fixative, the nuclei of the oocytes were stained with Hoechst (Number 33258, Sigma) by pipetting SOF containing 30 μg Hoechst ml^{-1} (approximately 30 μl) under the coverslip on each slide. After 30 min, oocyte nuclei were viewed under fluorescent light on an inverted microscope (200x magnification). Oocytes with a distinct germinal vesicle, as evidenced by the presence of a distinct nuclear envelope, were classified as GV stage oocytes (Figure 2.2a). Oocytes with a less distinct nuclear envelope or no visible nuclear envelope and the chromatin still in the area of the nucleus were classified as GVBD stage oocytes (Figure 2.2b). For the determination of oocytes at MII, oocytes were viewed under an inverted microscope immediately following the removal of cumulus cells. Oocytes with an extruded polar body were classified as MII oocytes (Figure 2.3).

2.7 ASSESSMENT OF EMBRYO MORPHOLOGY

2.7.1 Classification According to Stage of Development

Embryo morphology during development was assessed according to the number of distinct cells in the embryo (Figure 2.4). Cleavage stage embryos were classified as either 2-cell, 4-cell, 8-cell, or 9- to 16-cell (Figure 2.4a-d). Morulae were classified as either a compacting morula (embryos with 16- to 32-cells, in the initial stages of compaction, Figure 2.4e) or a compacted morula (fully compacted cells, Figure 2.4f).

Blastocysts were characterized as follows: early blastocyst (blastocoel cavity less than two thirds of the volume of the embryo, Figure 2.5a); mid blastocyst

Figure 2.2 Micrographs of cow oocytes at (a) the germinal vesicle (GV) stage and (b) the germinal vesicle breakdown (GVBD) stage.

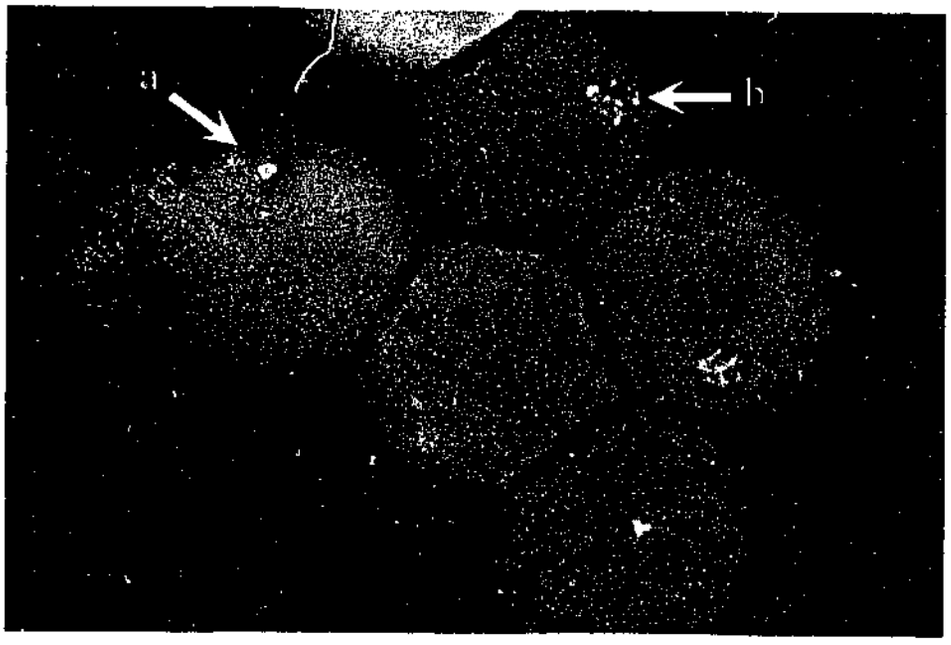

a
b

Figure 2.3 Micrograph of a cow oocyte at Metaphase II (MII).

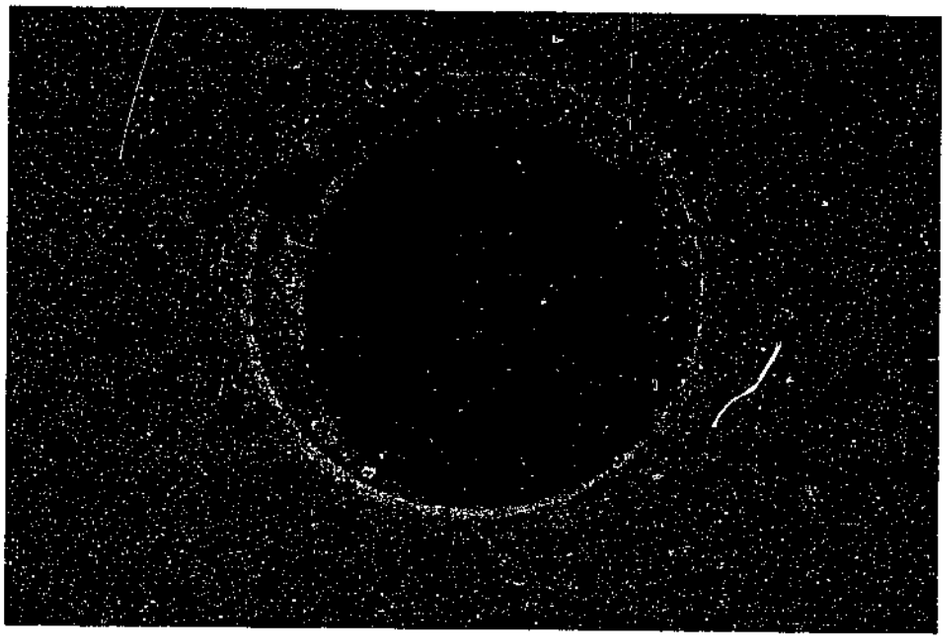


Figure 2.4 Micrographs of the morphological classification of cow embryos during culture: (a) 2-cell embryo; (b) 4-cell embryo; (c) 8-cell embryo; (d) 9- to 16-cell embryo; (e) early morula (compacting 16- to 32-cell embryo); and (f) morula.

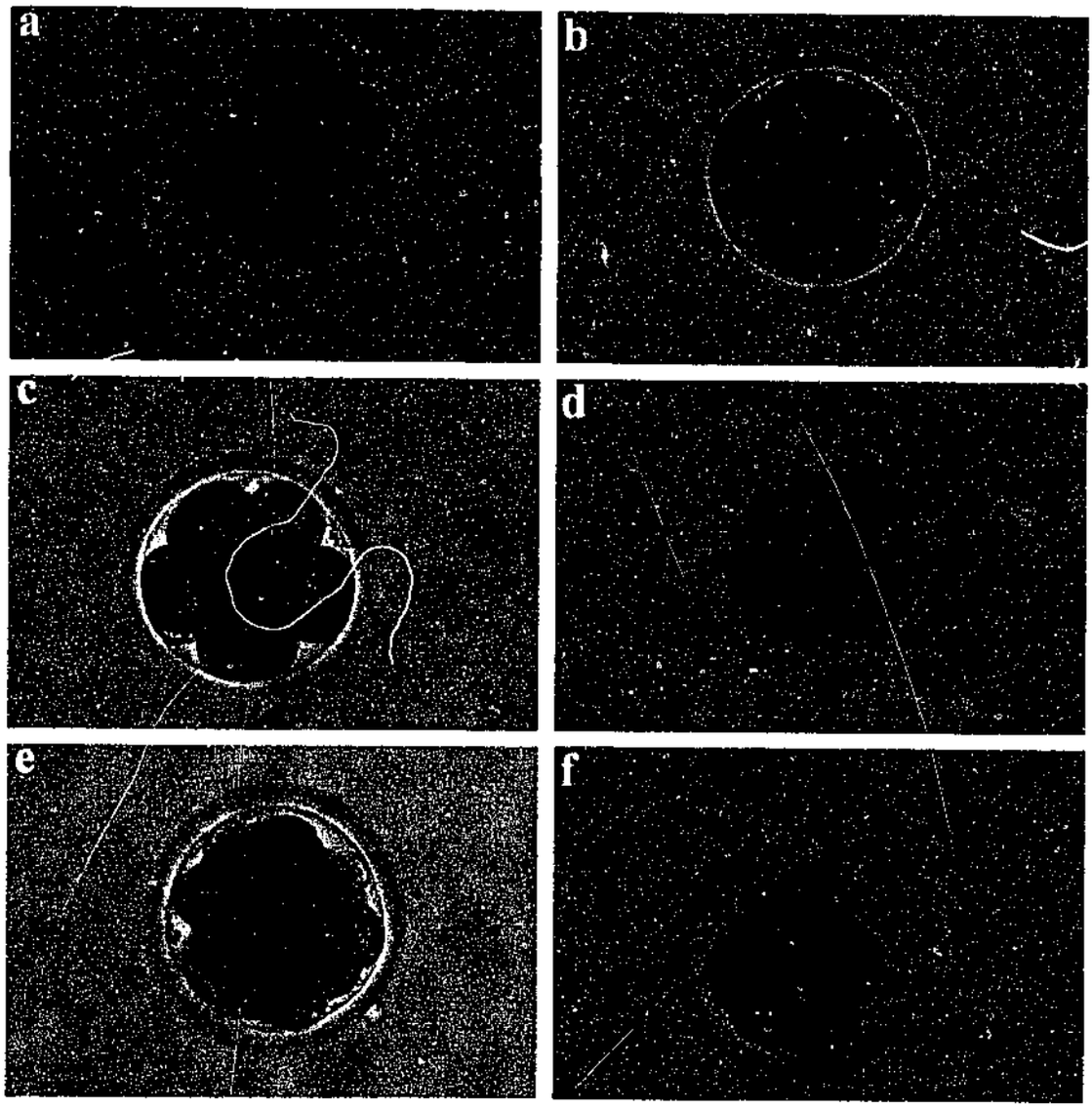
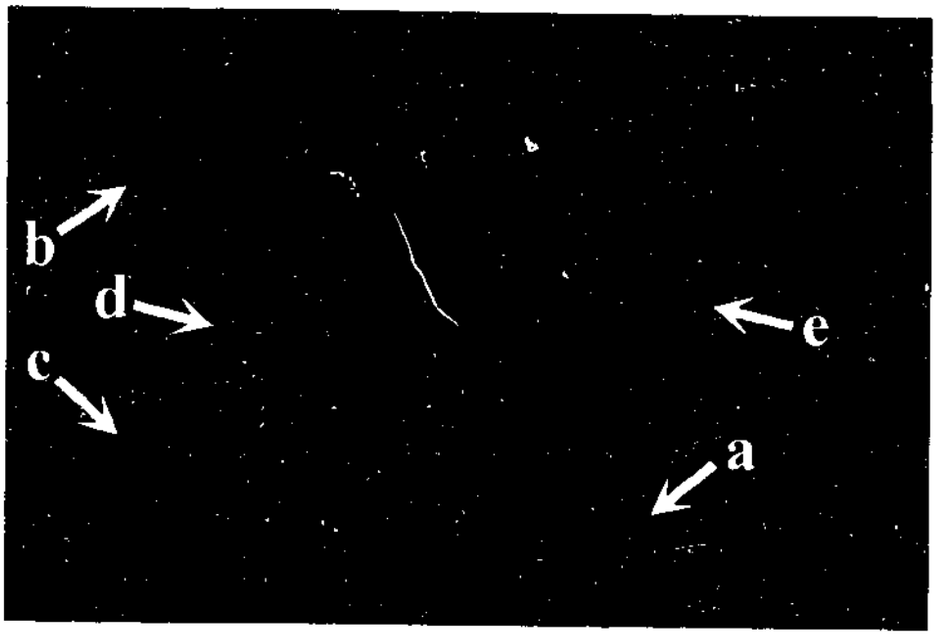

a
b
c
d
e
f

Figure 2.5 Micrographs of the morphological classification of cow blastocysts during culture: (a) early blastocyst; (b) mid blastocyst; (c) expanded blastocyst; (d) hatching blastocyst; (e) hatched blastocyst.

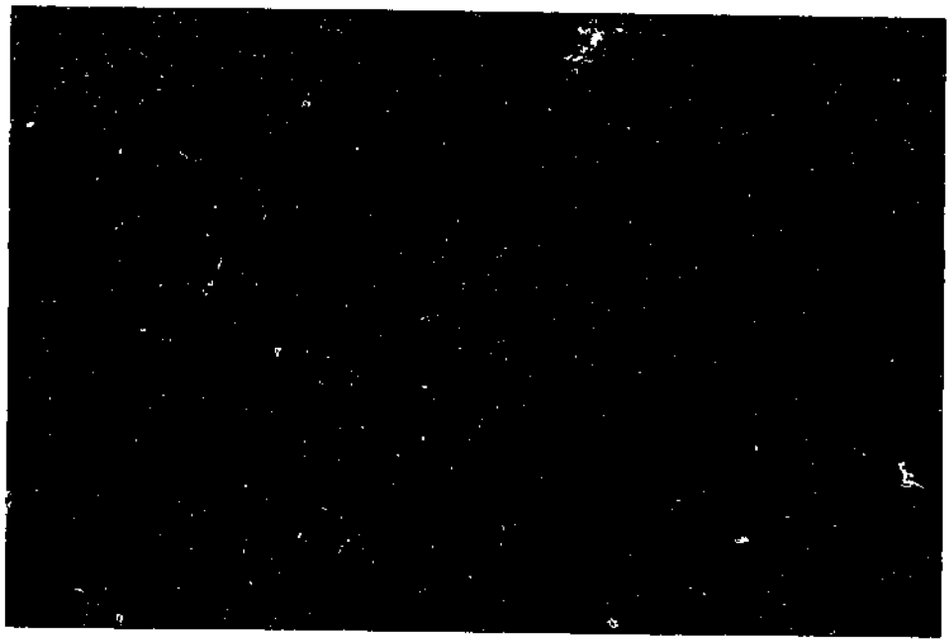

b
d
c
e
a

(blastocoel cavity equal to or greater than two thirds the volume of the embryo but width of the zona pellucida equal to that of an early blastocyst, Figure 2.5b); expanded blastocyst (blastocoel cavity greater than two thirds of the embryo volume, the zona pellucida thinner than a mid blastocyst and the diameter of the embryo greater than a mid blastocyst, Figure 2.5c); hatching blastocyst (TE escaping through a herniation of the zona pellucida, Figure 2.5d); and hatched blastocyst (an expanded blastocyst with no zona pellucida, Figure 2.5e).

2.7.2 Determination of Embryo Cell Number

Some embryos were stained to determine the precise number of cells at the blastocyst stage. At the end of the culture period, blastocysts were incubated for approximately 3 to 5 min in a 30 µl drop of protein-free H-SOF containing Pronase (0.5%, w/v; Sigma), under light mineral oil, at 39°C, to remove the zona pellucida. The zona pellucida was removed in order to allow blastomeres to separate after staining, making counting of individual cell nuclei easier. Blastocysts were washed once in 3 ml PBS and transferred to 500 µl ethanol, containing 25 µg Hoechst ml^{-1}. Embryos remained in Hoechst for at least 18 h in the dark at 4° C. Blastocysts were subsequently washed in ethanol before being mounted on a microscope slide in a small drop of glycerol. A coverslip was placed over the drop of glycerol and lightly depressed in order to separate the cells of the blastocyst. Embryos were then viewed on an inverted microscope under UV light (x 200 magnification). The filter used to view the embryos had an excitation wavelength of 340-380 nm. Cell nuclei appeared blue and could be individually counted (Figure 2.6).

Figure 2.6 Micrograph of a cow blastocyst (Day 7 post-insemination). Cell nuclei have been stained with Hoechst (Number 33258) to enable counting of total cell number.



2.7.3 Differential Nuclear Staining

Differential nuclear staining (or dual staining) of blastocysts enabled the determination of the number of cells differentiated into the ICM and the TE. The procedure used was a modification of that used by Hardy *et al.* (1989a). All steps of the procedure were performed in 30 µl drops of the specific solutions covered with light mineral oil.

Blastocysts were treated with Pronase (0.5% (w/v)) in protein-free H-SOF for 3 to 5 min, at 39°C to remove the zona pellucida. Blastocysts were then washed in protein-free H-SOF and incubated for 10 min on ice, in 10 mM picrysulfonic acid (Sigma) in SOF with 4 mg PVP ml^{-1} (Calbiochem Corporation, La Jolla, CA, USA). After being washed in protein-free H-SOF, blastocysts were incubated for 60 min in H-SOF containing 0.1 mg anti-dinitrophenol BSA ml^{-1} (ICN), at 39°C. Blastocysts were again washed in protein-free H-SOF and incubated in the dark for 12 to 15 min in a 1/10 dilution of guinea-pig complement (ICN) in protein-free H-SOF, containing 20 µg propidium iodide ml^{-1} (Sigma), at 39°C. Blastocysts were then washed briefly in PBS and transferred to ethanol containing 25 µg Hoechst ml^{-1}. Blastocysts remained in Hoechst for at least 18 h in the dark, at 4° C. Blastocysts were subsequently washed in ethanol before being mounted on a microscope slide in a small drop of glycerol. A coverslip was placed over the drop of glycerol and pushed down lightly to spread blastocyst cells out. Embryos were viewed on an inverted microscope under UV light. Two filters were used to count ICM and TE cells. Both ICM (appeared blue) and TE (appeared orange) cells could be viewed with filter number 1 (excitation wavelength of 340-380 nm; Figure 2.7a). Only TE cells could be viewed with filter number 2

Figure 2.7 Micrographs of a cow blastocyst (Day 7 post-insemination). Cell nuclei have been differentially stained in order to distinguish between the inner cell mass (ICM) and the trophectoderm (TE).

(a) Blastocyst viewed with UV filter. ICM cell nuclei appear blue and TE cell nuclei appear orange.

(b) Blastocyst viewed with blue/green filter. Only orange TE cell nuclei are visible.


a


b

(blue/green filter with an excitation wavelength of 350-460; Figure 2.7b), enabling TE cells to be counted more easily.

2.8 MEASUREMENT OF THE ACTIVITY OF METABOLIC PATHWAYS IN INDIVIDUAL OOCYTES AND EMBRYOS

The relative activities of metabolic pathways within individual oocytes or embryos was determined with the use of radiolabelled energy substrates. The procedure was adapted from Rieger *et al.* (1992a). Specific energy substrates labelled with either ^{14}C or ^{3}H were incorporated into culture media (metabolic media; Appendices 10 and 11). The use of two radioisotopes enabled the determination of the metabolism of two energy substrates simultaneously. A miniaturised enclosed culture system was set up in a 2 ml microcentrifuge tube to trap $^{14}CO_2$ and $^{3}H_2O$ liberated from the metabolism of the radiolabelled substrates (Figure 2.8). An individual oocyte or embryo was incubated in a 3 μl drop of metabolic medium in the lid of a tube. Within the tube, 1.5 ml of $NaHCO_3$ served as a reservoir to trap the liberated $^{14}CO_2$ and $^{3}H_2O$. Radiolabel was trapped because of the large size differential between incubation volume and reservoir volume.

2.8.1 Radiolabelled Energy Substrates for the Determination of Metabolic Activity

Three radiolabelled energy substrates were used to measure the activity of specific metabolic pathways in the oocyte and embryo. [5-^{3}H] glucose was chosen to reflect total glucose utilization. While the conversion of [5-^{3}H] glucose to 2-

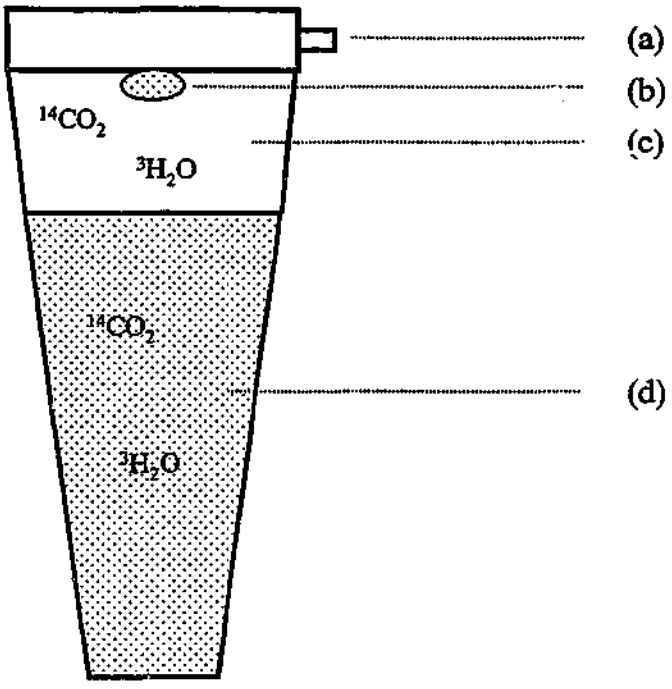


Figure 2.8 A microcentrifuge tube set up as a miniature incubation chamber to determine the metabolism of radiolabelled energy substrates by individual oocytes and embryos.

(a) Lid of tube

(b) Drop of metabolic medium containing oocyte or embryo

(c) Air space between metabolic medium and $NaHCO_3$ reservoir for the exchange of liberated $^{14}CO_2$ and ^3H_2O.

(d) $NaHCO_3$ reservoir to trap liberated $^{14}CO_2$ and ^3H_2O.

phosphoglycerate (resulting in the liberation of ^3H_2O) is a direct measurement of the flux of glucose through the EMP (Figure 1.1), it also is a reflection of total glucose metabolism by the oocyte or embryo (Tiffin *et al.*, 1991). This is because there is a reversible link between the EMP and the PPP: [5-^{3}H] glucose-6-phosphate that enters the PPP will re-enter the EMP as [2-^{3}H] glyceraldehyde-3-phosphate and will result in the liberation of ^3H_2O.

Two radiolabelled substrates were chosen to measure the activity of the TCA and thus mitochondrial function. The metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine are both indicators of different sections of the TCA cycle. Pyruvate enters the TCA cycle following its oxidative carboxylation to acetyl CoA (Figure 1.2) and the $^{14}CO_2$ liberated from the metabolism of [2-^{14}C] pyruvate is released prior to the entry of glutamine into the TCA cycle at α-ketoglutarate (Figure 1.2).

The radiolabelled substrates were purchased with the following specific activities: [5-^{3}H] glucose (15.7 mCi/mmol, Amersham International plc, Buckinghamshire, UK); [2-^{14}C] pyruvate (15.9 mCi/mmol, Pharmacia Biotech, Asia Pacific Ltd, Quarry Bay, Hong Kong); and [G-^{3}H] glutamine (54.0 Ci/mmol, Amersham). The desired specific activity of each radiolabelled substrate was 0.25 μCi/μl (Rieger *et al.*, 1992a). A specific activity of 0.25 μCi/μl for [2-^{14}C] pyruvate, however, would have resulted in a concentration of around 16 mM pyruvate which is nearly fifty times greater than the concentration of pyruvate in SOF (Table 2.3). Thus, [2-^{14}C] pyruvate was used at a specific activity of 0.005 μCi/μl. The final concentrations of radiolabelled substrates in the metabolic media were as follows: [5-^{3}H] glucose (0.017 mM), [2-^{14}C] pyruvate (0.33 mM) and [G-^{3}H] glutamine (0.005 mM).

2.8.2 Metabolic Incubation Procedure

The day prior to metabolic determinations, known volumes of radiolabelled substrates were added to 5 ml tubes. The number of tubes was dependent on the number of treatment groups (that is, different metabolic media). Individual tritiated and ^{14}C substrates were combined when determinations were made of the metabolism of two substrates simultaneously. Volumes were pre-determined in order to give the required specific activity of each radiolabelled substrate when reconstituted in metabolic media (Appendices 10 and 11). The radiolabelled substrates were then dried with nitrogen gas. A tube and glass pipette were attached to the regulator of a nitrogen gas cylinder. The glass pipette tip was placed inside the tube about half way down. The nitrogen gas was then turned on gently so as not to move the drop of radiolabelled substrates. When radiolabelled substrates were fully dried, the tube was sealed and kept at $4^{0}C$ until use.

On the morning of the metabolic determinations, the pre-determined volume of each pre-equilibrated metabolic medium was added to each vial of dried radiolabelled substrate. A drop of metabolic medium (maximum 60 µl) containing the radiolabel was then pipetted into a 35 mm culture dish containing 4.0 ml pre-equilibrated mineral oil. Dishes were then returned to the incubator and allowed to re-equilibrate for approximately 2 h.

For metabolic incubations embryos were washed five times in the pre-equilibrated metabolic medium without radiolabel and then transferred in less than 1 µl to the drop of metabolic medium containing radiolabel (typically ten embryos per 60 µl drop). Each embryo was then picked up in 3 µl medium and transferred to the lid of a 2.0 ml tube. Each tube was then filled with 1.5 ml pre-equilibrated $NaHCO_3$

(25 mM) and purged with gas (5% CO_2 and air or 5% CO_2, 7% O_2, 88% N_2, depending on experiment) before being sealed and placed in the incubator at 39°C. From the same drop of medium, three sham controls (no embryo in 3 µl drop) and three total counts (3 µl added directly to $NaHCO_3$ in tube) were set up. After 3 h incubation, 1 ml of the $NaHCO_3$ was removed and added to 200 µl of 0.1 mM NaOH in a 15 ml scintillation vial (super polyethylene vials, Packard Instrument Company, Meriden, CT, USA). The scintillation vials were stored at 4°C for at least 18 h, before the addition of 10 ml scintillation fluid (Optiphase Hisafe 3; Wallac, Milton Keynes, UK). Radioactivity was then measured in a liquid scintillation counter programmed for dual radioisotope counting (5 min per vial).

The amount of each radiolabelled substrate metabolized by individual oocytes and embryos (x) was calculated as pmoles/embryo/h for embryos and pmoles/oocyte/h for oocytes, by the following equation:

$$x = \frac{\text{(count for embryo - sham) x (mol of substrate present) x (recovery efficiency) x (concentration of labelled substrate + concentration of unlabelled substrate)}}{\text{(total counts) x (concentration of labelled substrate)}}$$

2.8.3 Calculation of Recovery Efficiencies for Radioisotopes

In order to accurately determine the proportion of liberated radiolabel trapped in the $NaHCO_3$ reservoir, it was necessary to determine the exchange rate or recovery efficiency of individual isotopes between the incubation drop and the $NaHCO_3$ reservoir. Known amounts of $NaH^{14}CO_3$ and ^3H_2O were added to pre-equilibrated SOF

medium to give specific activities of 0.005 μCi/μl and 0.25 μCi/μl, respectively (specific activity of each radioisotope incubated with individual oocytes and embryos). A total of forty five tubes was set up as in section 2.8.2. Five of the tubes were opened immediately (time 0 min) and 1 ml of the $NaHCO_3$ removed. A further five tubes were opened and $NaHCO_3$ was removed every 30 min for 4 h. Five tubes were also set up to determine total counts.

The recovery efficiency at each time point was calculated from the proportion of total radioactivity found in the $NaHCO_3$ reservoir. The recovery of both $^{14}CO_3$ and ^3H_2O increased rapidly up to 30 min and then more slowly to over 90% after 150 min (Figure 2.9). The recovery efficiency following a 3 h incubation was thus taken as 1.04 (100/96) for ^3H_2O and 1.10 (100/91) for $^{14}CO_2$.

2.9 DETERMINATION OF SUBSTRATE UPTAKE AND METABOLITE PRODUCTION BY INDIVIDUAL EMBRYOS

2.9.1 Assay Technique

The non-invasive technique of microfluorescence was used to determine the rates of nutrient uptake and metabolite production by individual embryos at various stages of development (Leese and Barton, 1984; Gardner *et al.*, 1993). The fluorometric technique was modelled on standard methods for enzymatic analysis, utilizing the pyridine nucleotides NADH and NADPH which are generated or consumed in coupled reactions (Bergmeyer and Gawehn, 1974). The reduced forms of the nucleotides fluoresce when exposed to UV light (340 nm).

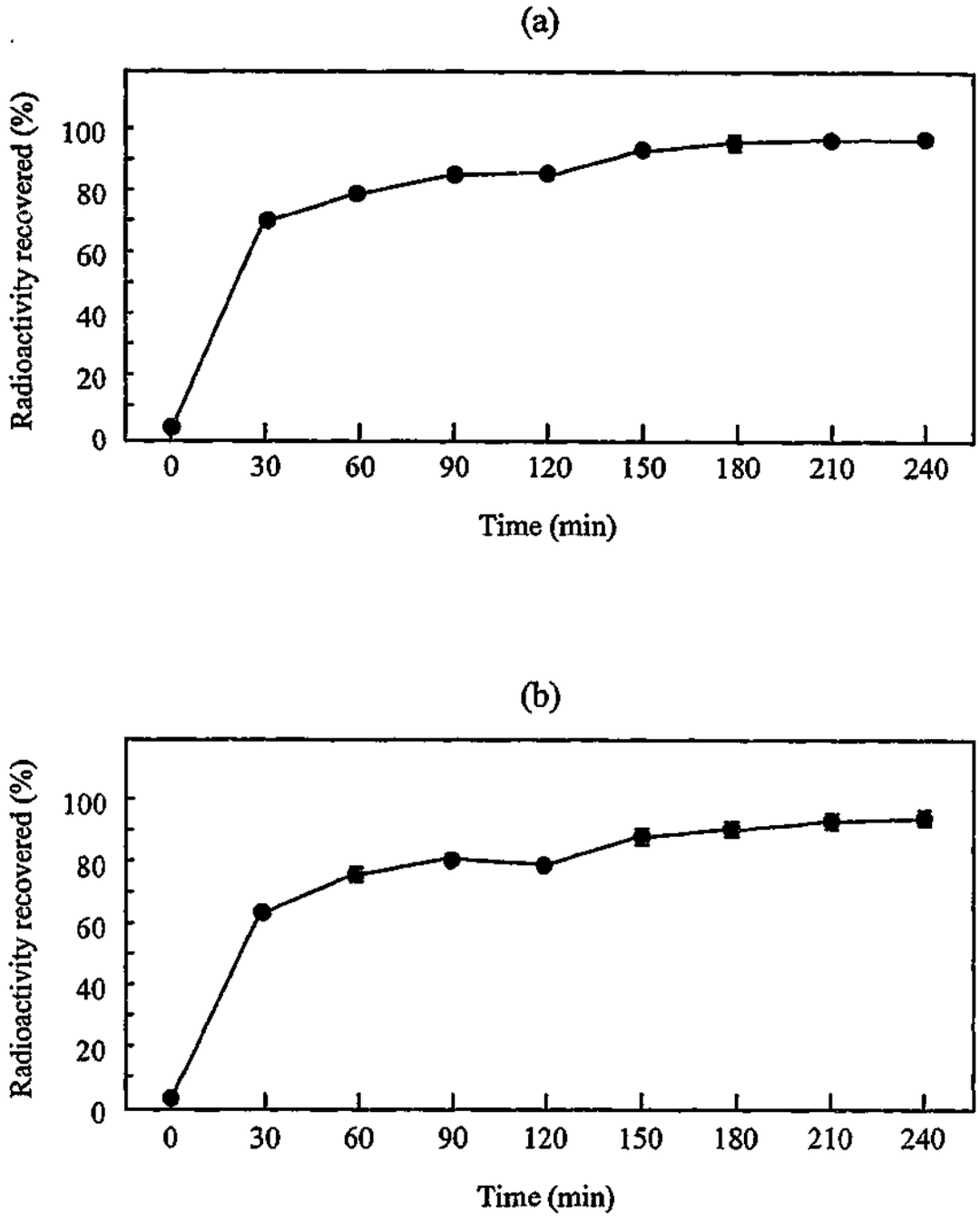


Figure 2.9 The recovery of $^{3}H_2O$ (a) and $^{14}CO_2$ (b) over a 4 h period into 1.5 ml $NaHCO_3$ (25 mM) from a 3 µl drop of SOF containing $^{3}H_2O$ at a specific activity of 0.25 µCi/µl and $NaH^{14}CO_3$ at a specific activity of 0.005 µCi/µl. Each point represents the mean ± SEM of five measurements.

Each biochemical assay was performed in a nanolitre sized drop (typically 10-20 nl) under heavy white mineral oil (Sigma) on a siliconized microscope slide. The fluorescence of each assay drop, containing enzymes and cofactors for a specific biochemical substrate assay, but minus the substrate, was initially measured using an inverted fluorescent microscope with photomultiplier and photometer attachments (Leitz, Fluorovert). The specific substrate was added (typically 1-2 nl depending on the pre-determined ratio of substrate solution to assay solution) and the biochemical reaction was allowed to proceed to completion before the assay drop was again measured to determine the level of fluorescence. The change in fluorescence following the addition of the substrate to the assay drop was quantified by the construction of a standard curve in which fluorescence increased or decreased linearly with an increase in substrate concentration (typically five serial dilutions of substrate from 0 to 0.5 mM; Figure 2.10). A new standard curve was constructed each day that assays were run. Each standard curve had to produce a regression value of at least 0.996 to be used for the quantification of fluorescence.

The biochemical assays used in the present study were as follows:

Glucose Assay

$$\text{(1) Glucose + ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate + ATP}$$

$$\text{(2) Glucose-6-phosphate + } NADP^{+} \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate + NADPH + } H^{+}$$

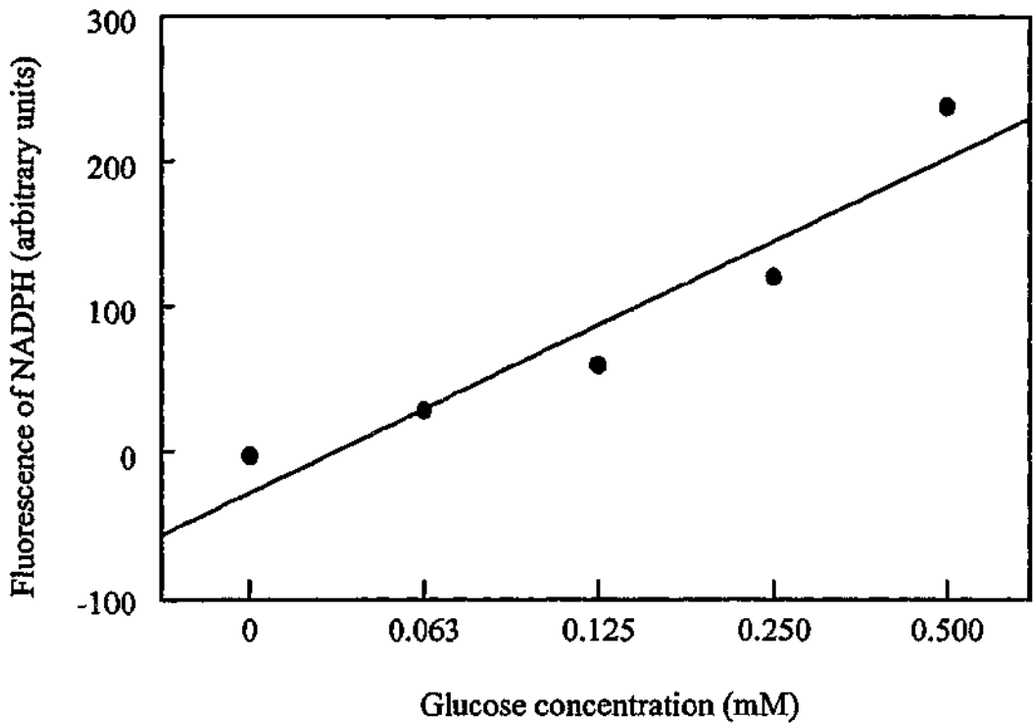


Figure 2.10 A calibration curve for the change in fluorescence of NADPH with an increase in the concentration of glucose (r = 0.9997).

The glucose assay involved a two-step reaction for the generation of fluorescent NADPH. An increase in glucose concentration thus equated to an increase in fluorescence. In the first reaction, hexokinase catalysed the phosphorylation of glucose to glucose-6-phosphate. In the second reaction, glucose-6-phosphate dehydrogenase catalysed the oxidation of glucose-6-phosphate to 6-phosphogluconate (G6PDH). The equilibrium of equation (2) lies far to the right.

The ratio of substrate solution to assay cocktail was 1:10 (v:v). The reaction took 3 min at room temperature to reach completion. The cocktail for the glucose assay contained Dithiothreitol (0.42 mM), $MgSO_4.7H_2O$ (3.1 mM), ATP (0.42 mM), NADP (1.25 mM), Hexokinase (EC 2.7.1.1: 12 U/ml), and glucose-6-phosphate dehydrogenase (EC: 1.1.1.49: 6 U/ml), in EPPS buffer pH 8.0 (Appendix 12).

Pyruvate Assay

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+$$

Pyruvate concentration was determined through the reduction of pyruvate to lactate by the enzyme lactate dehydrogenase. The fluorescent NADH was consumed in the reaction, thus a decrease in fluorescence was proportional to an increase in the concentration of pyruvate. The assay reaction was carried out in a neutral medium (pH 8.0), which means that the equilibrium of the equation was in favour of lactate production.

The ratio of substrate solution to assay cocktail was 1:10 (v:v). The reaction took 3 min at room temperature to reach completion. The cocktail for the pyruvate

assay contained NADH (0.1 mM) and lactate dehydrogenase (EC 1.1.1.27: 28 U/ml), in EPPS buffer pH 8.0 (Appendix 12).

Lactate Assay

$$\text{Lactate} + NAD^+ \xrightleftharpoons{\text{Lactate dehydrogenase}} \text{Pyruvate} + NADH + H^+$$

Lactate concentration was determined by the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase. Fluorescent NADH was generated in the reaction, thus an increase in fluorescence was proportional to an increase in the concentration of lactate. The assay reaction was carried out in a slightly alkaline medium (pH 9.4). The equilibrium of the equation was in favour of pyruvate production because the pyruvate formed was trapped as pyruvate-hydrazone.

The ratio of substrate solution to assay cocktail was 1:10 (v:v). The reaction took 3 min at room temperature to reach completion. The cocktail for the lactate assay contained NAD^+ (4.76 mM), EDTA (2.5 mM) and lactate dehydrogenase (EC 1.1.1.27: 100 U/ml) in a glycine hydrazine buffer, pH 9.4 (Appendix 12).

2.9.2 Construction and Calibration of Micropipettes

Micropipettes were used to accurately measure and sample nanolitre sized drops of solutions (Figure 2.11). The glass micropipettes were made on a microforge (Narishige Scientific Instrument Lab., Tokyo, Japan). Glass pipettes (Clark Electromedical Instruments, Middle Cove, NSW, Australia) were heated over a micro Bunsen burner flame until the glass was malleable and then the ends of the pipette

Figure 2.11 Micrograph of a 1 nl micropipette used to take serial samples of media to determine the uptake of substrates or the production of metabolites by individual embryos, using the microfluorometric technique.

were pulled horizontally so that the pulled section of the pipette had an outside diameter of less than 200 μm. The pulled section of glass was then broken and a small hook was made at the end by bending the glass while over a microburner flame. The pipette was then mounted on the microforge and the heated filament of the microforge was used to form a slight constriction in the walls of the pipette. A small weight was then attached to the hook at the bottom of the pipette and the heated filament was used to soften the glass just below the constriction. The weight on the hook pulled the heated glass down and a fine tip was made by breaking the glass with watchmaker's forceps. The large end of each pipette was mounted into a metal tube (approximately 7 cm long) and the join sealed with hot wax. Each pipette was washed with acetone and siliconized for 30 seconds prior to use. A 10 ml syringe with fine tubing was attached to the end of the mounted pipette to enable liquids to be taken up and expelled by suction.

The volume of each pipette was calibrated with tritiated water (^3H_2O; specific activity 5 mCi/ml, Amersham) before use. The ^3H_2O was diluted to a specific activity of 1 mCi/ml, drawn into each pipette up to the constriction and expelled into a 15 ml scintillation vial containing 0.2 ml water and 10 ml scintillation fluid. Three samples were taken from each pipette. A standard for the calibration was made by adding 0.1 ml ^3H_2O (specific activity of 0.1 μCi/ml) to each of four scintillation vials containing 0.1 ml water and 10 ml scintillation fluid, with a Gilson pipette. Blank controls were also made by adding 0.2 ml water to scintillation vials containing 10 ml scintillation fluid. Radioactivity was counted in a liquid scintillation counter. Each vial was counted for 5 min. The volume of each pipette was then calculated by the following equation:

0.1 ml ^3H_2O (for standard) = 10 nl ^3H_2O (for pipette) thus,

$$x\ \text{nl} = \frac{\text{pipette}}{((\text{standard} - \text{blank})/10)}$$

A pipette was used for the fluorometric technique only if each of the three measurements made for individual pipettes was within 5% of the mean of the three measurements.

2.9.3 Measurement of Substrate Uptake and Production by Individual Embryos

Embryos were incubated in drops of metabolic media with known concentrations of substrates (Appendix 13; media dependent on experiment), under light mineral oil in the lid of a 35 mm culture dish. The drops of media (80 - 400 nl) were pipetted under the oil with either micropipettes (80 - 200 nl drops) or a Gilson pipette (200 - 400 nl drops). The size of the drops for each stage of embryo development was pre-determined such that the concentration of carbohydrates was not allowed to fall below half of the initial concentration, as this has been shown to affect the rate of nutrient uptake by embryos (Gardner and Leese, 1988). Typically several drops were set up for embryos and one drop as a control in each lid. The drops of metabolic medium were then pre-equilibrated for at least 7 h before the addition of embryos. Embryos were washed three times in metabolic medium before individual embryos were added to drops with a finely pulled pipette. The tip of the pipette was just larger then the diameter of the embryo so that embryos were transferred in less than 3 nl medium (Gardner and Leese, 1987). Serial samples were taken from incubation drops on a warm stage (39°C) with a micropipette, approximately every 40

mins for up to 3 h, and assayed for carbohydrate concentration. The incubating embryos were returned to the incubator immediately after a sample was taken. Linear rates of uptake or production were determined for individual embryos and expressed as pmoles/embryo/h.

2.10 DETERMINATION OF INTRACELLULAR pH OF EMBRYOS

The method used to measure the intracellular pH (pHi) of embryos was the method used by Edwards *et al.* (1998a).

2.10.1 Measurement of Intracellular pH

Measurements of intracellular pH were made on embryos using the fluorescent pH indicator SNARF-1 (Molecular Probes, Portland, Oregon, USA). Cow embryos were loaded with SNARF-1 by adding 1.5 µl SNARF-1 stock solution (Appendix 14) to a 30 µl drop of culture medium which was pre-equilibrated to 5% CO_2, 7% O_2, 88% N_2, and 39°C. The composition of the medium was dependent on experimental treatments. Each drop of medium contained up to ten embryos, to give a final concentration of 5 µM SNARF-1. Embryos were incubated with SNARF-1 for 10 min at 5% CO_2, 7% O_2, 88% N_2 , and 39°C. Embryos were then washed three times in culture medium to remove excess SNARF-1 from the surrounding medium. Embryos loaded with SNARF-1 appeared pink. Embryos were then transferred to a drop of H-SOF based medium, pH 7.4, at 39°C (approximately 60 µl) on a poly-l-lysine coated slide.

Embryos were viewed on an inverted Nikon Diaphot 300 (FSE Pty Ltd, Blackburn, Victoria, Australia) connected to a Bio-Rad MRC 1000 confocal

microscope (Bio-Rad Microscience Ltd, Hemel, Hempstead, UK). In order to measure the SNARF-1 content of embryos, SNARF-1 was excited at a wavelength of 488 nm with an argon laser. The emitted fluorescence was split with a dichroic mirror (595 nm) and two photomultiplier tubes were used to collect emissions of fluorescence less than 595 nm and greater than 595 nm, separately. In order to ensure that both emissions were coming from images of the same thickness from the equatorial plane of the embryo, the confocal apertures were set identically for each emission pathway. Arbitrary measurements of SNARF-1 were then determined by dividing the signal from the channel collecting emissions less than 595 nm by the signal from the channel collecting emissions greater than 595 nm.

2.10.2 Calibration of SNARF-1

The arbitrary measurements of SNARF-1 were calibrated to the pHi of embryos by equilibrating the pHi of a group of embryos (from same group as treatment embryos) to that of extracellular pH (6.9, 7.0, 7.1, 7.2, 7.3 and 7.4) with the potassium ionophore, nigericin (Molecular Probes). This procedure was used by Edwards *et al.* (1998a), adapted from House (1994). Embryos were incubated as above with SNARF-1 and then transferred to 40 µm nigericin in 500 µl calibration buffer (Appendix 15) for 10 min, at 37°C. The solution was made by adding 484.5 µl calibration buffer (pH 6.9, 7.0, 7.1, 7.2, 7.3 and 7.4 in individual wells of 40-well dishes) to 15.5 µl nigericin stock solution (Appendix 14). SNARF-1 emissions were then calculated using the same microscope settings as for treatment embryos. Approximately four embryos were measured for each pH calibration.

The SlideWrite Plus for Windows software (Advanced Graphics Software, Inc., Carlsbad, CA, USA) was used to determine values of pHi for treatment embryos from each calibration curve (Figure 2.12).

2.11 STATISTICAL ANALYSIS

Each experiment was designed with the null hypothesis of no treatment effect. All statistical analyses were performed using the Graphpad Instat statistical package (Graphpad Software, San Diego, CA,USA). A probability of $P \leq 0.05$ was considered to be significant for all statistical tests.

2.11.1 Parametric and Non Parametric Tests

When the experiment tested the equality of more than two groups of data, the homogeneity of variances among treatment groups was analysed using Bartlett's test, to determine whether the data came from populations with equal standard deviations. When variances were found to be homogenous data were analysed by an ANOVA (see section 2.11.1.1). When the experiment tested the equality of two groups of data, the homogeneity of variances between treatment groups was analysed using an F-test to determine whether the data were from populations with equal standard deviations. When variances were found to be homogenous, data were analysed using an unpaired, two-tailed Student's t-test.

When variances were found to be heterogenous, data were transformed (log x or log [x +1]). If variances were not homogenous following transformation of data, data were analysed using a non parametric test (see section 2.11.1.2). Data presented as

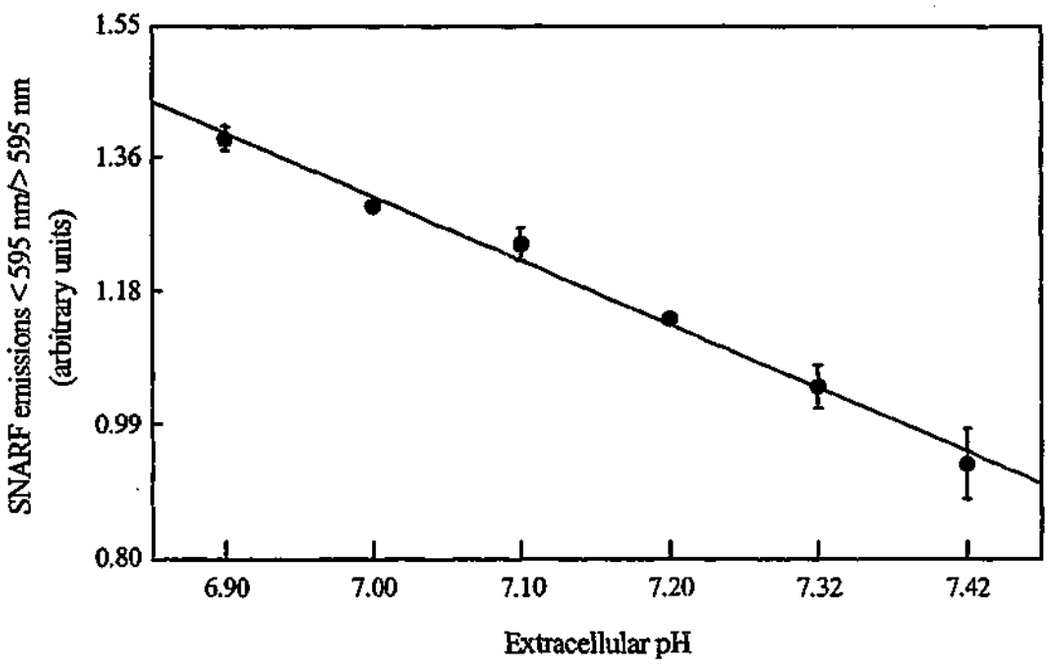


Figure 2.12 A calibration curve for SNARF-1 and extracellular pH. Extracellular pH was a reflection of the intracellular pH of the embryo following exposure of embryos to buffers of varying pH with a high potassium concentration, in the presence of nigericin. SNARF-1 measured in arbitrary units as emissions < 595 nm divided by emissions > 595 nm. Values are mean ± SEM from four embryos.

percentages were converted to proportions and transformed (arc sine) before a parametric or non parametric test was performed.

2.11.1.1 Parametric analysis of data

When more than two groups were compared and data came from populations with equal standard deviations, an ANOVA was used to analyse the statistical difference among treatment means. If the ANOVA revealed a significant difference in the data set, the Tukey-Kramer Multiple Comparison Test was used to determine the statistical difference between pairs of treatment means. The Tukey-Kramer test was chosen as it is more conservative than the Student-Newman-Keuls test (that is, less likely to produce Type I errors) and less conservative than the Bonferroni test (that is, less likely to produce Type II errors). Dunnett's test was used when individual treatment means were compared to an experimental control and not to other treatment means.

An unpaired, two-tailed Student's t-test was used to analyse the statistical difference between treatment means when only two groups of data with equal standard deviations were compared.

2.11.1.2 Non parametric analysis of data

When more than two groups were compared and data came from populations with unequal standard deviations, the Kruskal-Wallis test was used to analyse the statistical difference among group means. If the test revealed a significant difference in the data set, Dunn's test was used to perform multiple comparisons to determine the statistical difference between pairs of group means.

When only two groups of data with unequal standard deviations were compared, the Mann-Whitney U-statistic was used to determine the statistical difference between group means.

2.11.2 Chi-squared Test

Data presented as percentages that represented total observations and not means, were analysed by the Chi-square test, using a 2 x 2 contingency table and Yates' correction for discontinuity (Minium, 1978).

2.11.3 Correlation of Variables

When the degree of association between two variables was determined, the correlation coefficient (r) was calculated, using SlideWrite Plus software. The statistical significance of a correlation coefficient was determined from a table of the probability distribution of the correlation coefficient (Minium, 1978).

CHAPTER 3

IN VITRO DEVELOPMENT AND NUTRIENT UPTAKE BY EMBRYOS DERIVED FROM OOCYTES OF PRE-PUBERTAL AND ADULT COWS

3.1 INTRODUCTION

The in vitro production of oocytes and embryos from pre-pubertal cows has the potential to increase the rate of genetic gain in cows, by significantly reducing the generation interval (Lohuis, 1995; Duby *et al.*, 1996). Further, the ovaries of pre-pubertal animals have a larger number of oocytes than the ovaries of adult cows (Erickson, 1966b). The use of pre-pubertal ovaries may, therefore, be advantageous for the in vitro production of embryos for transfer, research or other assisted reproductive technologies. Progress in this direction, however, has been hindered because developmental competence is often impaired in oocytes retrieved from the ovaries of pre-pubertal animals (Onuma and Foote, 1969; Kajihara *et al.*, 1991; Palma *et al.*, 1993; Levesque and Sirard, 1994; Revel *et al.*, 1995). This phenomenon has also been reported for pre-pubertal sheep (O'Brien *et al.*, 1996; O'Brien *et al.*, 1997) and pigs (Pinkert *et al.*, 1989). It has not been established why material from pre-pubertal animals is often developmentally incompetent.

Determination of the uptake and/or metabolism of specific energy substrates by mammalian embryos at different stages of development has given valuable insight into the developmental requirements of embryos (Gardner and Leese, 1986; Rieger *et al.*, 1992b; Gardner and Lane, 1993b; Gardner *et al.*, 1993; Gardner, 1998b). Furthermore, the uptake and utilization of energy substrates by embryos has been shown to be a valuable indicator of developmental competence and viability. Hardy *et al.* (1989b) found that the uptake of pyruvate by early cleavage stage human embryos was indicative of subsequent developmental competence, while the uptake of glucose by abnormal blastocysts (high degree of fragmentation) was significantly lower than in normal blastocysts. In the mouse, glycolytic activity of early cleavage stage

embryos was found to reflect developmental competence (Gardner and Lane, 1993b). In addition, the uptake of glucose at the blastocyst stage has been correlated with viability following uterine transfer in the cow (Renard *et al.*, 1980) and mouse (Gardner and Leese, 1987). Furthermore, the metabolism of glucose (the proportion of glucose taken up by the embryo and converted to lactate) by mouse blastocysts was strongly correlated with embryo viability after transfer of embryos to recipient mice (Lane and Gardner, 1996).

The aim of the present study was two-fold. Firstly, the study aimed to determine the uptake of glucose and pyruvate by the developing cow embryo. To date, linear rates of nutrient uptakes by individual developing cow embryos have not been established under routine culture conditions. Secondly, the study was designed to compare the uptake of pyruvate and glucose by embryos from pre-pubertal and adult cows, to determine whether developmental competence was reflected in the uptake of energy substrates. Linear rates of nutrient uptake were determined non-invasively for individual embryos at successive stages of development, under the conditions in which embryos were cultured from the zygote to the blastocyst.

3.2 MATERIALS AND METHODS

3.2.1 Source of Oocytes

Pre-pubertal oocytes were retrieved from stimulated Friesian heifers at five to seven months of age, via TVR (see section 2.1.2). At each TVR session, all follicles equal to or greater than 2 mm diameter were aspirated. Follicular aspirates from each calf were collected into separate 50 ml tubes containing 5 ml H-199, supplemented with 50 μg heparin ml^{-1}, at 39°C. Tubes containing follicular aspirates were placed in a water bath (39°C) for up to 1 h before aspirates were searched for COC's. Each follicular aspirate was poured into a nylon filter apparatus and COC's were washed briefly with PBS at 39° to remove blood. PBS containing COC's was immediately poured into a 10 cm dish to enable searching and removal of COC's. COC's were classified as having at least five layers of compact cumulus (grade A), two to four layers of compact cumulus (grade B), partially denuded or one layer of cumulus (grade C), denuded (grade D) or expanded cumulus (grade E). Retrieved COC's were washed four times in 2.5 ml H-199 and then transferred to a holding dish containing 0.5 ml maturation medium, pre-equilibrated to 5% CO_2 and air, at 39°C. COC's aspirated from each calf were pooled and were from five to sixteen calves for each replicate. For each of five replicates, up to 50 COC's (grade A and B only) were removed from the pool and transferred to a 5 ml tube containing 0.5 ml maturation medium with a 200 μl overlay of mineral oil, pre-equilibrated to 5% CO_2 and air, at 39°C.

On the morning of each TVR, ovaries from adult cows were collected from a local abattoir. Aspiration of follicles of abattoir ovaries was carried out

simultaneously with the TVR of the calves, using identical settings on a separate vacuum apparatus. COC's were aspirated from follicles of 2 - 5 mm in diameter, into a 50 ml tube containing 5 ml H-199 supplemented with 50 μg heparin ml^{-1}, in a water bath at 39°C. Follicular aspirates containing COC's were then processed identically to those from calves (above). COC's from calves and adult cows were processed simultaneously to ensure that they were placed in maturation medium at the same time. As with the calves, up to 50 COC's were removed from the pool of COC's from adult cows and transferred to a 5 ml tube containing 0.5 ml maturation medium with a 200 μl overlay of mineral oil, pre-equilibrated to 5% CO_2 and air, at 39°C.

After being re-gassed with 5% CO_2 and air, tubes containing immature COC's from pre-pubertal and adult cows in maturation medium were sealed and transported to another laboratory, approximately 1 h away. In order to maintain a constant temperature during transportation, tubes were secured in a 50 ml tube attached to the side of a sealed thermos flask containing water at 39°C. Once at the laboratory, the temperature of the water in the flask was measured to ensure that it was still at 39°C and tubes containing COC's were immediately removed from the thermos. The lids of the tubes were loosened and tubes were placed in an incubator (5% CO_2 and air, at 39°C) for the completion of maturation. Standard in vitro maturation and fertilization procedures were then followed (see sections 2.3 and 2.4).

3.2.2 Embryo Culture

Embryos were cultured in groups of five in 30 μl drops of 20aa for six days from the zygote (18 h pi) to the blastocyst (Day 7 pi), at 5% CO_2, 7% O_2 and 88% N_2, at 39°C. Embryos were changed into fresh medium after 72 h culture.

3.2.3 Determination of Cell Number

Blastocysts (Day 7 pi) were stained with Hoechst (Number 33258) to determine total cell number (see section 2.7.2).

3.2.4 Determination of Nutrient Uptake During Culture

Non-invasive microfluorescence was used to determine the uptake of glucose and pyruvate by individual embryos from pre-pubertal and adult cows, on successive days of development (Day 1 pi to Day 7 pi). For each replicate, up to six embryos with equivalent cell numbers were selected from each group of cows (pre-pubertal and adult) per day, as follows: presumptive zygote (Day 1 pi), 2- to 4-cell (Day 2 pi), 5- to 8-cell (Day 3 pi), 8- to 16-cell (Day 4 pi), 16-cell to morula (Day 5 pi), morula (Day 6 pi), blastocyst (Day 7 pi). Selected embryos were washed three times and subsequently incubated in a modification of 20aa, containing 0.5 mM glucose, 0.5 mM pyruvate and 4 mg BSA ml^{-1} (cmet20aa: see Appendix 13). Slight alterations in the concentrations of glucose, pyruvate and BSA were the only modifications made to 20aa for the determination of nutrient uptakes by embryos. Individual embryos were transferred to pre-equilibrated drops of cmet20aa (80 nl drops for embryos Day 1 to Day 3 pi; 160 nl drops for Day 4 to Day 5 pi; 250 nl drops for Day 6 pi and 400 nl drops for Day 7 pi), in the lid of a 35 mm primaria culture dish, with a mineral oil overlay, at 5% CO_2, 7%O_2 and air, at 39^{0}C. Incubating embryos were briefly removed from the incubator and kept on a warm stage (39^{0}C) while a 1 nl sample of medium was taken from each drop. A sample of each incubation drop was taken within 60 seconds of the removal of each dish from the incubator and then embryos were immediately returned to the incubator. Serial 1 nl samples of incubation drops were

taken approximately every 40 mins, for up to 3 h. The concentrations of pyruvate and glucose in media samples were then determined by microfluorimetry (see section 2.9).

At the completion of the determination of nutrient uptake, embryos were returned to drops of 20aa for continued culture (5% CO_2, 7%O_2 and 88% N_2, at 39^{0}C) in groups of five embryos per 30 µl medium. In order to ensure that nutrient uptake values at the 1-cell stage were from zygotes and not from unfertilized oocytes, presumptive zygotes were cultured individually following the determination of nutrient uptake, in 10 µl drops of 20aa until the 2-cell stage. Cleaved embryos were then transferred to 30 µl drops of media and cultured in groups of five.

3.2.5 Statistical Analysis

In order to obtain homogeneity of variances, values for the uptake of nutrients by individual embryos were transformed log (y + 1) where y was equal to the rate of nutrient uptake. Differences in the rates of nutrient uptake between embryos derived from pre-pubertal and adult cows were determined by an unpaired, two-tailed t-test, for each stage of development. Differences in rates of nutrient uptake among different stages of development were determined separately for pre-pubertal and adult embryos by an ANOVA, followed by the Tukey-Kramer Multiple Comparison Test. The degree of association between day of development and nutrient uptake was determined by calculation of the correlation coefficient (r). Differences between means for fertilization, blastocyst development and blastocyst cell number were determined by a two-tailed t-test.

3.3 RESULTS

3.3.1 Fertilization, Development and Cell Number of Pre-Pubertal and Adult Embryos

There was no observed difference in the ability of oocytes from pre-pubertal and adult cows to be fertilized in vitro ($P > 0.05$, Table 3.1). Subsequent blastocyst development, however, was significantly lower in embryos from pre-pubertal cows ($P < 0.05$, Table 3.1). Despite differences in development, embryos from pre-pubertal animals that developed to the blastocyst stage by Day 7 pi had equivalent numbers of cells to blastocysts from adult cows ($P > 0.05$, Table 3.1).

3.3.2 Nutrient Uptake by Individual Embryos from Pre-Pubertal and Adult Cows

3.3.2.1 Glucose

The uptake of glucose by embryos from both pre-pubertal and adult cows was exponential during culture from Day 1 to Day 7 pi ($r = 0.82$ and 0.82, respectively, $P < 0.05$; Figure 3.1a). In both groups of embryos, glucose uptake was low and constant from Day 1 to Day 4 pi and then steadily increased from Day 4 to Day 7 pi (Figure 3.1a and Table 3.2). The greatest increase in glucose uptake was between the morula and blastocyst stages of development (5.0 to 20.8 pmoles/embryo/h for pre-pubertal and 6.7 to 17.6 pmoles/embryo/h for adult, $P < 0.05$). The rate of glucose uptake was equivalent in embryos from pre-pubertal and adult cows at every stage of development, except for the 2- to 4-cell stages (Day 2 pi) when glucose uptake was

Table 3.1 Fertilization, development and total cell number of embryos from pre-pubertal and adult cows.

Treatment	Oocytes (n)	Fertilized (%)	Blastocyst[A] (%)	Total cell number[B] (n)
Pre-Pubertal	216	70.4[a]	9.8[a]	85.9 ± 6.3[a] (15)
Adult	239	70.8[a]	33.7[b]	88.7 ± 3.7[a] (55)

[ab]Values in same column with different letters are significantly different ($P < 0.05$).

[A]Percentage blastocyst calculated from cleaved ova.

[B]Total cell number of blastocysts, Day 7 post-insemination. Values are mean ± SEM. Data are from five replicates.

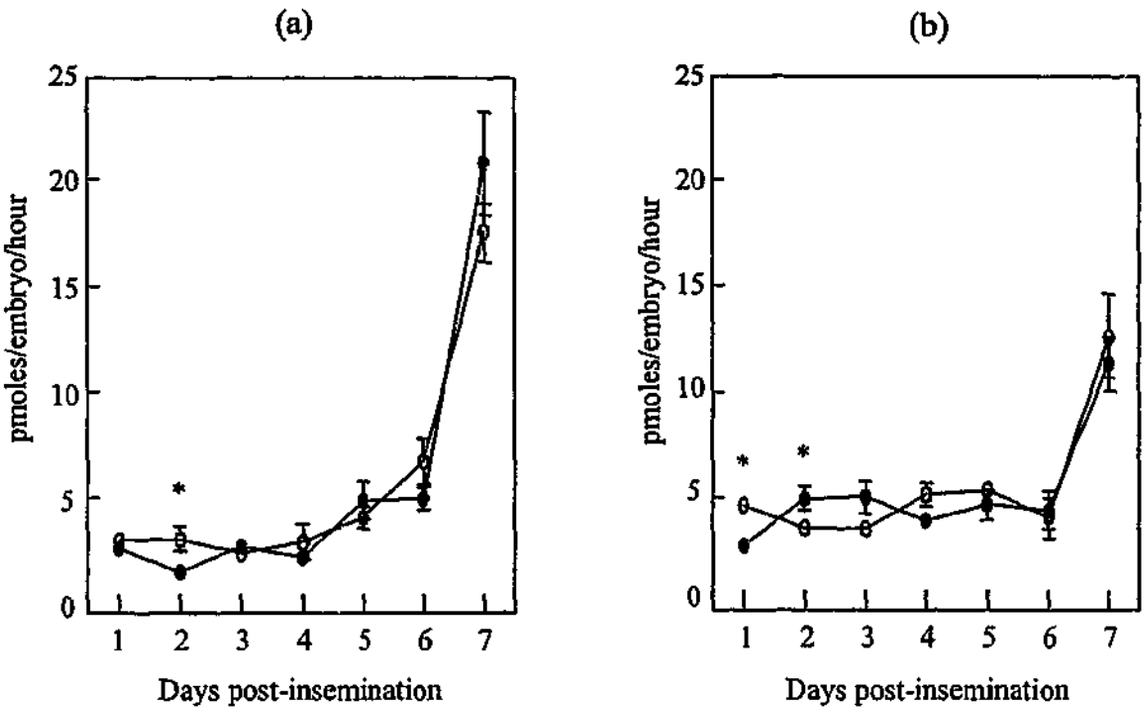


Figure 3.1 The mean ± SEM uptake of glucose (a) and pyruvate (b) by individual embryos from pre-pubertal (closed circle) and adult (open circle) cows, on successive days of development from the zygote (Day 1 post-insemination) to the blastocyst (Day 7 post-insemination). Exponential correlation (r) between nutrient uptake and days post-insemination is significant at $P < 0.05$. For glucose uptake, $r = 0.82$, $P < 0.05$ (pre-pubertal) and $r = 0.82$, $P < 0.05$ (adult). For pyruvate uptake, $r = 0.71$, $P < 0.05$ (pre-pubertal) and $r = 0.66$, $P > 0.05$ (adult). *Nutrient uptake was significantly different between pre-pubertal and adult cow, for the same day ($P < 0.05$). Data are from five replicates.

Table 3.2 Glucose and pyruvate uptake by individual embryos from pre-pubertal and adult cows, from the 1-cell stage to the blastocyst.

Days post-insemination	Stage of development	Pre-Pubertal glucose uptake (n)	Adult glucose uptake (n)	Pre-Pubertal pyruvate uptake (n)	Adult pyruvate uptake (n)
1	1-cell	2.6 ± 0.4^{ab} (18)	3.0 ± 0.4^{a} (15)	2.7 ± 0.3^{a} * (19)	4.6 ± 0.4^{a} (17)
2	2- to 4-cell	1.5 ± 0.2^{a} * (13)	3.0 ± 0.5^{a} (11)	4.9 ± 0.5^{b} * (14)	3.6 ± 0.5^{a} (17)
3	8-cell	2.7 ± 0.3^{abc} (11)	2.4 ± 0.4^{a} (11)	4.9 ± 0.7^{b} (13)	3.5 ± 0.3^{a} (15)
4	8- to 16-cell	2.2 ± 0.3^{a} (17)	2.9 ± 0.8^{a} (14)	3.9 ± 0.4^{ab} (16)	5.1 ± 0.6^{a} (15)
5	16-cell to morula	4.8 ± 0.9^{bc} (10)	4.1 ± 0.5^{ab} (11)	4.6 ± 0.7^{ab} (11)	5.3 ± 0.5^{a} (10)
6	morula	5.0 ± 0.5^{c} (14)	6.7 ± 1.1^{b} (15)	4.4 ± 0.9^{ab} (14)	4.1 ± 0.9^{a} (10)
7	blastocyst	20.8 ± 2.4^{d} (14)	17.6 ± 1.3^{c} (17)	11.3 ± 1.3^{c} (15)	12.6 ± 1.9^{b} (11)

[abcd]Values in same column with different letters are significantly different, ($P < 0.05$). Values are mean ± SEM. Means are pmoles/embryo/h. Data are from four replicates.

*Significantly different to value for adult, for same nutrient in same row, ($P < 0.05$).

significantly lower in embryos from pre-pubertal cows (1.5 pmoles/embryo/h versus 3.0 pmoles/embryo/h for adult, $P < 0.05$). The rate of glucose uptake was not significantly different in blastocysts derived from pre-pubertal and adult cows (20.8 versus 17.6 pmoles/embryo/h, respectively, $P > 0.05$).

3.3.2.2 Pyruvate

The rate of pyruvate uptake in embryos from the adult cow was low and constant from Day 1 to Day 6 pi (1-cell to morula, $P > 0.05$), increasing at the blastocyst stage (4.1 to 12.6 pmoles/embryo/h for the morula and blastocyst, respectively, $P < 0.05$, Table 3.2). Pyruvate uptake during development of embryos from adult cows was not quite exponential ($r = 0.66$, $P > 0.05$; Figure 3.1b). Pyruvate uptake by embryos from pre-pubertal cows however, was exponential during development ($r = 0.71$, $P < 0.05$; Figure 3.1b). The pattern and rate of pyruvate uptake by embryos from pre-pubertal animals was found to be different to that of embryos from adult cows (Table 3.2 and Figure 3.1b). Pyruvate uptake was not constant during development of the early cleavage stage pre-pubertal embryo, with a significantly lower uptake of pyruvate on Day 1 pi compared with Day 2 and Day 3 pi (2.7 versus 4.9 and 4.9 pmoles/embryo/h, respectively, $P < 0.05$). In fact, the rate of pyruvate uptake by embryos from pre-pubertal animals on Day 1 and Day 2 pi (1-cell and 2- to 4-cell stages) was significantly different than the uptake of pyruvate by embryos from adult cows (Day 1 pi: 2.7 versus 4.6 pmoles/embryo/h, respectively; and Day 2 pi: 4.9 versus 3.6 pmoles/embryo/h, respectively, $P < 0.05$). Figure 3.1a shows how the pattern of pyruvate uptake differs in early cleavage stage embryos from pre-pubertal and adult cows. When pyruvate uptake increased in the embryos from pre-pubertal animals, uptake decreased in the embryos from adult cows. The uptake of pyruvate by

later stage embryos from pre-pubertal cows was equivalent to that of embryos from adult cows, with uptake low up to the morula stage and then significantly increasing at the blastocyst stage (4.4 to 11.3, respectively, $P < 0.05$). The rate of pyruvate uptake was not significantly different in blastocysts derived from pre-pubertal and adult cows (11.3 versus 12.6 pmoles/embryo/h, respectively, $P > 0.05$; Table 3.2).

3.4 DISCUSSION

This is the first report of the non-invasive measurement of the linear uptake of nutrients by individual, in vitro produced cow embryos on successive days of development. Furthermore, the conditions in which nutrient consumption was assessed were almost identical to those in which the embryo was cultured from the zygote to the blastocyst.

Glucose uptake was low and constant during development of the early cleavage stages and increased with formation of the morula and development to the blastocyst. The rates of glucose uptake by the cow embryo at various developmental stages in the present study, are similar to those reported by Thompson *et al.* (1996) for embryos in HEPES-buffered SOF in the absence of amino acids. The glucose uptake values are also comparative to values reported for the uptake of radiolabelled glucose by the cow embryo in HEPES-buffered complex culture medium (Rieger *et al.*, 1992b). Further, the pattern of glucose uptake in the cow is qualitatively similar to that reported for other species, including the mouse (Leese and Barton, 1984; Gardner and Leese, 1986), sheep (Gardner *et al.*, 1993), goat (Gardner *et al.*, 1994a) and human (Gott *et al.*, 1990).

The low utilization of glucose by the early cleavage stage mammalian embryo is believed to be due to a blockade in glycolysis, resulting from inhibition of PFK (Barbehenn *et al.*, 1974; Barbehenn *et al.*, 1978) by a high ratio of ATP:ADP (Leese *et al.*, 1984; Gardner and Lane, 1997b). There is a very high ATP:ADP ratio in the early cleavage stage mouse embryo which decreases with development from the 8- to 16-cell stage to the blastocyst (Leese *et al.*, 1984; Gardner and Lane, 1997b). The increase in glucose uptake in the cow embryo after the 8- to 16-cell stage is concurrent

with the major onset of activation of the embryonic genome (Telford *et al.*, 1990) and a significant increase in protein synthesis (Frei *et al.*, 1989), metabolic activity (Rieger *et al.*, 1992a, 1992b), oxygen consumption and ATP production (Thompson *et al.*, 1996).

The uptake of pyruvate by the early cleavage stage cow embryo was also low and did not increase until formation of the blastocyst. Gardner *et al.* (1993) found a similar pattern of pyruvate uptake in the sheep, however, several studies have indicated that pyruvate uptake starts to increase with formation of the morula in both sheep (Butler and Williams, 1991) and cows (Rieger *et al.*, 1992b; Thompson *et al.*, 1996). Furthermore, there are vast differences in the reported rates of pyruvate uptake by ruminant blastocysts. The rate of 11.3 pmoles/embryo/h obtained in the present study was similar to rates reported for the cow by Rieger *et al.* (1992b) (approximately 12 pmoles/embryo/h) and Donnay and Leese (1999) (8 pmoles/embryo/h) but lower than reported for the cow by Thompson *et al.* (1996) (20.5 pmoles/embryo/h). The observed discrepancy in the rate of pyruvate uptake may be due to pyruvate uptake measured in media containing different concentrations of lactate and pyruvate and in the presence or absence of amino acids. Thompson *et al.* (1993) found that the addition of glucose, lactate and amino acids to culture medium decreased the rate of pyruvate uptake by sheep blastocysts. Consideration of culture conditions must be taken when comparing results from different studies. Different studies have used different culture systems, with nutrient uptakes often not measured in the same medium that embryos were cultured in. Culture systems are known to greatly affect the uptake and utilization of nutrients (Gardner and Leese, 1990; Dorland *et al.*, 1991; Gardner and Sakkas, 1993; Leppens *et al.*, 1996; Lane and

Gardner, 2000a). In the present study, nutrient uptakes were measured non-invasively for individual embryos, in the same medium in which embryos were cultured (except for a slight change in glucose concentration from 1.5 mM to 0.5 mM and pyruvate concentration from 0.33 mM to 0.5 mM). Therefore, the nutrient uptake values in the present study most likely reflect the actual requirements of the embryo for glucose and pyruvate during culture in 20aa, under 7% O2, 5% CO2 and 88% N_2, at 39°C.

Pyruvate is an important nutrient for the early cleavage stage embryo and unlike glucose, will support development of the cow embryo in the absence of other energy substrates (Takahashi and First, 1992; Kim *et al.*, 1993b; Rosenkrans *et al.*, 1993; Pinyopummintr and Bavister, 1996b). In the present study, there was an evident difference in the pattern of pyruvate uptake by early cleavage stage embryos from pre-pubertal and adult cows. The uptake of pyruvate by embryos from pre-pubertal animals was significantly lower than that of embryos from adult cows at the 1-cell stage, but was significantly higher at the 2-cell stage. This suggests that there may be a delay in the uptake of pyruvate by developmentally incompetent embryos. Hardy *et al.* (1989b) found that developmentally incompetent human embryos (those that arrested and did not continue development to the blastocyst stage) had a significantly lower uptake of pyruvate in the early cleavage stages than those that successfully developed to the blastocyst stage.

The oxidation of pyruvate yields large amounts of ATP. The production of ATP by early cleavage stage cow embryos, however, is relatively low and constant with respect to production from the 16-cell stage onwards (Thompson *et al.*, 1996). It is likely that the role of pyruvate during the early cleavage stages extends beyond energy production. In support of this, Rieger *et al.* (1992b) reported that less than 50% of

pyruvate taken up by the early cleavage stage cow embryo was actually oxidized. In somatic cells pyruvate functions as an antioxidant through its direct reaction with hydrogen peroxide (Andrae *et al.*, 1985). Hydrogen peroxide is a product of normal cellular metabolism, however, it is extremely toxic to cells (Fridovich, 1976). Pyruvate has been shown to reduce levels of hydrogen peroxide in mouse embryos (Kouridakis and Gardner, 1995; O'Fallon and Wright, 1995). Studies in the mouse (Cross and Brinster, 1973) and the sheep (Thompson *et al.*, 1993) have indicated that pyruvate is required by embryos for the maintenance of an appropriate redox state ($NADH:NAD^+$), with the ratio of pyruvate:lactate being a critical parameter for embryo development. Butcher *et al.* (1998) made a similar hypothesis as to the role of pyruvate in the maintenance of an appropriate redox state during development of the early cleavage stage human embryo, as only 56% of pyruvate taken up by the embryo was oxidized. Perturbations in the uptake of pyruvate by embryos could ultimately affect embryo metabolism by altering the intracellular ratio of pyruvate:lactate and thus the intracellular ratio of $NADH:NAD^+$. While development of the cow embryo has been shown to be affected by the concentrations of pyruvate and lactate in the culture medium (Rosenkrans *et al.*, 1993; Thompson *et al.*, 1993; Edwards *et al.*, 1997), there is evidence indicating that cow embryos are not as sensitive to the ratio of pyruvate:lactate (Rosenkrans *et al.*, 1993; Edwards *et al.*, 1997) as are sheep and mouse embryos. Thus, the abnormal pattern of pyruvate uptake by early cleavage stage embryos from pre-pubertal cows may reflect perturbations in energy production and a reduced ability to counteract the toxic effects of hydrogen peroxide.

The uptake and/or metabolism of nutrients during embryo development is not only an indicator of the requirements of the embryo during culture but is also an

indicator of developmental competence (Hardy *et al.*, 1989b; Gardner and Lane, 1993b) and viability following transfer (Renard *et al.*, 1980; Gardner and Leese, 1987; Lane and Gardner, 1996). Morphology alone is often used as an indicator of embryo viability, however, its reliability has been challenged (Bavister, 1995). Lane and Gardner (1996) demonstrated the variability in the viability of morphologically identical mouse blastocysts after transfer of embryos to recipient mice. In the present study, differences were seen in the uptake of nutrients during development of early cleavage stage embryos from pre-pubertal and adult cows. Considering the fact that development to the blastocyst stage was extremely low in embryos from pre-pubertal animals, observed differences in nutrient uptakes by the early cleavage stages were most likely due to a higher proportion of developmentally incompetent embryos from pre-pubertal cows than from adult cows.

The observed differences in the rates of nutrient uptakes by embryos from pre-pubertal cows were possibly due to factors inherited from the oocyte. Up until the 8- to 16-cell stage, development of the cow embryo is largely regulated by the maternal genome (Telford *et al.*, 1990). Developmental competence of the embryo is acquired during oocyte growth and maturation (Levesque and Sirard, 1994; Blondin *et al.*, 1995). Interestingly, Gandolfi *et al.* (1998) reported a significantly lower metabolism of pyruvate and glutamine by pre-pubertal oocytes compared with oocytes from adult cows.

Despite poor development in embryos from pre-pubertal cows, it is significant that those embryos that developed to the blastocyst stage were not compromised with respect to cell number or the uptake of nutrients. Lane and Gardner (1997a) found that the number of cells in the mouse blastocyst was positively correlated with fetal

development following transfer. Furthermore, previous studies have demonstrated that the uptake of glucose by the blastocyst is indicative of embryo viability following transfer of both mouse (Gardner and Leese, 1987) and cow (Renard *et al.*, 1978; Renard *et al.*, 1980) embryos. In contrast to glucose, there has been minimal research on the role of pyruvate as a viability marker. Pyruvate uptake in the cow embryo increased at a time when the embryo requires large amounts of ATP for protein synthesis, blastocyst development (Thompson *et al.*, 2000) and blastocoel formation (Donnay and Leese, 1999). The metabolism of pyruvate yields large amounts of ATP. Thus the uptake of pyruvate by the cow blastocyst, warrants further investigation as a potential marker of blastocyst viability.

In conclusion, the values reported in the present study for the uptake of glucose and pyruvate at various stages of development, were linear rates of nutrient uptakes by individual embryos under conditions similar to those in which the embryos were cultured from the zygote to the blastocyst. Thus, the reported nutrient uptakes are likely to represent the actual uptakes of glucose and pyruvate by the cow embryo during culture in SOF with amino acids (20aa). Glucose appears to be an important nutrient for the cow embryo, with the rate of uptake increasing after Day 4 pi and with development of the morula and blastocyst. The embryo's requirement for pyruvate increased with formation of the blastocyst, albeit at a lower rate than the uptake of glucose by the blastocyst. A large proportion of embryos from pre-pubertal cows was found to be developmentally incompetent and this was possibly reflected in the uptake of nutrients by the early cleavage stage embryos. Observed perturbations in the uptake of nutrients could be indicative of a reduced ability to counteract oxidative stress. Similar rates of nutrient uptake by blastocysts from pre-pubertal and adult cows and

equivalent blastocyst cell numbers, suggest that the viability of blastocysts from pre-pubertal cows was not compromised.

CHAPTER 4

THE METABOLISM OF GLUCOSE, PYRUVATE AND GLUTAMINE DURING THE MATURATION OF OOCYTES DERIVED FROM PRE-PUBERTAL AND ADULT COWS

4.1 INTRODUCTION

Blastocyst development is often low following the in vitro production of embryos from oocytes retrieved from the ovaries of pre-pubertal cows (Onuma and Foote, 1969; Kajihara *et al.*, 1991; Palma *et al.*, 1993; Levesque and Sirard, 1994; Revel *et al.*, 1995). A comparison of the developmental potential of oocytes from pre-pubertal and adult cows suggests that material from pre-pubertal animals is deficient in some aspect pertaining to developmental competence. In the previous chapter it was determined that the differences between embryos from pre-pubertal and adult cows, with respect to nutrient uptakes, were confined to the early cleavage stages of development. That is, differences were observed between embryos from pre-pubertal and adult cows during regulation by the maternal genome but not following activation of the embryonic genome. This suggests that the reason for the poor developmental competence of oocytes from pre-pubertal animals may reside in the oocyte.

Several studies have shown comparative development between material from pre-pubertal and adult ruminants when various ovarian hyperstimulation techniques were applied (Armstrong *et al.*, 1992; Armstrong *et al.*, 1994). Other studies, however, have found no difference in embryo development from stimulated versus unstimulated pre-pubertal ovaries (Revel *et al.*, 1995; O'Brien *et al.*, 1997). These conflicting reports indicate that the hormonal environment may be critical for the stimulation of the appropriate follicular dynamics and thus growth of the pre-pubertal oocyte. Developmental competence of the oocyte and/or embryo is in fact acquired during the growth phase of the oocyte and is correlated with the size of both the follicle and the oocyte (Motlik and Fulka, 1986; Pavlok *et al.*, 1992; Lonergan *et al.*, 1994; Blondin *et al.*, 1995; Fair *et al.*, 1995; Fair and Hytell, 1997). Interestingly,

oocytes from pre-pubertal cows have been reported to be significantly smaller than oocytes from adult cows (Duby *et al.*, 1996; Gandolfi *et al.*, 1998).

Successful development of the oocyte requires both nuclear and cytoplasmic maturation. Undoubtedly an important aspect of cytoplasmic maturation is the activation of and continued activity of metabolic pathways for the production of energy (in the form of ATP) and precursors for biosyntheses. Preliminary findings indicate that developmental competence may be reflected in the energy metabolism of the oocyte (Krisher and Bavister, 1998). Further, it has been shown that energy substrates have a key role in nuclear maturation (Fagbohun and Downs, 1992; Downs and Mastropolo, 1994).

Thus, the purpose of the present study was to determine whether there were differences in the activities of intracellular energy generating pathways during maturation of oocytes retrieved from unstimulated pre-pubertal and adult cows. The metabolism of [5-^{3}H] glucose, [2-^{14}C] pyruvate and [G-^{3}H] glutamine was determined during maturation and rates and patterns of metabolism for oocytes from pre-pubertal and adult cows were compared. In addition, the progression of nuclear maturation during maturation of oocytes from pre-pubertal and adult cows was determined.

4.2 MATERIALS AND METHODS

4.2.1 Source of Oocytes

Pre-pubertal oocytes were retrieved from the ovaries of twelve two to three month old calves (Hereford x Angus) which were slaughtered at an abattoir over a three week period (see section 2.1.2). Ovaries were collected simultaneously from adult cows of unknown breed and age. Oocytes were retrieved from the pre-pubertal and adult ovaries using standard aspiration procedures (see section 2.2.1). Follicles with a diameter of 2 - 5 mm were aspirated from the ovaries of adult cows. All follicles were aspirated from the pre-pubertal ovaries, in which the majority of follicles were approximately 2 mm in diameter.

4.2.2 In Vitro Maturation of Oocytes for Metabolic Determinations

For each of three replicates, up to sixty COC's with at least five layers of compact cumulus cells were collected from the ovaries of both pre-pubertal and adult cows to determine metabolic activity during maturation. COC's from each group were divided into three sub-groups (up to twenty COC's in each sub-group per replicate), in order to compare the metabolism of oocytes from pre-pubertal and adult cows, at three time points during maturation (0 h, 12 h and 24 h). Oocytes were matured in maturation medium for either 12 h or 24 h following standard in vitro maturation procedures (see section 2.3). COC's allocated to the 0 h maturation group were not cultured in maturation medium but remained in H-199 for up to 30 min prior to the measurement of oocyte metabolism.

4.2.3 Determination of Metabolism During Oocyte Maturation

At 0 h, 12 h and 24 h maturation, up to twenty COC's from each of the pre-pubertal and adult cow groups were transferred to H-199 containing 1 mg hyaluronidase ml^{-1} (Sigma), at 39^0C for 30 seconds. COC's were then vortexed in H-199 to remove all cumulus and corona cells. Any remaining corona cells were removed by pipetting up and down with a fine pulled glass pipette. Denuded oocytes were then washed in a modification of 20aa, containing no pyruvate, 0.5 mM glucose and 4 mg BSA ml^{-1} (mmet20aa: see Appendix 10), equilibrated to 5% CO_2 and air, at 39^0C. Oocytes from each animal group were divided between two metabolic media: 1) mmet20aa1 which was mmet20aa containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [5-^{3}H] glucose (at a concentration of 0.015 mM and specific activity of 0.25 μCi/μl), and 2) mmet20aa2 which was mmet20aa containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [G-^{3}H] glutamine (at a concentration of 0.004 mM and specific activity of 0.25 μCi/μl). Thus, measurements of glutamine, glucose and pyruvate metabolism were made concurrently.

Determinations of oocyte metabolism were carried out as described in section 2.8. Individual oocytes were cultured in 3 μl drops of either mmet20aa1 or mmet20aa2 for 3 h, equilibrated to 5% CO_2 and air, at 39^0C, in the lid of individual 2 ml tubes. Rates of nutrient metabolism by each oocyte at different time points during maturation were calculated from the liberation of ^3H_2O and $^{14}CO_2$ (see section 2.8.2).

4.2.4 Measurement of Oocyte Diameter and Volume

At the conclusion of each metabolic incubation the diameter of each oocyte was measured in μm (excluding zona pellucida,), on an inverted microscope (Leitz) with an eyepiece graticule (200x magnification). The volume of each oocyte was calculated according to the formula for the volume of a sphere, $V = 4/3\pi r^3$. Where oocyte metabolism was calculated for the volume of the oocyte, substrate metabolism (pmoles/oocyte/h) was divided by oocyte volume (pl) to give pmoles/pl/h for individual oocytes.

4.2.5 Determination of GV, GVBD and MII

Following the initial metabolic incubation (0 h maturation group; approximately 5 h post-aspiration) oocytes were stained with Hoechst (Number 33258) in order to determine what proportion of pre-pubertal and adult oocytes were at the GV stage versus what proportion were at the GVBD stage. After the final metabolic incubation (24 h maturation group; approximately 29 h post-aspiration) oocytes were examined for the extrusion of a polar body to determine what proportion of pre-pubertal and adult oocytes had progressed to MII. Procedures for determining nuclear maturation are outlined in section 2.6.2.

4.2.6 Production of Embryos In Vitro from Pre-Pubertal and Adult Cows

In only one replicate out of three, sufficient additional COC's were retrieved from the pre-pubertal ovaries in order to compare developmental potential of the pre-pubertal and adult oocytes. After standard in vitro maturation and fertilization

procedures (see sections 2.3 and 2.4), embryos were cultured for six days in 20aa at 5% CO_2, 7% O_2 and 88% N_2, at 39°C, with a change into fresh medium after 72 h culture.

4.2.7 Statistical Analysis

Comparisons of mean metabolic rates and mean oocyte sizes between pre-pubertal and adult cows at 0 h, 12 h and 24 h maturation were analysed using an unpaired, two-tailed t-test. When standard deviations were found to be significantly different between the two groups, data were transformed (1/y) prior to the t-test, where y represents the mean metabolic rate. Differences between mean metabolic rate and mean oocyte size at different times of oocyte maturation were determined for both pre-pubertal and adult cows by an ANOVA followed by the Tukey-Kramer Multiple Comparison Test. When variances were found to be heterogenous, data were transformed (1/y) prior to the ANOVA. For cell cycle stage data expressed as percentages, an unpaired, two-tailed t-test was performed after arc sine transformation of original data expressed as proportions. The distribution of oocyte diameter was analysed by the Chi-square test using a 2 x 2 contingency table and Yates' correction for discontinuity. The degree of association between maturation time and metabolic rate was determined by calculation of the correlation coefficient (r).

4.3 RESULTS

4.3.1 Developmental Potential of Oocytes from Pre-Pubertal and Adult Cows

Fertilization was equivalent in oocytes retrieved from the ovaries of pre-pubertal and adult cows ($P > 0.05$, Table 4.1). Subsequent development however to the morula and blastocyst stages and the blastocyst stage alone was significantly lower in embryos from pre-pubertal cows than from adult cows ($P < 0.05$, Table 4.1).

4.3.2 Metabolism of Oocytes from Pre-Pubertal and Adult Cows During Maturation

The activities of the main energy generating pathways were different during maturation of oocytes from pre-pubertal and adult cows.

4.3.2.1 [2-^{14}C] pyruvate

During maturation of oocytes from pre-pubertal and adult cows, the metabolism of [2-^{14}C] pyruvate peaked at 12 h maturation and then decreased to initial metabolic rates by 24 h maturation (Figure 4.1a and Table 4.2). [2-^{14}C] pyruvate metabolism was significantly greater at 12 h maturation than 0 h or 24 h maturation ($P < 0.05$, Table 4.2). Pyruvate metabolism was not linear during oocyte maturation (pre-pubertal: 0.16, $P > 0.05$; and adult: $r = 0.08$, $P > 0.05$; Table 4.2). Peak metabolism of [2-^{14}C] pyruvate at 12 h maturation was significantly lower in oocytes from pre-pubertal cows, than in oocytes from adult cows ($P < 0.05$, Figure 4.1a and Table 4.2).

Table 4.1 Fertilization and subsequent development of embryos produced in vitro from oocytes retrieved from pre-pubertal and adult cows.

Treatment	Oocytes (n)	Fertilized (%)	Morula and blastocyst[A] (%)	Blastocyst[A] (%)
Pre-Pubertal	20	75.0[a]	26.7[a]	13.3[a]
Adult	20	80.0[b]	56.3[a]	43.8[a]

[ab]Like pairs of letters are significantly different within a column, $P < 0.05$.

[A]Calculated from cleaved ova.

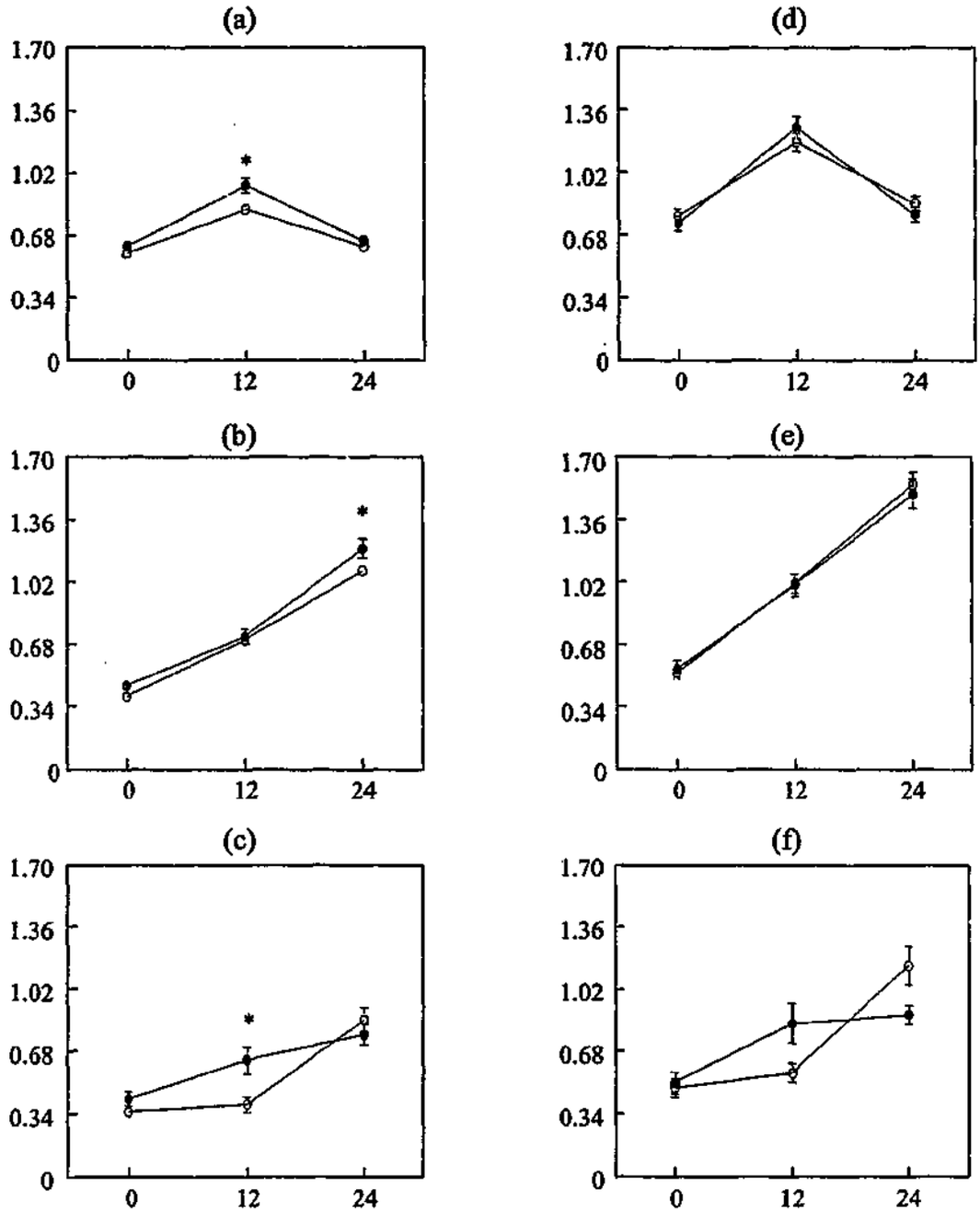


Figure 4.1 Substrate metabolism during maturation of oocytes from pre-pubertal (open circles) and adult cows (closed circles). X-axis indicates the number of hours oocytes had been in maturation medium. (a-c) pmoles/oocyte/h (pmoles/o/h); (d-f) pmoles/pl/h where metabolic rates were adjusted for oocyte volume. Values are mean ± SEM. *Means for pre-pubertal and adult cows significantly different at same time point ($P < 0.05$). Data are from three replicates. Note: graphs do not show significant differences between means at different maturation times.

Table 4.2 Metabolism of [2-^{14}C] pyruvate, [G-^{3}H] glutamine and [5-^{3}H] glucose during maturation of oocytes derived from pre-pubertal and adult cows.

Maturation[A] (h)	[2-^{14}C] pyruvate (pmoles/oocyte/h)[†]		[G-^{3}H] glutamine (pmoles/oocyte/h)[†]		[5-^{3}H] glucose (pmoles/oocyte/h)[†]	
	pre-pubertal	adult	pre-pubertal	adult	pre-pubertal	adult
0	0.58 ± 0.03^{a}	0.62 ± 0.02^{a}	0.39 ± 0.02^{a}	0.45 ± 0.03^{a}	0.35 ± 0.03^{a}	0.42 ± 0.04^{a}
12	0.82 ± 0.03^{ab}*	0.95 ± 0.04^{ab}	0.70 ± 0.03^{a}	0.72 ± 0.04^{a}	0.39 ± 0.04^{b}*	0.63 ± 0.08
24	0.62 ± 0.02^{b}	0.65 ± 0.03^{b}	1.08 ± 0.03^{a}*	1.20 ± 0.05^{a}	0.85 ± 0.07^{ab}	0.77 ± 0.06^{a}
r	0.16	0.08	0.99**	0.99**	0.90	0.99**

[A]Indicates length of time oocytes had been in maturation medium.

[ab]Like pairs of letters are significantly different within a column, $P < 0.05$.

*Significantly different to cow oocyte in same row, for individual nutrient, $P < 0.05$. **Correlation coefficient (r) significant, $P < 0.05$.

[†]Values are mean ± SEM; n was 48 - 60 oocytes per mean for pyruvate and 22 - 30 oocytes per mean for glutamine and glucose. Data are from three replicates.

4.3.2.2 [G-^{3}H] glutamine

The metabolism of [G-^{3}H] glutamine increased linearly during maturation of oocytes from both pre-pubertal and adult cows (Figure 4.1b) with a significant correlation between maturation time and metabolic activity in both groups (pre-pubertal: $r = 0.99$, $P < 0.05$; adult: $r = 0.99$, $P < 0.05$; Table 4.2). There was a significant difference among metabolic rates at each time point during maturation of oocytes within both groups of cows ($P < 0.05$, Table 4.2). The peak metabolism of [G-^{3}H] glutamine in the 24 h maturation group, was significantly lower in oocytes obtained from pre-pubertal cows than in those from adult cows ($P < 0.05$, Figure 4.1b and Table 4.2).

4.3.2.3 [5-^{3}H] glucose

Glycolytic activity (the metabolism of [5-^{3}H] glucose) increased steadily during maturation of oocytes from adult cows (Figure 4.1c), with a significant correlation between maturation time and metabolic rate ($r = 0.99$, $P < 0.05$; Table 4.2). Oocytes from pre-pubertal cows, however, had a different pattern of glycolytic activity during maturation. After 12 h maturation the metabolism of [5-^{3}H] glucose was significantly lower in oocytes from pre-pubertal cows ($P < 0.05$, Figure 4.1c and Table 4.2) and metabolic rate did not increase linearly with maturation time ($r = 0.89$, $P > 0.05$). Further, there was a significant increase in metabolic rate between 12 h and 24 h maturation in oocytes from pre-pubertal cows ($P < 0.05$, Table 4.2), however, this increase was not significant in oocytes from adult cows ($P > 0.05$). [5-^{3}H] glucose metabolism was not significantly different in oocytes from pre-pubertal and adult cows after 24 h maturation ($P > 0.05$; Figure 4.1c and Table 4.2).

4.3.3 Oocyte Diameter and Volume

The diameter and volume of oocytes from adult cows significantly decreased between 0 h and 12 h maturation and then significantly increased to initial values by 24 h maturation ($P < 0.05$, Table 4.3). While oocytes from pre-pubertal cows also showed a significant decrease in size from 0 h to 12 h maturation ($P < 0.05$), oocyte size at 24 h maturation was not significantly different to oocyte size at 0 h or 12 h maturation ($P > 0.05$). In fact, oocyte diameter and volume of oocytes from pre-pubertal cows were significantly smaller than those from adult cows at 0 h, 12 h and 24 h maturation ($P < 0.05$, Table 4.3).

Significantly more oocytes from pre-pubertal cows were less than 110 μm compared with oocytes from adult cows at 12 h maturation (67.9% versus 36.2%, respectively, $P < 0.05$) and 24 h maturation (46.7% versus 15.2%, respectively). Furthermore, significantly more oocytes from pre-pubertal cows were less than 115 μm compared with oocytes from adult cows at 0 h maturation (54.0% versus 31.6%, respectively, $P < 0.05$), 12 h maturation (83.0% versus 55.3%, respectively, $P < 0.05$) and 24 h maturation (63.3% versus 25.4%, respectively, $P < 0.05$).

4.3.4 Oocyte Metabolism Following Correction for Oocyte Volume

After correction of metabolic rates for oocyte volume, there were no significant differences between pre-pubertal and adult cows in the metabolism of [2-^{14}C] pyruvate, [G-^{3}H] glutamine [5-^{3}H] or glucose at 0 h, 12 h, or 24 h maturation ($P > 0.05$; Figure 4.1 d-f and Table 4.4). Correction for oocyte volume, however, did not change the observed differences in the pattern of glycolytic activity during maturation

Table 4.3 The diameter and volume of oocytes derived from pre-pubertal and adult cows during maturation.

Maturation[A] (h)	Pre-Pubertal diameter (μm)[†]	Adult diameter (μm)[†]	Pre-Pubertal volume (*p*l)[†]	Adult volume (*p*l)[†]
0	113.3 ± 0.80[a]*	117.6 ± 0.93[a]	0.77 ± 0.02[a]**	0.86 ± 0.02[a]
12	110.1 ± 0.66[a]*	113.1 ± 0.74[ab]	0.70 ± 0.01[a]**	0.76 ± 0.01[ab]
24	112.2 ± 0.88*	117.0 ± 0.83[b]	0.75 ± 0.02**	0.85 ± 0.02[b]

[A]Indicates length of time oocytes had been in maturation medium. Measurements of size were recorded approximately 3 hours after the time given (following metabolic measurements).

[ab]Like pairs of letters are significantly different within a column, $P < 0.05$.

*Significantly different to diameter of cow oocytes in same row, $P < 0.05$.

**Significantly different to volume of cow oocytes in same row, $P < 0.05$.

[†]Values are mean ± SEM; n was 47 - 60 oocytes per mean. Data are from three replicates.

Table 4.4 Metabolism of [2-^{14}C] pyruvate, [G-^{3}H] glutamine and [5-^{3}H] glucose during maturation of oocytes derived from pre-pubertal and adult cows, after correction for oocyte volume.

Maturation[A] (h)	[2-^{14}C] pyruvate (pmoles/pl/h)[†]		[G-^{3}H] glutamine (pmoles/pl/h)[†]		[5-^{3}H] glucose (pmoles/pl/h)[†]	
	pre-pubertal	adult	pre-pubertal	adult	pre-pubertal	adult
0	0.78 ± 0.04^{a}	0.74 ± 0.04^{a}	0.52 ± 0.03^{a}	0.54 ± 0.05^{a}	0.48 ± 0.05^{a}	0.51 ± 0.06^{ab}
12	1.18 ± 0.05^{ab}	1.26 ± 0.06^{ab}	1.01 ± 0.05^{a}	1.00 ± 0.06^{a}	0.56 ± 0.06^{b}	0.83 ± 0.12^{a}
24	0.85 ± 0.04^{b}	0.79 ± 0.04^{b}	1.55 ± 0.07^{a}	1.50 ± 0.08^{a}	1.15 ± 0.11^{ab}	0.88 ± 0.05^{b}
r	0.16	0.09	0.99*	0.99*	0.92	0.92

[A]Indicates length of time oocytes had been in maturation medium.

[ab]Like pairs of letters are significantly different within a column, $P < 0.05$.

*Correlation coefficient (r) significant, $P < 0.05$.

[†]Values are mean ± SEM; n was 47 - 60 oocytes per mean for pyruvate and 24 - 30 oocytes per mean for glutamine and glucose. Data are from three replicates.

of oocytes from pre-pubertal and adult cows. During maturation of oocytes from adult cows, [5-^{3}H] glucose metabolism increased significantly from 0 h to 12 h maturation ($P < 0.05$, Table 4.4), but did not increase significantly between 12 h and 24 h maturation ($P > 0.05$). During maturation of oocytes from pre-pubertal animals, however, there was no observed significant increase in the metabolism of [5-^{3}H] glucose between 0 h and 12 h maturation ($P > 0.05$), but there was a significant increase between 12 h and 24 h maturation ($P < 0.05$).

4.3.5 Determination of GV, GVBD and MII During Oocyte Maturation

Following the 0 h maturation metabolic incubation (approximately 5 h post-aspiration), there were significantly more pre-pubertal oocytes still at the GV stage than adult oocytes (88.03% versus 39.02%, respectively, $P < 0.05$; Figure 4.2a). Thus, significantly more oocytes from adult cows were at GVBD than oocytes from pre-pubertal cows (61.0% versus 11.9%, respectively, $P < 0.05$; Figure 4.2a). After 24 h maturation (following 3 h metabolic incubation), there was no significant difference in the percentage of oocytes reaching MII, between oocytes from pre-pubertal and adult cows (76.7% versus 78.3%, respectively, $P > 0.05$; Figure 4.2b).

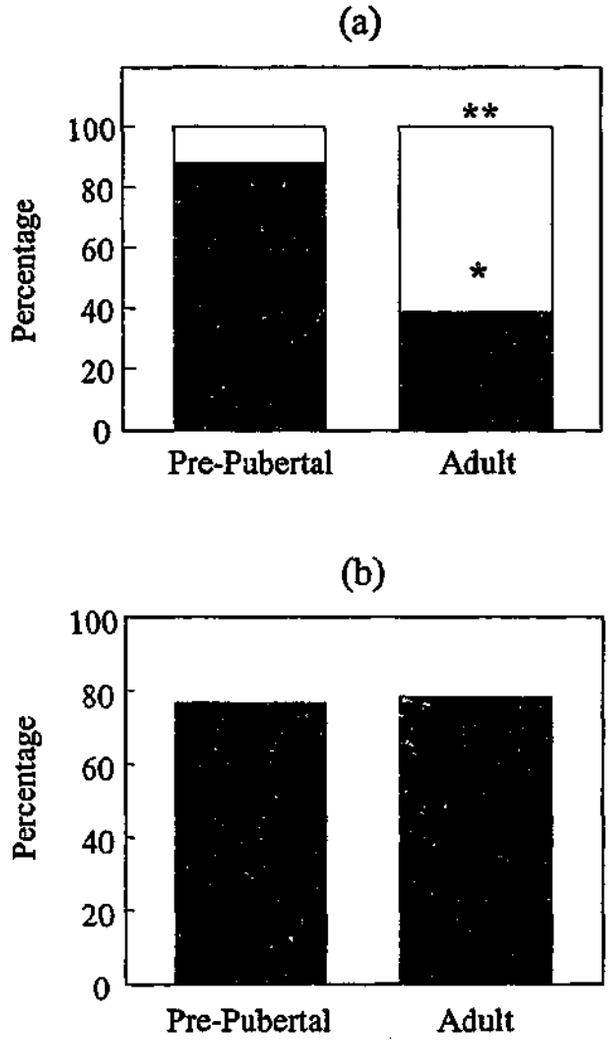


Figure 4.2 The meiotic stage of oocytes derived from pre-pubertal and adult cows: (a) the percentage of oocytes at the germinal vesicle stage (GV, solid bars) and at germinal vesicle breakdown (GVBD, open bars) following a 3 h metabolic incubation (approximately 5 h post-aspiration). The percentage of pre-pubertal oocytes at GV (*) and GVBD (**) was significantly different to adult oocytes, $P < 0.05$; (b) the percentage of oocytes with an extruded polar body after 24 hours maturation followed by a 3 h metabolic incubation. Data are from three replicates; (a) $n \geq 40$ oocytes per group (note some oocytes were lost during cell cycle determination) and (b) 59 oocytes per group. Data are from three replicates.

4.4 DISCUSSION

This study revealed that oocytes from pre-pubertal cows were different to oocytes from adult cows with respect to the activity of the main intracellular, energy generating pathways, oocyte size and initiation of germinal vesicle breakdown during maturation in vitro. It also highlighted the oocyte's differential metabolism of the carbohydrates glucose and pyruvate and the amino acid glutamine, during maturation.

The metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine are both indicators of the activity of TCA cycle and thus indicators of mitochondrial function. Pyruvate enters the TCA cycle following its oxidative carboxylation to acetyl CoA (Figure 1.2) and the $^{14}CO_2$ liberated from the metabolism of [2-^{14}C] pyruvate is released prior to the entry of glutamine into the TCA cycle at α-ketoglutarate (Figure 1.2). Thus the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine reflects the relative activities of two different sections of the TCA cycle. In the present study, the metabolism of pyruvate in the maturing cow oocyte peaked at 12 h maturation and then decreased to initial levels of metabolism by 24 h maturation. Gandolfi *et al.* (1998) reported that pyruvate metabolism increased significantly during oocyte maturation, however, metabolism was determined only at 0 and 21 h maturation and oocytes were matured to 21 h in the absence of pyruvate. In support of the present findings, Rieger and Loskutoff (1994) also found that pyruvate metabolism peaked at 12 h maturation and that pyruvate metabolism at 0 h was not significantly different to 24 h maturation. The values for pyruvate metabolism were five to ten fold higher in the studies by Rieger and Loskutoff (1994) and Gandolfi *et al.* (1998) than reported in the present study. These differences are most likely due to the fact that the concentration of pyruvate was six to twenty times higher during metabolic determinations in the studies by

Rieger and Loskutoff (1994) and Gandolfi *et al.* (1998), compared with the present study. In the present study, oocyte metabolism was determined with the concentration of pyruvate within the range used in oocyte maturation media (0.2 to 1.0 mM) (Rieger *et al.*, 1992b; Takahashi and First, 1992; Levesque and Sirard, 1994; Dominko and First, 1997).

The linear increase in the rate of glutamine metabolism from 0 h to 24 h maturation, further illustrates the importance of oxidative metabolism during maturation of the cow oocyte. Rieger and Loskutoff (1994) and Gandolfi *et al.* (1998) also observed an increase in glutamine metabolism during maturation, however, Rieger and Loskutoff (1994) found that glutamine metabolism did not increase until 18 h maturation. It is unknown why the pattern of glutamine metabolism is different in the two studies but it is plausibly due to the use of different metabolic incubation media and maturation media.

Thus, it appears as though oxidative metabolism is an important means of energy production for the maturing cow oocyte. Studies on energy metabolism during maturation of mammalian oocytes are few. Magnusson *et al.* (1977) found that oxygen consumption increased steadily in maturing rat oocytes and Tsutsumi *et al.* (1992) observed that the activity of the TCA cycle enzyme malate dehydrogenase (MDH) increased during maturation of in vivo rat oocytes. In light of this, it is interesting that pyruvate metabolism decreased when glutamine metabolism was still significantly increasing. Rieger *et al.* (1992b) also reported a discrepancy in TCA activity when they found that glutamine metabolism increased in the cow blastocyst, while pyruvate metabolism remained constant. There are several possibilities to consider. With respect to enzyme activity, MDH catalyses the conversion of malate to

oxaloacetate, long after the liberation of $^{14}CO_2$ from [2-^{14}C] pyruvate. Thus an increase in the activity of MDH (Tsutsumi *et al.*, 1992) may be a reflection of increased glutamine metabolism during oocyte maturation and may have no reflection on pyruvate metabolism. One possible explanation for differences in activity within the TCA cycle is that the pool of [2-^{14}C] pyruvate could have been diluted by the formation of endogenous pyruvate through the EMP. Rieger and Loskutoff (1994) found that glucose oxidation increased at 6 h and 18 h maturation (from zero oxidation), representing 39% of total glucose metabolism. There was, however, no detectable oxidation of glucose by 24 h maturation when pyruvate oxidation was low in both the present study and the study by Rieger and Loskutoff (1994), indicating that it is unlikely that the pool of [2-^{14}C] pyruvate was diluted. Rieger *et al.* (1992b) suggested that differences in the oxidation of pyruvate and glutamine could be due to differences in the sensitivity of NAD^+-substrate-dehydrogenases to inhibition by NADH, in different sections of the TCA cycle. That is, there is greater sensitivity to inhibition in the section from oxaloacetate to α-ketoglutarate (liberation of $^{14}CO_2$ from [2-^{14}C] pyruvate) than there was in the section from α-ketoglutarate to oxaloacetate (liberation of ^3H_2O from [G-^{3}H] glutamine). Thus it appears that glutamine may be a very important energy substrate for the oocyte as energy demands increase. In support of this, Krisher and Bavister (1998) demonstrated that maturation of cow oocytes with glutamine and glucose as the only energy substrates supported a significantly higher development to the blastocyst stage than maturation with pyruvate and glucose. Gwatkin and Haidri (1973) found that glutamine was important for the maturation of hamster oocytes, while there have been many studies highlighting the significant role of glutamine in the development of embryos in vitro (Chatot *et al.*, 1989; Gardner and

Lane, 1993a; Carney and Bavister, 1987a). While glutamine is used as an energy source (Rieger and Guay, 1988), it may also have an important role in the oocyte as a biosynthetic precursor, an osmolyte (Biggers *et al.*, 1993; Dawson and Baltz, 1997) or a buffer of intracellular pH (Bavister and McKiernan, 1993; Edwards *et al.*, 1998b).

Peak pyruvate and glutamine metabolism were significantly lower in oocytes obtained from the ovaries of pre-pubertal animals. When metabolic rates were corrected for oocyte volume, there were no longer significant differences in the patterns or levels of oxidative metabolism in oocytes from pre-pubertal and adult cows. Gandolfi *et al.* (1998) also reported a significantly lower metabolism of pyruvate and glutamine in pre-pubertal oocytes (at 0 h maturation). Despite a significant difference in oocyte size between pre-pubertal and adult cows, metabolic rates were not corrected for oocyte volume. The results from the present study reveal that the TCA cycle has been appropriately activated in oocytes from pre-pubertal cows and that observed differences in oxidative metabolism were simply a function of oocyte size.

In the present study glycolytic activity increased steadily during oocyte maturation with a significant increase from 0 h to 24 h maturation in both pre-pubertal and adult oocytes. Previous studies have indicated that glucose metabolism does not significantly increase during cow oocyte maturation (Rieger and Loskutoff, 1994; Gandolfi *et al.*, 1998). Although not significant, there was an increase in glucose metabolism between 0 h and 24 h maturation in the study by Rieger and Loskutoff (1994). Sample sizes, however, were lower than in the present study and may not have allowed for the detection of a significant increase in glucose metabolism (Rieger and Loskutoff, 1994). Furthermore, in the previous studies, metabolic determinations were

made in HEPES-buffered media with a much higher concentration of pyruvate, as mentioned above (Rieger and Loskutoff, 1994; Gandolfi *et al.*, 1998). It is possible that the higher rate of pyruvate metabolism reported in those studies could have led to the allosteric inhibition of PFK because of an increase in ATP and citrate production. Interestingly, the activities of several enzymes of the EMP were reported to increase during maturation of the rat oocyte (hexokinase and PFK) (Tsutsumi *et al.*, 1992) and the mouse oocyte (glucose-6-phosphate dehydrogenase) (Mangia and Epstein, 1975).

Compared with the oocytes from adult cows, glucose metabolism was significantly lower in oocytes from pre-pubertal cows after 12 h maturation. When metabolic rates were corrected for oocyte volume, however, there were no significant differences in the rates of glucose metabolism between pre-pubertal and adult oocytes. Despite correction for oocyte size, the pattern of activity of glucose metabolism was different in oocytes from pre-pubertal and adult cows. While glucose metabolism increased significantly between 0 and 12 h maturation in oocytes from adult cows, it did not increase until 12 to 24 h maturation in the pre-pubertal oocyte. Thus, there appeared to be a delay in the increase in the metabolism of glucose in oocytes from pre-pubertal animals. This finding is important because it could have implications for the normal maturation of oocytes from pre-pubertal cows and subsequent developmental competence. Glucose is necessary not only for energy production in the oocyte (aerobic glycolysis) but also for the generation of ribose moieties (for incorporation into nucleic acids and nucleotides) and NADPH (for reductive biosyntheses) through the PPP (Rieger *et al.*, 1992b; Gardner, 1998a). The conversion of [5-^{3}H] glucose to 2-phosphoglycerate, resulting in the liberation of ^3H_2O, not only

reflects the activity of the EMP but also the metabolism of glucose through the PPP by the oocyte (Tiffin *et al.*, 1991).

The values obtained in the present study for the diameter of oocytes aspirated from the ovaries of unstimulated adult cows, were similar to those reported in other studies (Arlotto *et al.*, 1996; Otoi *et al.*, 1997). In the present study, there was a significant decrease in oocyte size in both groups of oocytes, at 12 h maturation. This phenomenon has also been reported in other studies (Suzuki *et al.*, 1994; Suzuki *et al.*, 1996). Further, Tsutsumi *et al.* (1992) found a decrease in the weight of rat oocytes during maturation in vivo. The physiological significance of these volumetric changes is undetermined. An increase in the size of the perivitelline space was not due to the extrusion of the polar body as oocyte size increased between 12 h and 24 h maturation. It is possible that volumetric changes may function to facilitate the reorganisation of organelles in the ooplasm. Suzuki *et al.* (1994) suggested that a decrease in the size of the oocyte may be due to physiological contractions of the ooplasm, which may aid in disconnecting the cytoplasmic projections between cumulus cells and the oocyte.

Oocytes retrieved from the ovaries of pre-pubertal cows were significantly smaller than those from adult cows at all time points during maturation. The 4.1% difference in oocyte diameter observed in the present study, after 24 h maturation, actually represents a difference of 11.8% in oocyte volume. This has implications for the viability of oocytes from pre-pubertal animals as an increase in oocyte volume has been correlated with an increase in protein content (Schultz and Wassarman, 1977). Levesque and Sirard (1994) found that oocytes from the ovaries of adult cows that were 'defective' (development to morula and blastocyst was impaired) had similar

protein profiles to oocytes from pre-pubertal cows, which may account for the low developmental competence of these oocytes. Fair *et al.* (1995) found that oocytes with a diameter less than 110 μm were still involved in RNA synthesis, indicating that they were still in their growth phase (with respect to oocytes with a diameter greater than 110 μm). In the present study, there were significantly more oocytes less than 110 μm at 12 h and 24 h maturation, in the oocytes retrieved from the ovaries of pre-pubertal animals with respect to those from adult cows.

It has previously been shown that oocyte size is critical for maturation, fertilization and subsequent embryo development (Fair *et al.*, 1995; Arlotto *et al.*, 1996; Fair and Hytell, 1997; Otoi *et al.*, 1997). In the present study, there were significantly more oocytes less than 115 μm from the ovaries of pre-pubertal cows than from adult cows. Arlotto *et al.* (1996) found that subsequent development to morula and blastocyst stages was severely compromised in oocytes with a diameter less than 115 μm. In the present study, there was no difference in the ability of oocytes from pre-pubertal and adult cows to reach MII. This confirms the findings of other studies from oocytes of a similar size (Fair *et al.*, 1995; Arlotto *et al.*, 1996; Hytell *et al.*, 1997). It is important to note that extrusion of the polar body was determined after 24 h maturation (approximately 29 h after aspiration, following metabolic incubation). Several studies have demonstrated that there is a correlation between oocyte size and the speed at which the oocyte matures (Tsafriri and Channing, 1975; Fukui and Sakuma, 1980; Arlotto *et al.*, 1996). In addition, it has been shown that oocytes reaching MII earlier result in embryos of improved developmental competence (Dominko and First, 1997). Thus, the fact that oocytes from pre-pubertal animals were able to reach MII after 24 h maturation may not be an

accurate indicator of subsequent developmental competence. Further studies are thus required to time the progression of pre-pubertal and adult oocytes to MII.

The fact that only 11.9% of pre-pubertal oocytes were at GVBD at approximately 5 h post-aspiration versus 61% of adult oocytes, indicates that nuclear maturation was indeed slower in pre-pubertal oocytes. Rieger and Loskutoff (1994) reported similar proportions of adult cow oocytes beyond the GV stage following a 3 h metabolic incubation. The observed delay in nuclear maturation in pre-pubertal oocytes may also be linked to the observed delay in glucose metabolism as energy substrates play a crucial role in nuclear maturation (Fagbohun and Downs, 1992; Downs and Mastropolo, 1994; Lim *et al.*, 1999). It has been suggested that the metabolism of glucose through the PPP is related to the initiation of GVBD (Downs *et al.*, 1996).

The measurements of energy metabolism in the present study were made on denuded oocytes. It has been suggested that glycolytic and TCA cycle intermediates, along with amino acids and ATP may be transferred from cumulus cells to the oocyte via gap junctions between cumulus cell projections and the oolemma (Eppig, 1991; Downs *et al.*, 1996). This would render any observed perturbations in metabolic activity in denuded oocytes meaningless. Gap junctions between the cow oolemma and cumulus cell projections, however, start to disconnect as early as 3 h culture (Hytell *et al.*, 1986) and can completely disappear as early as 6 h culture (Sutovsky *et al.*, 1993). Thus, by 6 h maturation the oocyte would be largely dependent on the activity of its own energy generating pathways. It has in fact been suggested that the gap junctions may disappear earlier in pre-pubertal oocytes (Gandolfi *et al.*, 1998). The present study allows for a valid comparison of energy substrate metabolism

between the oocytes of pre-pubertal and adult cows and likely reflects the changing patterns of metabolism and energy demands in the cumulus-enclosed oocyte during maturation.

In conclusion this chapter identified several important differences in oocytes from pre-pubertal and adult cows as well as highlighting the increasing demand for both glucose and glutamine, during maturation of the cow oocyte. Observed differences in metabolic rates between oocytes from pre-pubertal and adult cows were a function of the smaller size of oocytes from pre-pubertal ovaries. The fact that oocytes from pre-pubertal cows were significantly smaller than those from adult cows, suggests that cytoplasmic maturation may be incomplete, potentially affecting the subsequent developmental competence of the pre-pubertal oocytes. Irrespective of oocyte size, there was a delay in the increase in glucose metabolism of oocytes from pre-pubertal animals. This delay may be related to the observed delay in GVBD in pre-pubertal oocytes. Thus, a delay in the generation of NADPH and ribose moieties through the PPP may contribute to the observed poor developmental competence of oocytes from pre-pubertal animals. In light of the present findings, studies are required to determine the exact timing of polar body formation in the pre-pubertal oocyte and to determine the relationship between glucose metabolism and GVBD in the cow oocyte.

CHAPTER 5

TEMPORAL AND DIFFERENTIAL EFFECTS OF AMINO ACIDS ON THE DEVELOPMENT OF COW EMBRYOS DURING CULTURE

5.1 INTRODUCTION

The culture of the cow embryo in simple, defined media has enabled the determination of the embryo's requirements for specific substrates such as glucose, pyruvate, lactate and glutamine (Takahashi and First, 1992; Kim *et al.*, 1993b; Matsuyama *et al.*, 1993; Rosenkrans *et al.*, 1993; Pinyopummintr and Bavister, 1996b). A major step towards improving media for the culture of the cow embryo was the discovery that culture in the presence of Eagle's non-essential and Eagle's essential amino acids improved embryo development to the morula and blastocyst stages (Takahashi and First, 1992; Kim *et al.*, 1993b; Gardner, 1994; Rosenkrans and First, 1994). Much of our knowledge of the effect of amino acids on mammalian embryo development and subsequent viability has come from studies in the hamster (Carney and Bavister, 1987a; Bavister and Arlotto, 1990; Bavister and McKiernan, 1993; McKiernan *et al.*, 1995), mouse (Mehta and Kiessling, 1990; Gardner and Lane, 1993a; Lane and Gardner, 1994; Gardner and Lane, 1996; Lane and Gardner, 1997a, 1997b) and rat (Zhang and Armstrong, 1990). These studies have shown that specific amino acids can either stimulate or inhibit embryo development and that the presence of amino acids in culture media has a significant effect on post-implantation development.

Partridge and Leese (1996) determined the depletion of 19 amino acids from culture media during culture of cow embryos from the zygote to the blastocyst stage. It was found that the rate of depletion of individual amino acids changed with developmental stage, suggesting that the cow embryo changes its requirements for amino acids during development. Subsequently, Lane and Gardner (1997a) reported that the mouse embryo changed its requirements for amino acids as it developed from

the zygote to the blastocyst. Development of the early cleavage stages was stimulated by Eagle's non-essential amino acids and glutamine but was not affected by Eagle's essential amino acids. The presence of the essential amino acids during culture of the early cleavage stages did, however, decrease subsequent blastocyst cell number and embryo viability following transfer. Furthermore, the presence of the essential amino acids in culture medium negated the beneficial effects of the non-essential group (Gardner and Lane, 1993a; Lane and Gardner, 1997a, 1997b). During development from the 8-cell stage to the blastocyst, the non-essential amino acids and glutamine stimulated blastocyst formation and hatching while the essential amino acids increased blastocyst cell number and allocation of cells to the ICM. The study by Lane and Gardner (1997a) supports the use of a two-step culture system in evaluating the requirements of the developing embryo (Gardner and Sakkas, 1993). It also demonstrates the need to assess the affect of amino acids not only on embryo development but also on possible indicators of viability such as blastocyst cell number and the allocation of cells to the TE and ICM.

The main objective of the present study was, therefore, to determine whether the cow embryo changes its requirements for amino acids as it develops from the zygote to the blastocyst. A two-step culture system was used to determine the effect of glutamine, Eagle's non-essential amino acids and Eagle's essential amino acids during culture of both the early cleavage stages (zygote to Day 4 pi) and the later stages of development (Day 4 to Day 7 pi). Embryo development, blastocyst cell number and allocation of cells to the TE and ICM were assessed. In light of the fact that the essential amino acids in Eagle's essential amino acids group are at a higher concentration than are found in the reproductive tract of the cow (Table 1.3), the study

also determined the effect of altering the concentration of Eagle's essential amino acids during culture. Furthermore, experiments were performed to assess the embryo's requirement for glutamine and the effect of substituting glutamine for the organic osmolyte betaine, during development of both the early cleavage stages and the later stages of embryo development.

5.2 MATERIALS AND METHODS

The exact composition of media for the following experiments is listed in Appendix 6. Embryos were cultured in groups of five in 30 μL drops of media, under 7 ml light mineral oil, in a 60 mm culture dish (non-pyrogenic) at 39°C, in 7% O_2, 5% CO_2 and 88% N_2.

5.2.1 The Temporal Effect of Amino Acid Groups on Development, Cell Number and Differentiation During Culture.

The first set of experiments was designed to determine the effect of glutamine, Eagle's non-essential amino acids and Eagle's essential amino acids on development, blastocyst cell number and allocation of cells to the TE and ICM, during the first 72 h and the second 72 h culture of the cow embryo, from the zygote to the blastocyst. Note that Eagle's essential amino acids did not contain glutamine. See Table 2.4 for the composition of Eagle's non-essential and Eagle's essential amino acids.

5.2.1.1 Zygote to Day 4 post-insemination.

Presumptive zygotes (18 h pi) were cultured for 72 h to Day 4 pi in one of five media: 1) SOF (control), 2) Gln (SOF with 1 mM glutamine), 3) NeGln (SOF with 1 mM glutamine and Eagle's non-essential amino acids), 4) EssGln (SOF with 1 mM glutamine and Eagle's essential amino acids), or 5) 20aa (SOF with 1 mM glutamine and Eagle's non-essential and Eagle's essential amino acids). The stage of embryo development in each medium was scored at 72 h culture. Embryos were then transferred in their respective treatment groups to 20aa for a further 72 h culture to Day 7 pi. The development of embryos was scored and blastocysts were differentially

stained to determine total cell number and allocation of cells to the TE and ICM (see section 2.7.3).

5.2.1.2 Day 4 to Day 7 post-insemination.

Due to the results of experiment 5.2.1.1, presumptive zygotes (18 h pi) were cultured in NeGln for 72 h to Day 4 pi. Embryo development was scored and embryos were divided equally among five groups with each group containing equivalent numbers of embryos less than the 8-cell stage and greater than or equal to the 8-cell stage. Each group of embryos was randomly transferred to one of five media: 1) SOF, 2) Gln, 3) NeGln, 4) EssGln, or 5) 20aa. Embryos were cultured for a further 72 h to Day 7 pi. The development of embryos was scored and blastocysts were differentially stained to determine total cell number and allocation of cells to the TE and ICM (see section 2.7.3).

The following experiment was carried out to determine the role of individual groups of amino acids in blastocyst formation, cleavage and differentiation in the cow embryo. Embryos were cultured in NeGln as above to Day 4 pi and then transferred in equal groups to one of four media: 1) Ne (SOF with Eagle's non-essential amino acids), 2) NeGln (SOF with 1 mM glutamine and Eagle's non-essential amino acids), 3) Ess (SOF with Eagle's essential amino acids), or 4) EssGln (SOF with 1 mM glutamine and Eagle's essential amino acids). Embryos were then cultured for a further 72 h to Day 7 pi. The development of embryos was scored and blastocysts were differentially stained to determine total cell number and allocation of cells to the TE and ICM (see section 2.7.3).

5.2.2 The Effect of Reducing the Concentration of Eagle's Essential Amino Acids During Culture, on Development and Cell Number.

The following experiments examined the effect of the concentration of Eagle's essential amino acids on development and cell number during the first 72 h and the second 72 h culture of cow embryos, from the zygote to the blastocyst. Note that Eagle's essential amino acids did not contain glutamine (see Table 2.4).

5.2.2.1 Zygote to Day 4 post-insemination

Presumptive zygotes (18 h pi) were randomly allocated to SOF with 1 mM glutamine and Eagle's non-essential amino acids, plus Eagle's essential amino acids at one of three concentrations (single-, half-, or quarter-strength essential amino acids). Embryos were cultured for 72 h to Day 4 pi and development was scored. Embryos were then transferred in their respective groups to 20aa for a further 72 h culture to Day 7 pi. The development of embryos was scored and blastocysts were differentially stained to determine total cell number and allocation of cells to the TE and ICM (see section 2.7.3).

5.2.2.2 Day 4 to Day 7 post-insemination

Presumptive zygotes (18 h pi) were cultured for 72 h to Day 4 pi in NeGln. Embryos were thus cultured in the absence of Eagle's essential amino acids. At 72 h culture, embryos were divided equally into three groups (as for section 5.2.1.2) and transferred to SOF with 1 mM glutamine and Eagle's non-essential amino acids, plus Eagle's essential amino acids at one of three concentrations (single-, half-, or quarter-strength essential amino acids) for a further 72 h culture to Day 7 pi. The development

of embryos was scored and blastocysts were differentially stained to determine total cell number and allocation of cells to the TE and ICM (see section 2.7.3).

5.2.3 The Effect of Glutamine and Betaine During Culture.

The following set of experiments determined the effect of the presence and absence of glutamine and the substitution of glutamine with betaine on embryo development and total cell number, during culture of cow embryos with other amino acid groups from the zygote to the blastocyst.

5.2.3.1 Zygote to Day 4 post-insemination

Presumptive zygotes (18 h pi) were randomly allocated to one of four media: 1) NeGln (control, SOF with 1 mM glutamine and Eagle's non-essential amino acids), 2) Ne (SOF with Eagle's non-essential amino acids), 3) NeBet (SOF with 1 mM betaine and Eagle's non-essential amino acids), or 4) Bet (SOF with 1 mM betaine). Embryos were cultured for 72 h to Day 4 pi and development was scored. Embryos were then transferred in their respective groups to 20aa for a further 72 h culture to Day 7 pi. Development of embryos was scored and blastocysts were stained with Hoechst (Number 33258) to determine total cell numbers (see section 2.7.2).

5.2.3.2 Day 4 to Day 7 Post-Insemination

Presumptive zygotes (18 h pi) were cultured for 72 h to Day 4 pi in NeGln. Embryos were then divided equally among three groups (as for section 5.2.1.2) and transferred to one of three media: 1) 20aa (control, SOF with 1 mM glutamine and Eagle's non-essential and Eagle's essential amino acids), 2) 19aa (SOF with Eagle's non-essential and Eagle's essential amino acids), or 3) 19aaBet (19aa with 1 mM

betaine). Embryos were cultured for a further 72 h to Day 7 pi. Development of embryos was scored and blastocysts were stained with Hoechst (Number 33258) to determine total cell numbers (see section 2.7.2).

5.2.4 Statistical Analysis

Differences among treatment means in each experiment were determined by an ANOVA, followed by the Tukey-Kramer Multiple Comparison Test. Bartlett's test was used to check for homogeneity of variances. For data expressed as percentages, an ANOVA was performed after arc sine transformation of original data was expressed as proportions. When the degree of association between two variables was determined, the correlation coefficient (r) was calculated. A probability of $P < 0.05$ was considered to be significant for all statistical tests.

5.3 RESULTS

5.3.1 The Temporal Effect of Amino Acid Groups on Development, Cell Number and Differentiation During Culture.

5.3.1.1 The Zygote to Day 4 post-insemination

Culture of embryos in NeGln for 72 h from the zygote significantly increased the number of embryos reaching the 8- to 16-cell stage, compared with culture in EssGln ($P < 0.05$) or in the absence of amino acids (SOF: $P < 0.05$, Table 5.1). Culture of embryos in EssGln neither stimulated nor inhibited embryo development with respect to culture in SOF ($P > 0.05$). The addition of the essential amino acids to media containing glutamine and Eagle's non-essential amino acids (20aa) did not further affect embryo development ($P > 0.05$).

Development of embryos to the 8- to 16-cell stage in media containing different groups of amino acids was predictive of subsequent blastocyst development. Blastocyst development was significantly higher when embryos had been cultured for the first 72 h in media containing glutamine and Eagle's non-essential amino acids (NeGln and 20aa), compared with culture in EssGln or SOF ($P < 0.05$, Table 5.1). The early cleavage stage embryo showed a requirement for the Eagle's non-essential amino acids, as culture for the first 72 h in NeGln resulted in significantly more embryos reaching the blastocyst stage than culture of embryos in Gln ($P < 0.05$). Culture in Gln, however, significantly increased the number of embryos reaching the morula stage compared with culture in SOF ($P < 0.05$, Table 5.1).

Table 5.1 Effect of culture of the early cow embryo with different groups of amino acids for the first 72 h, on development and subsequent developmental competence.

			Development[A] to various stages after		
			72 h culture	144 h culture	
Media* 1st 72 h	Media** 2nd 72 h	Cleaved ova	≥ 8-cell	Morula and blastocyst	Blastocyst
SOF	20aa	161	35.4^{ac}	32.3^{abc}	20.9^{ab}
Gln	20aa	200	44.8	53.8^{a}	28.7^{e}
NeGln	20aa	178	62.7^{ab}	57.5^{b}	41.5^{ace}
EssGln	20aa	196	43.4^{b}	46.1	24.6^{cd}
20aa	20aa	187	55.3^{c}	57.7^{c}	39.2^{bd}

*Media for culture 0-72 h (0 = 18-20 h pi). Media are defined in section 5.2.1.

**Media for culture 72-144 h pi.

$^{a-e}$Like pairs of letters are significantly different within a column, $P < 0.05$.

^{A}Percentage of cleaved ova. Data are from seven replicates.

Culture with amino acids during the first 72 h did not significantly affect subsequent blastocyst cell number when compared to the control (SOF versus Gln, NeGln, EssGln and 20aa; $P > 0.05$, Figure 5.1a). Initial culture, however, in EssGln depressed subsequent blastocyst cell number and resulted in blastocysts with significantly fewer cells than after culture in Gln or NeGln ($P < 0.05$, Figure 5.1a). Determination of the allocation of cells in the blastocyst indicated that the depression in total cell number, after initial culture in EssGln, was in both the TE and ICM with significantly fewer cells in the ICM ($P < 0.05$, Figure 5.1a). There was, however, no significant difference between any of the amino acid treatments during the first 72 h culture, in the proportion of cells subsequently allocated to the ICM ($P > 0.05$, Figure 5.1b).

5.3.1.2 Day 4 to Day 7 post-insemination

From Day 4 to Day 7 pi, the cow embryo showed a requirement for both Eagle's non-essential and Eagle's essential amino acids and glutamine. While development to the morula and blastocyst stages combined was not significantly different following culture in Gln, NeGln, EssGln and 20aa ($P > 0.05$), culture in 20aa resulted in the formation of significantly more blastocysts than did any other treatment (20aa versus SOF, Gln, NeGln and EssGln, $P < 0.05$; Table 5.2). In addition, culture in 20aa resulted in a significantly higher blastocyst cell number ($P < 0.05$, Figure 5.2a). Analysis of the allocation of cells in the blastocyst revealed that culture with 20aa significantly increased the number of cells in both the TE and ICM ($P < 0.05$, Figure 5.2a). Further, culture with 20aa significantly increased the proportion of total cells allocated to the ICM, when compared to embryos cultured in SOF, Gln or EssGln ($P <$

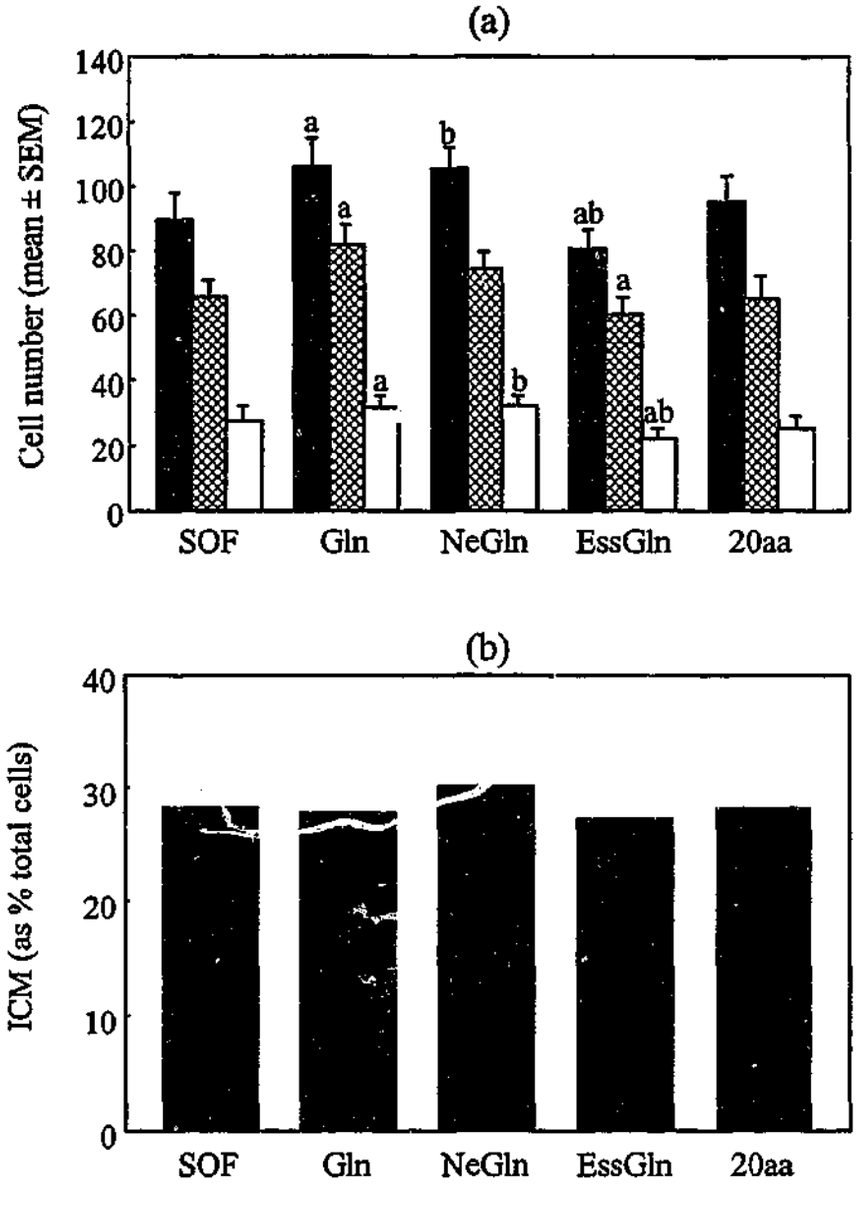


Figure 5.1 Allocation of cells in the blastocyst following culture of cow embryos from 0-72 h (0 = 18-20 h post-insemination) in SOF, Gln, NeGln, EssGln or 20aa, followed by culture from 72-144 h in 20aa (media are defined in section 5.2.1). (a) total cell number (solid bars) and allocation of cells to the trophectoderm (TE; hatched bars) and inner cell mass (ICM; open bars). (b) percentage of total cells in the ICM. Data are from four replicates with at least 100 cleaved ova per treatment. [ab]Like pairs of letters within a cell number group are significantly different between treatments ($P < 0.05$).

Table 5.2 Effect of culture of the early cow embryo with different groups of amino acids for 72 h from Day 4 to Day 7 post-insemination, on development.

Media* 1st 72 h	Media** 2nd 72 h	Cleaved ova	Development[A] to various stages after 144 h culture	
			Morula and blastocyst	Blastocyst
NeGln	SOF	159	35.8[a]	18.2[a]
NeGln	Gln	153	45.6	29.0[b]
NeGln	NeGln	158	49.6	32.5[a]
NeGln	EssGln	160	39.2	29.0[c]
NeGln	20aa	158	56.4[a]	46.3[abc]

*Media for culture 0-72 h (0 = 18-20 h pi). Media are defined in section 5.2.1.

**Media for culture 72-144 h pi.

[a-c]Like pairs of letters are significantly different within a column, $P < 0.05$.

[A]Percentage of cleaved ova. Data are from six replicates.

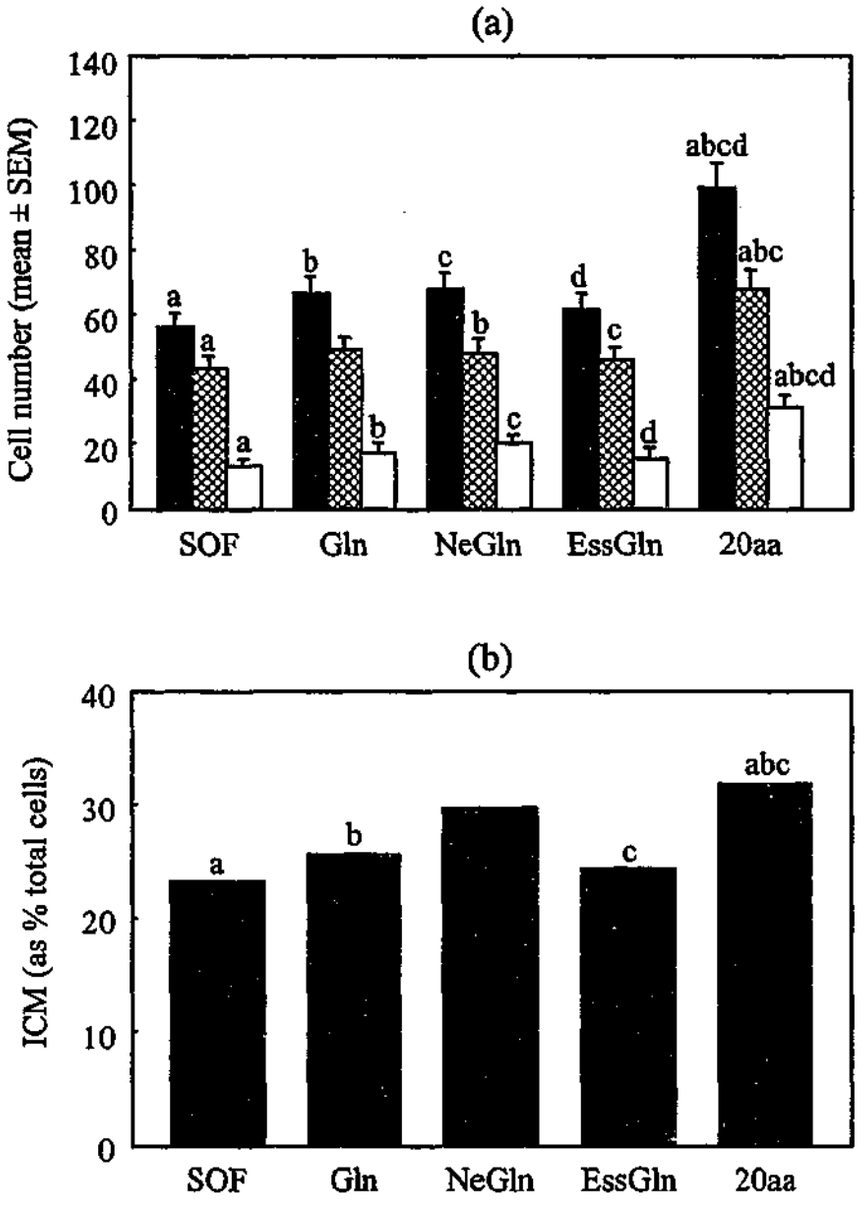


Figure 5.2 Allocation of cells in the blastocyst following culture of cow embryos from 0-72 h (0 = 18-20 h post-insemination) in NeGln followed by culture from 72-144 h in SOF, Gln, NeGln, EssGln or 20aa (media are defined in section 5.2.1). (a) total cell number (solid bars) and allocation of cells to the trophectoderm (TE; hatched bars) and inner cell mass (ICM; open bars). (b) percentage of total cells in the ICM. Data are from four replicates with at least 100 cleaved ova per treatment. [abcd]Like pairs of letters within a cell number group are significantly different between treatments ($P < 0.05$).

0.05, Figure 5.2b). Proportions of cells in the ICM of embryos cultured in NeGln and 20aa were not significantly different ($P > 0.05$).

Blastocyst formation and development to the morula stage were significantly higher after culture with Eagle's non-essential amino acids (Ne and NeGln) than after culture with Eagle's essential amino acids in the absence of glutamine (Ess, $P < 0.05$; Table 5.3) but not in the presence of glutamine (EssGln, $P > 0.05$). There was no significant difference between the effect of Eagle's non-essential and Eagle's essential amino acids on total cell number or the allocation of cells to the TE and ICM (Ne versus Ess, $P > 0.05$; Figure 5.3a). Furthermore, neither the non-essential nor the essential amino acids on their own affected the proportion of cells in the ICM ($P > 0.05$, Figure 5.3b). The addition of glutamine to media containing Eagle's non-essential or Eagle's essential amino acids did not significantly affect blastocyst development, cell number or cell allocation to the TE and ICM (Ne versus NeGln; and Ess versus EssGln, $P > 0.05$; Table 5.3 and Figure 5.3a and b).

5.3.2 The Effect of the Concentration of Eagle's Essential Amino Acids During Culture on Development and Cell Number.

5.3.2.1 The Zygote to Day 4 Post-Insemination

Culture of cow embryos with various concentrations of Eagle's essential amino acids (single-, half- and quarter-strength) during the first 72 h culture did not have a significant effect on development to the 8- to -16-cell stage or subsequent development to the morula or blastocyst stages ($P > 0.05$, Table 5.4). Further, total blastocyst cell number and the allocation of cells to the TE and ICM were not

Table 5.3 Effect of culture of the early cow embryo with Eagle's[T] non-essential or essential amino acids in the presence and absence of glutamine from Day 4 to Day 7 post-insemination, on development.

			Development[A] to various stages after 144 h culture	
Media* 1st 72 h	Media** 2nd 72 h	Cleaved ova	Morula and blastocyst	Blastocyst
NeGln	Ne	146	58.3[a]	42.9[a]
NeGln	NeGln	146	55.3[b]	36.1[b]
NeGln	Ess	146	33.9[ab]	16.5[ab]
NeGln	EssGln	146	46.6	27.3

[T]Listed in Table 1.3.

*Media for culture 0-72 h (0 = 18-20 h pi). Media are defined in section 5.2.1.

**Media for culture 72-144 h pi.

[a-b]Like pairs of letters are significantly different within a column, $P < 0.05$.

[A]Percentage of cleaved ova. Data are from five replicates.

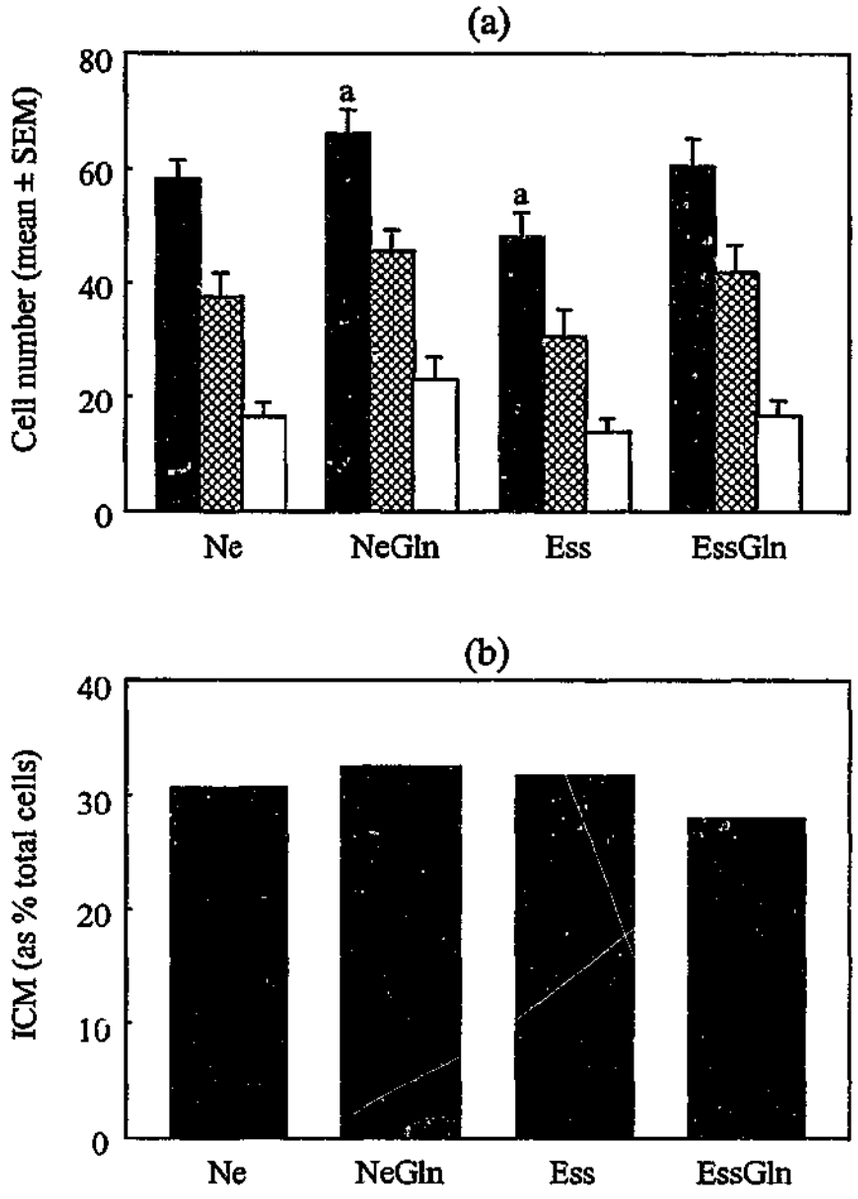


Figure 5.3 Allocation of cells in the blastocyst following culture of cow embryos from 0-72 h (0 = 18-20 h post-insemination) in NeGln followed by culture from 72-144 h in Ne, NeGln, Ess or EssGln (media are defined in section 5.2.1). (a) total cell number (solid bars) and allocation of cells to the trophectoderm (TE; hatched bars) and inner cell mass (ICM; open bars). (b) percentage of total cells in the ICM. Data are from three replicates with at least 90 cleaved ova per treatment. [a]Like pairs of letters within a cell number group are significantly different between treatments ($P < 0.05$).

Table 5.4 Effect of culture of the early cow embryo with various concentrations of Eagle's essential amino acids for the first 72 h, on development and subsequent developmental competence.

			Development[A] to various stages after		
			72 h culture	144 h culture	
Concentration (x) of essential amino acids in media* 1st 72 h	Media** 2nd 72 h	Cleaved ova	≥ 8-cell[B]	Morula and blastocyst[B]	Blastocyst[B]
1x	20aa	95	46.7	49.8	38.2
0.5x	20aa	95	50.9	51.3	43.8
0.25x	20aa	93	53.6	59.3	48.4

*Media for culture 0-72 h (0 = 18-20 h pi) was SOF with glutamine and Eagle's non-essential amino acids plus Eagle's essential amino acids at single-, half- and quarter-strength.

**Media for culture 72-144 h. Media are defined in section 5.2.2.

[A]Percentage of cleaved ova. Data are from four replicates.

[B]No significant difference among means within a column, $P > 0.05$.

significantly affected by the concentration of essential amino acids during the first 72 h culture ($P > 0.05$, Figure 5.4a and b).

There was, however, a significant negative correlation between the concentration of Eagle's essential amino acids during the first 72 h culture and (1) development to the 8- to 16-cell stage ($r = -0.99$, $P < 0.01$; Figure 5.5a); and (2) subsequent blastocyst formation ($r = -0.99$, $P < 0.01$; Figure 5.5a). There was no correlation between the concentration of Eagle's essential amino acids and total blastocyst cell number ($r = -0.66$, $P > 0.05$; Figure 5.5b).

5.3.2.2 Day 4 to Day 7 Post-Insemination

Culture of cow embryos with various concentrations of Eagle's essential amino acids (single-, half- and quarter-strength) during the second 72 h culture did not have a significant effect on development to the morula or blastocyst stages ($P > 0.05$, Table 5.5). Total blastocyst cell number and the allocation of cells to the TE and ICM were not significantly affected by the concentration of Eagle's essential amino acids during the second 72 h culture ($P > 0.05$, Figure 5.6a and b).

There was, however, a significant negative correlation between the concentration of the essential amino acids in media during the second 72 h culture and blastocyst formation ($r = -0.99$, $P < 0.01$; Figure 5.5c). There was no correlation between the concentration of essential amino acids and total blastocyst cell number ($r = -0.70$, $P > 0.05$; Figure 5.5d).

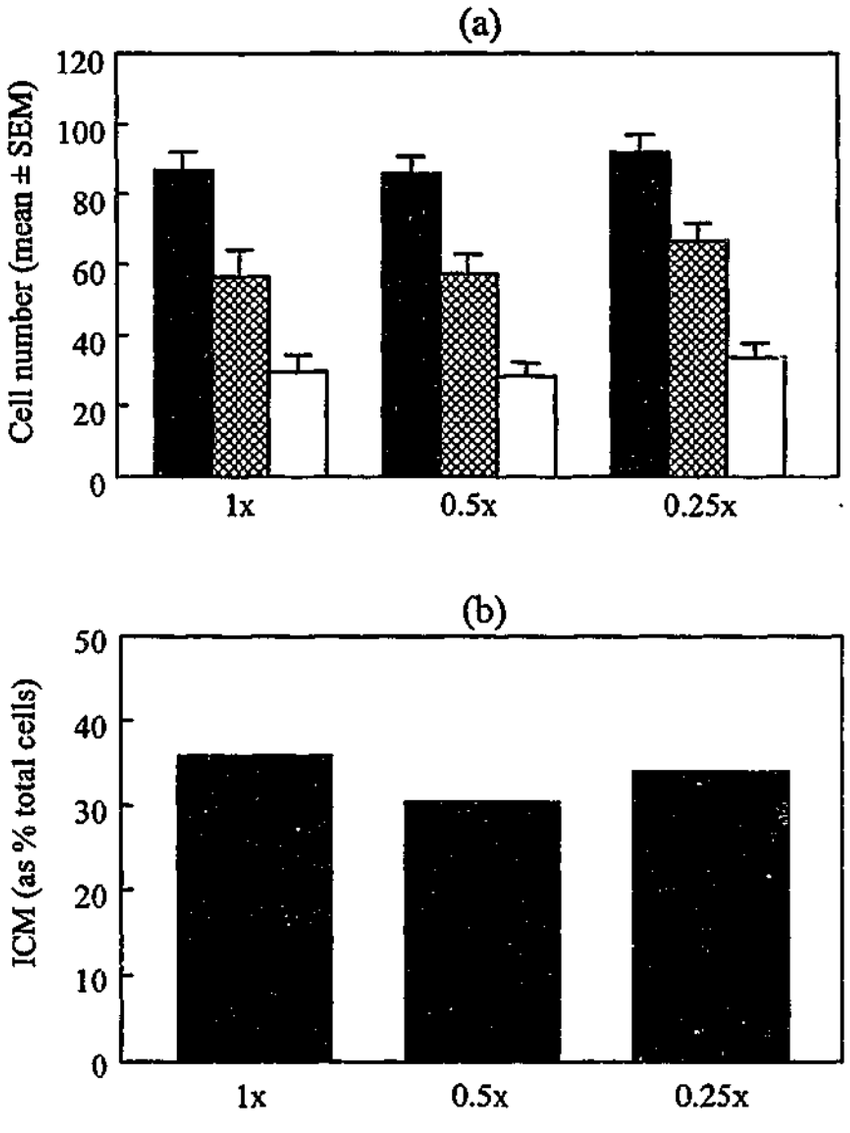


Figure 5.4 Allocation of cells in the blastocyst following culture of cow embryos from 0-72 h (0 = 18-20 h post-insemination) in SOF with glutamine and Eagle's non-essential amino acids plus Eagle's essential amino acids at single-, half- and quarter-strength (x-axis), followed by culture from 72-144 h in 20aa. (a) total cell number (solid bars) and allocation of cells to the trophectoderm (TE; hatched bars) and inner cell mass (ICM; open bars). (b) percentage of total cells in the ICM. Data are from four replicates with at least 95 cleaved ova per treatment. Note that Eagle's essential amino acids did not contain glutamine. No significant differences among treatments within a cell number group ($P > 0.05$).

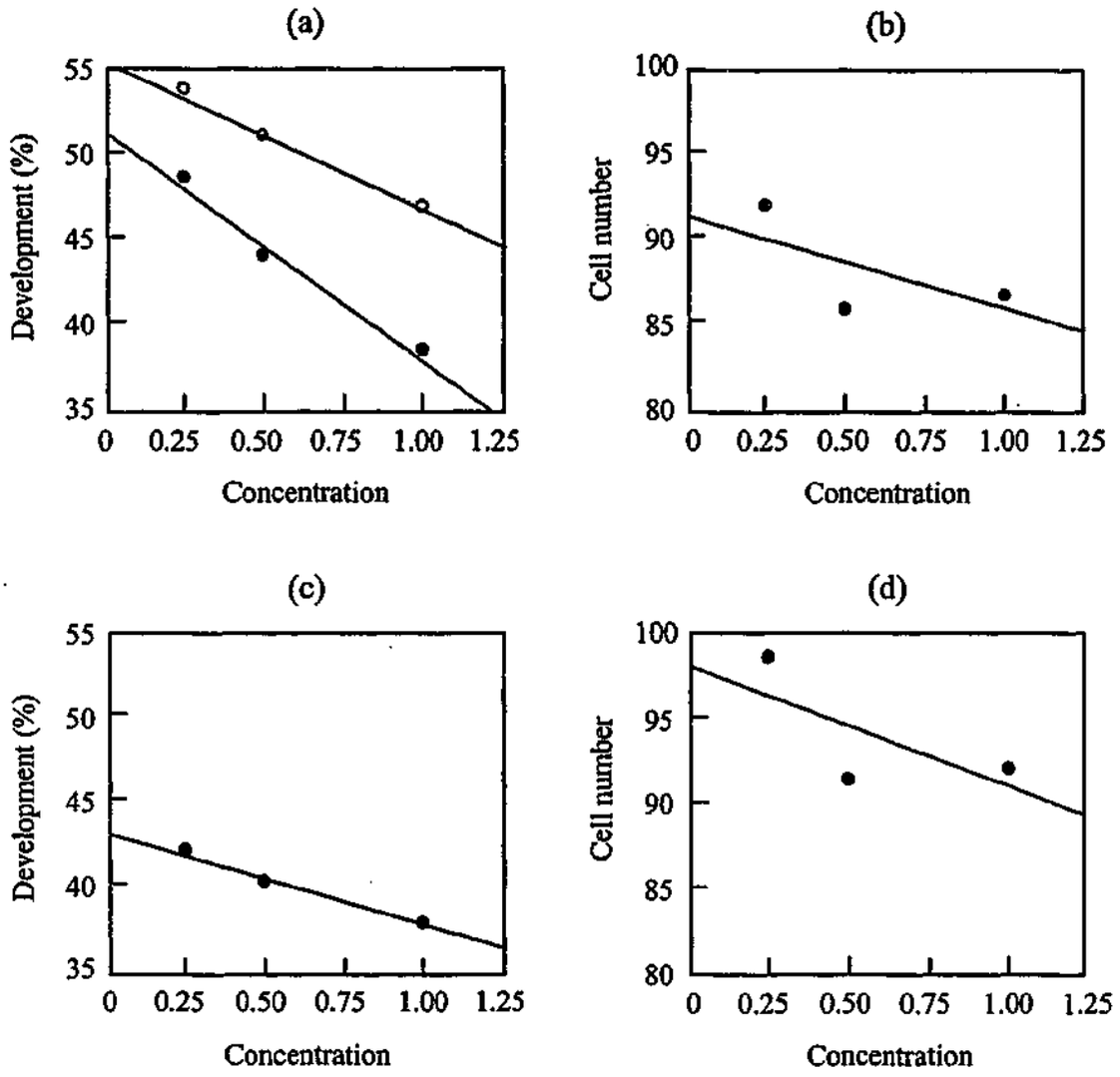


Figure 5.5 Correlations between the concentration of Eagle's essential amino acids in media and embryo development and blastocyst total cell number. (a) Development to the 8- to 16-cell stage (open circles, $r = -0.99$, $P < 0.01$) and subsequent blastocyst formation (closed circles, $r = -0.99$, $P < 0.01$) following culture of cow zygotes to Day 4 post-insemination in media with various concentrations of essential amino acids (see Table 5.4) and then culture to Day 7 post-insemination in 20aa; (b) mean number of cells in blastocysts following culture conditions for (a), ($r = -0.66$, $P > 0.05$); (c) blastocyst formation following culture to Day 4 post-insemination with NeGln and then culture to Day 7 post-insemination in media with various concentrations of essential amino acids (see Table 5.5; $r = -0.99$, $P < 0.01$); (d) mean blastocyst cell number following culture conditions for (c), ($r = -0.70$, $P > 0.05$). (a) and (b) data are from four replicates with at least 93 cleaved ova per treatment. (c) and (d) data are from three replicates with at least 60 cleaved ova per treatment.

Table 5.5 Effect of culture of the early cow embryo with various concentrations of Eagle's essential amino acids from Day 4 to Day 7 post-insemination, on development.

Media* 1st 72 h	Concentration (x) of essential amino acids in media** 2nd 72 h	Development[A] to various stages after 144 h culture	
		Morula and blastocyst[B]	Blastocyst[B]
NeGln	1x	52.6	37.6
NeGln	0.5x	55.7	40.1
NeGln	0.25x	50.2	41.9

*Media for culture 0-72 h (0 = 18-20 h pi). Media are defined in section 5.2.2.

**Media for culture 72-144 h pi was SOF with glutamine and Eagle's non-essential amino acids plus Eagle's essential amino acids at single-, half- and quarter-strength.

[A]Percentage of cleaved ova. Data are from three replicates; n = 60 cleaved ova per treatment.

[B]No significant difference among means within a column, $P > 0.05$.

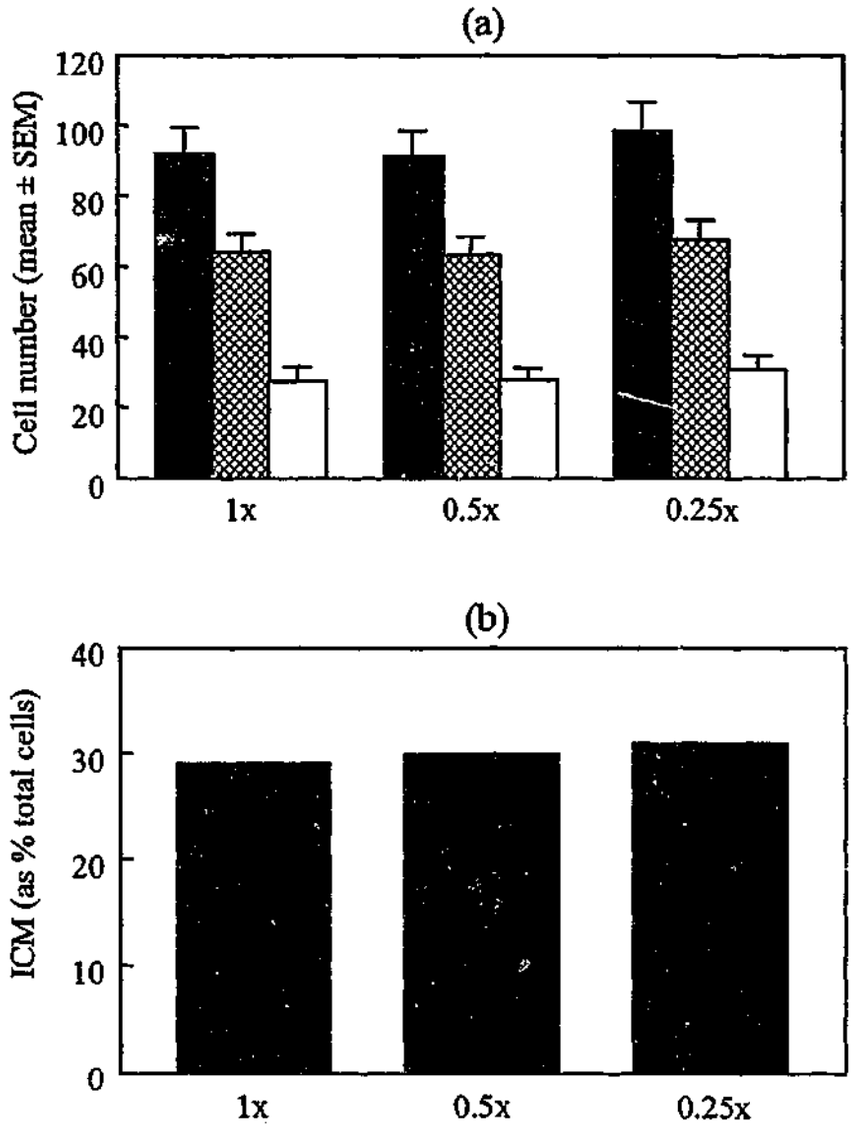


Figure 5.6 Allocation of cells in the blastocyst following culture of cow embryos from 0-72 h (0 = 18-20 h post-insemination) in NeGln, followed by culture from 72-144 h in 20aa containing various concentrations of Eagle's essential amino acids (x-axis). (a) total cell number (solid bars) and allocation of cells to the trophectoderm (TE; hatched bars) and inner cell mass (ICM; open bars). (b) percentage of total cells in the ICM. Data are from three replicates with at least 60 cleaved ova per treatment. Note that Eagle's essential amino acids did not contain glutamine. No significant differences among treatments within a cell number group ($P > 0.05$).

5.3.3 The Effect of Glutamine and Betaine During Culture.

5.3.3.1 Zygote to Day 4 Post-Insemination

The cow embryo had a requirement for glutamine during the first 72 h culture. Culture in NeGln resulted in significantly more embryos reaching the 8- to 16-cell stage than culture in Ne ($P < 0.05$, Table 5.6). Subsequent blastocyst development was also higher after culture in the presence of glutamine during the first 72 h ($P < 0.05$), however, cell number was not affected ($P > 0.05$, Table 5.6). Subsequent development to the morula stage was not affected by the absence of glutamine during the first 72 h culture, as indicated by morula and blastocyst development combined (Ne versus NeGln: $P > 0.05$; Table 5.6). In the presence of Eagle's non-essential amino acids, betaine did not have a stimulatory effect during the first 72 h culture, with development to the 8- to 16-cell stage equivalent in Ne and NeBet ($P > 0.05$). Subsequent blastocyst development, however, from embryos cultured in NeBet was not significantly different to that of embryos cultured in the presence of glutamine (NeGln, $P > 0.05$). In the absence of Eagle's non-essential amino acids, culture with betaine resulted in poor development to the 8- to 16-cell stage and morula and blastocyst stages (Bet versus Ne, NeGln and NeBet, $P < 0.05$).

5.3.3.2 Day 4 to Day 7 Post-Insemination

The cow embryo had a requirement for glutamine from Day 4 to Day 7 pi. Culture in 20aa resulted in significantly more embryos reaching the blastocyst stage and the morula and blastocyst stages combined than culture in 19aa in the absence of betaine (20aa versus 19aa and 19aaBet, $P < 0.05$; Table 5.7). Glutamine did not affect blastocyst cell number (19aa versus 20aa, $P > 0.05$; Table 5.7).

Table 5.6 Effect of culture of the early cow embryo for the first 72 h with non-essential amino acids in the presence and absence of glutamine and betaine, and the effect on subsequent developmental competence.

			Development[A] to various stages			
			72 h culture	144 h culture		
Media* 1st 72 h	Media** 2nd 72 h	Cleaved ova	≥ 8-cell	Morula and blastocyst	Blastocyst	Total cell number[‡]
Ne	20aa	165	41.9[a]	43.3[a]	32.1[a]	98.9 ± 6.4
NeGln	20aa	178	54.9[ab]	56.6[b]	48.2[ab]	97.1 ± 6.8
NeBet	20aa	171	41.5[b]	52.6[c]	42.4[c]	89.8 ± 7.4
Bet	20aa	161	16.6[ab]	24.3[abc]	20.4[bc]	77.3 ± 6.4

*Media for culture 0-72 h (0 = 18-20 h pi). Media are defined in section 5.2.3.

**Media for culture 72-144 h pi.

[a-c]Like pairs of letters are significantly different within a column, $P < 0.05$.

[A]Percentage of cleaved ova. Data are from six replicates.

[‡]Blastocyst total cell number (mean ± SEM). Data are from three replicates (at least 90 cleaved ova per treatment).

Table 5.7 Effect of culture of the early cow embryo with Eagle's[τ] non-essential and essential amino acids in the presence and absence of glutamine and betaine from Day 4 to Day 7 post-insemination.

			Development[A] to various stages after 144 h culture		
Media* 1st 72 h	Media** 2nd 72 h	Cleaved ova	Morula and blastocyst	Blastocyst	Total cell number[†]
NeGln	20aa	194	61.9[ab]	54.6[ab]	90.6 ± 5.5
NeGln	19aa	194	50.1[a]	37.6[a]	77.3 ± 5.2
NeGln	19aaBet	194	48.8[b]	36.4[b]	93.5 ± 7.1

[τ]Listed in Table 1.3.

*Media for culture 0-72 h (0 = 18-20 h pi). Media are defined in section 5.2.3.

**Media for culture 72-144 h pi.

[a-b]Like pairs of letters are significantly different within a column, $P < 0.05$.

[A]Percentage of cleaved ova. Data are from six replicates.

[†]Blastocyst total cell number (mean ± SEM). Data are from four replicates (at least 120 cleaved ova per treatment).

5.4 DISCUSSION

It is established that the addition of amino acids to culture media significantly improves development of the cow embryo (Takahashi and First, 1992; Kim *et al.*, 1993b; Gardner, 1994; Keskintepe *et al.*, 1995; Liu and Foote, 1995b). The present study revealed that the cow embryo not only has a requirement for amino acids, but that amino acids have both a temporal and differential effect during development from the zygote to the blastocyst.

Culture for the first 72 h in NeGln stimulated cleavage to the 8- to 16-cell stage and subsequent blastocyst development. During this period, EssGln or glutamine as the sole amino acid had no measurable effect on cleavage. When present with the non-essential amino acids, however, glutamine was found to be an important amino acid for development of the early embryo. The fact that NeGln increased cleavage is important for the optimization of culture media, as an increased rate of development of the early cleavage stage embryo has been correlated with an increase in viability (Van Soom *et al.*, 1992; McKiernan and Bavister, 1994; Lane and Gardner, 1997a). Previous studies have illustrated the stimulatory effects of the combination of glutamine and the non-essential amino acids in development of mammalian embryos in vitro. Culture of mouse embryos from the zygote with Eagle's non-essential amino acids and glutamine significantly increased blastocyst formation, cell number, hatching and post-implantation development (Gardner and Lane, 1993a; Lane and Gardner, 1994). The non-essential amino acids and glutamine were found to decrease the time of the first three cleavage divisions in the mouse embryo and thus, stimulated the rate of embryo development (Lane and Gardner, 1997b). When added during the entire culture period, the non-essential amino acids and glutamine have also been

shown to stimulate development of the ruminant embryo (Gardner *et al.*, 1994b; Liu and Foote, 1995b).

While it has been determined that the early cleavage stage mouse embryo has a requirement for the non-essential amino acids and glutamine (Lane and Gardner, 1997a), only one other study has looked at the effect of amino acid groups during culture of the early cow embryo. Pinyopummintr and Bavister (1996a) reported that development of the cow embryo to the 8- to 16-cell stage (72 h pi) was equivalent following culture in the presence of non-essential amino acids (media also contained glutamine), essential amino acids or glutamine. The present study, however, showed that NeGln stimulated cleavage to the 8- to 16-cell stage (90 h pi), while EssGln and Gln had no effect on development. A feasible explanation for this apparent contrast is that the stimulatory effects of the non-essential amino acids and glutamine may not be evident until 90 h pi. This is supported by the fact that subsequent blastocyst development was higher after initial culture with non-essential amino acids and glutamine than after culture with the essential amino acids (Pinyopummintr and Bavister, 1996a). Pinyopummintr and Bavister (1996a), found that culture with glutamine or the non-essential amino acids and glutamine to the 8- to 16-cell stage, resulted in equivalent development to the blastocyst stage (Day 8 pi). In the present study, subsequent blastocyst development (Day 7 pi) was significantly lower after culture in Gln to the 8- to 16-cell stage when compared to culture in NeGln. Either culture in the presence of serum during the second culture period or the extra day of culture in the study of Pinyopummintr and Bavister (1996a) could explain the observed contrariety in results. In the present study, culture of the early cleavage

stages in Gln or NeGln resulted in equivalent proportions of embryos at the morula/blastocyst stages on Day 7 pi.

After Day 4 pi, the cow embryo showed a requirement for glutamine, non-essential amino acids and essential amino acids. The combination of all twenty amino acids stimulated blastocyst development, total cell number, the number of cells in the TE and ICM and the proportion of total cells allocated to the ICM. This is a significant finding for the optimization of culture media as the total number of cells in the blastocyst and the ICM have been positively correlated with blastocyst viability in the mouse (Papaioannou and Ebert, 1986; Lane and Gardner, 1997a). Further, Iwasaki *et al.* (1990) reported that in vitro produced cow blastocysts had lower total and ICM cell numbers and a smaller proportion of cells in the ICM compared with in vivo produced blastocysts. While the mouse embryo showed similar requirements for amino acid groups beyond the 8-cell stage, it was found that each group had quite specific functions in the development of the mouse blastocyst (Lane and Gardner, 1997a). The non-essential amino acids and glutamine stimulated blastocyst formation and hatching, while the essential amino acids stimulated cleavage, allocation of cells to the ICM and fetal development following transfer. From the present study, it is evident that the function of the amino acid groups is not as defined in the development of the cow blastocyst. With respect to the function of the essential amino acids, the non-essential amino acids stimulated blastocyst formation, however, blastocyst formation was highest after culture with a combination of glutamine and the non-essential and the essential amino acids. Neither the non-essential nor the essential group of amino acids on their own affected blastocyst cell number or the allocation of cells to the TE and ICM in the blastocyst. The essential amino acids did not stimulate

development of the ICM as was found in the mouse (Lane and Gardner, 1997a). In fact, blastocysts with the highest proportion of cells in the ICM resulted from embryos that had been cultured for the second 72 h in the presence of glutamine and the non-essential amino acids. In support of the observed differences in the role of amino acids in development of blastocysts of the mouse and cow, Lamb and Leese (1994) and Partridge and Leese (1996) reported differences between the two species in the depletion of individual amino acids from culture media containing blastocysts. The cow blastocyst depleted significant amounts of aspartate, glutamate, threonine and lysine (Partridge and Leese, 1996) while the depletion of amino acids by the mouse blastocyst included aspartate, glutamate and a larger number of essential amino acids: tyrosine, methionine, valine, phenylalanine, isoleucine and leucine (Lamb and Leese, 1994).

While the mammalian embryo requires essential amino acids beyond the third cleavage division, it is believed that they may be detrimental to development of the early cleavage stages. Culture of the mouse embryo up to the 8-cell stage with the essential amino acids negated the stimulatory effect of the non-essential amino acids and glutamine, reducing subsequent blastocyst cell number and post-implantation development (Gardner and Lane, 1993a; Lane and Gardner, 1997a). The essential amino acids methionine, phenylalanine and isoleucine inhibited development of the hamster 1-cell embryo (McKiernan *et al.*, 1995) but were not inhibitory beyond the 8-cell stage (Bavister *et al.*, 1983; Carney and Bavister, 1987a). This may be due to competition for transporters between non-essential and essential amino acids. Lewis and Kaye (1992) found that isoleucine, leucine, methionine and tryptophan, all reduced the uptake of glutamine in mouse 2-cell embryos. In the present study,

however, the essential amino acids did not counteract the stimulatory effects of the non-essential amino acids during culture of the early cleavage stage cow embryo. Development to the 8- to 16-cell stage, subsequent blastocyst development, cell number and the allocation of cells to the ICM and TE were all equivalent for embryos that had been cultured for the first 72 h in 20aa or NeGln. Previous studies in the cow (Liu and Foote, 1995b) and sheep (Gardner *et al.*, 1994b) have also reported that the presence of the essential amino acids did not negate the stimulatory effects of the non-essential amino acids and glutamine. This indicates that the transport mechanisms for non-essential and essential amino acids may differ in the sheep, cow, mouse and hamster.

The amino acids present in Eagle's essential amino acids are at higher concentrations than are found in the ruminant reproductive tract (Fahning *et al.*, 1967; Nancarrow *et al.*, 1992; see Table 1.3). Bavister and McKiernan (1993) found that a reduction in the concentration of several essential amino acids for culture of the hamster embryo changed them from inhibitory to stimulatory amino acids. Liu and Foote (1995b) reported that culture of cow embryos from the 4-cell stage to the blastocyst with half the concentration of essential amino acids, resulted in a significant increase in the proportion of hatching blastocysts. In the present study, it was found that a reduction in the concentration of the essential amino acids (when in combination with the non-essential amino acids and glutamine) was beneficial for both the early cleavage stages and the later stages of development. When the concentration of the essential amino acids was decreased during the first 72 h culture there was a significant negative correlation with embryo cleavage and subsequent blastocyst development. Interestingly, this effect was not restricted to the early

cleavage stages. When embryos were not exposed to the essential amino acids until Day 4 pi there was still a significant negative correlation with concentration and blastocyst development. Cleavage, however, was not affected by the concentration of essential amino acids as there was no correlation between concentration and blastocyst cell number.

Glutamine has been shown to have an important role in the in vitro development of embryos from several species. Culture with glutamine helped random-bred mouse embryos through the 2-cell block (Chatot *et al.*, 1989; Gardner and Lane, 1996), while the hamster embryo required glutamine for development beyond the 8-cell stage (Carney and Bavister, 1987a). In the present study, glutamine was found to be an important amino acid for development of the cow embryo from the zygote through to the blastocyst. During the first 72 h culture, the removal of glutamine from media containing non-essential amino acids decreased cleavage to the 8- to 16-cell stage. Similarly, Gardner and Lane (1996) found that the removal of glutamine from culture medium reduced the stimulatory effects of Eagle's non-essential amino acids, in helping CF1 mouse embryos through the first cleavage division. Glutamine is likely to be important as an energy source for the early embryo (Petters *et al.*, 1990; Rieger *et al.*, 1992a, 1992b). Rieger *et al.* (1992a, 1992b) found that glutamine metabolism by the cow embryo was highest at the 2- to 4-cell stages and the expanding blastocyst, with almost 80% of the glutamine taken up by the early cleavage stages metabolized through the TCA cycle.

As well as stimulating development of the early cleavage stages, glutamine was required by the cow embryo beyond Day 4 pi. Partridge and Leese (1996) measured the uptake of individual amino acids by Day 7 cow blastocysts in SOF and

did not find a significant depletion of glutamine, indicating that glutamine was not required by the cow blastocyst. Likewise, the uptake of glutamine by the mouse blastocyst was not significant (Lamb and Leese, 1994). The present study, however, showed that although glutamine did not affect embryo cleavage or blastocyst cell number, the removal of glutamine from medium containing non-essential and essential amino acids significantly reduced blastocyst development. Considering the findings of Partridge and Leese (1996), it would appear that glutamine is required after Day 4 pi but prior to development of the blastocyst. Rieger *et al* (1992b), however, reported that glutamine uptake was constant from the 8-cell stage to the blastocyst, increasing significantly with blastocyst expansion and hatching. In addition, between 43 % and 73 % of the glutamine taken up by the embryo from the 8-cell stage to the blastocyst was metabolized through the TCA cycle (Rieger *et al.*, 1992b). This indicates that glutamine is used for energy production beyond Day 4 pi. Thompson *et al.* (2000) recently reported that ATP production via oxidative phosphorylation was in fact critical for development of cow embryos from Day 5 to Day 7 pi. In addition, Leese and Donnay (1999) revealed that oxidative metabolism has a role in the production of energy for blastocoel expansion in the cow. It is unknown why there are inconsistencies between the findings of Rieger *et al.* (1992b) and Partridge and Leese (1996), however, several other studies have shown that glutamine is utilized by the cow blastocyst (Rieger and Guay, 1988; Tiffin *et al.*, 1991; Rieger *et al.*, 1992a).

As well as being a source of energy for the embryo, glutamine can act as an osmolyte to protect the embryo from osmotic stress (Dawson and Baltz, 1997). Regulation of osmotic pressure is a critical factor for embryo development (Beckmann

and Day, 1993; Lim *et al.*, 1994; Li and Foote, 1996; Liu and Foote, 1996). Osmolytes maintain proper cell function by protecting protein structure, particularly the structure of enzymes. Betaine, a non-metabolizable, organic osmolyte, has also been found to protect the developing mouse embryo from detrimental increases in osmolarity (Biggers *et al.*, 1993; Dawson and Baltz, 1997). In the present study, the role of glutamine as a potential osmolyte during culture of the cow embryo was examined by substituting betaine for glutamine. Unlike glutamine, betaine did not stimulate cleavage of the early embryo. Betaine did, however, have a beneficial effect during the first 72 h culture, but this was not evident until the blastocyst stage. These results indicate that, as well as being an energy source, glutamine may function as an osmolyte for the early cleavage stage cow embryo. It is not surprising that culture with betaine in the absence of any amino acids resulted in poor embryo development. Biggers *et al* (1993) showed that the stimulatory effect of betaine was dependent on the concentration of glutamine in mouse embryo culture medium. The present study indicates that the stimulatory effect of betaine during the first 72 h culture was dependent on the presence of the non-essential amino acids. While several of the non-essential amino acids (glycine, proline and alanine) have been shown to be effective osmolytes for the mouse embryo (Dawson and Baltz, 1997), culture in Ne was inferior to culture in NeBet or NeGln. In the study by Dawson and Baltz (1997), however, the non-essential amino acids were tested at ten times the concentration present in Eagle's non-essential amino acids.

Glutamine did not appear to function in the capacity of an osmolyte during development from Day 4 pi. Betaine, unlike glutamine, was not able to stimulate blastocyst formation. Liu and Foote (1996) found that betaine could not protect the

developing cow embryo from an increase in the concentration of NaCl (95-122 mM). Glutamine was also present in the culture medium, indicating that glutamine was not acting as an osmolyte. Liu and Foote (1996), however, looked only at the effect of osmolytes from the 4-cell stage onwards. Betaine and glutamine may function as osmolytes during the first two cell divisions, when glutamine uptake has been reported to be high in the cow embryo (Rieger *et al.*, 1992a; Partridge and Leese, 1996). Although there was no observed effect of betaine in the presence of the non-essential amino acids on cleavage to the 8- to 16-cell stage, in the present study, an increase in subsequent blastocyst development indicates that betaine may protect other cellular functions during development of the early cleavage stages.

Thus, while required by the cow embryo from the zygote through to the blastocyst, the function of glutamine may change. This is not surprising as the physiology of the embryo changes with development. In fact, Lewis and Kaye (1992) found a switch in the transport mechanisms for glutamine, from systems L and *gly* in the mouse 2-cell embryo to system $B^{0,+}$ in the blastocyst.

In conclusion, this study determined the temporal and differential role of amino acids during development of the cow embryo from the zygote to the blastocyst in vitro. Development of the early cleavage stages was stimulated by the non-essential amino acids and glutamine while development beyond Day 4 pi was stimulated by a combination of the non-essential amino acids, the essential amino acids and glutamine. Culture with all twenty amino acids increased blastocyst formation, cell number and allocation of cells to the ICM and TE. Furthermore, the non-essential amino acids and glutamine increased the proportion of cells allocated to the ICM. The study also revealed the embryo's requirement for glutamine from the zygote through

to the blastocyst and the partial role of glutamine as an osmolyte during the first 72 h culture. Further, the study suggests a reduction in the concentration of the essential amino acids for culture of the cow embryo from the zygote to the blastocyst stage.

CHAPTER 6

THE ROLE OF AMINO ACIDS IN THE REGULATION OF METABOLISM AND INTRACELLULAR pH DURING CULTURE OF THE COW EMBRYO

6.1 INTRODUCTION

Culture of mammalian embryos in vitro can induce metabolic perturbations (Menke and McLaren, 1970; Gardner and Leese, 1990; Thompson *et al.*, 1991; Gardner and Lane, 1993b; Gardner *et al.*, 1994b; Gardner, 1998b, Lane and Gardner, 1998; Krisher *et al.*, 1999) which have been associated with a 'developmental block' (Gardner and Lane, 1993b) and a reduction in viability (Gardner and Sakkas, 1993; Lane and Gardner, 1998). Furthermore, exposure to weak acids and bases such as lactate, which is present in embryo culture media, can alter the intracellular pH (pHi) of the early embryo (Edwards *et al.*, 1998a, 1998b). Despite the presence of the Na^+/H^+ antiporter for the regulation of pHi in the acid to neutral range, mouse embryos had difficulty in regulating pHi against an acid load prior to compaction (Edwards *et al.*, 1998a, 1998b). Similarly, despite the presence of the HCO_3^-/Cl^- exchanger in the cleavage stage cow embryo for the regulation of pH in the alkaline to neutral range, embryos were unable to recover from a slight alkaline load (Lane and Bavister, 1999). The regulation of pHi by the embryo is essential for normal cellular functions including metabolism (Busa and Nuccitelli, 1984; Edwards *et al.*, 1998a), protein and DNA synthesis (Aerts *et al.*, 1985), meiosis (Ben-Yosef *et al.*, 1996; Edwards *et al.*, 1999) and mitosis (Saunders *et al.*, 1989; Grandin and Charbonneau, 1990). An increase in the pHi of cow embryos at the 8- to 16-cell stage, following a slight alkaline load, in fact arrested embryo development (Lane and Bavister, 1999). Thus, changes in pHi under various culture conditions could be detrimental to the development and viability of the early cleavage stage embryo. To date, there have been no reports of the pHi of the cow embryo following an extended culture period under different conditions.

While it is known that amino acids affect the development and viability of the in vitro produced mammalian embryo, there has been little research into the intracellular mechanisms responsible for the observed effects. Amino acids are thought to have several roles in the support of embryo development in culture (Gardner and Lane, 1997a). Amino acids undoubtedly have an important biosynthetic role in development, particularly beyond the 8- to 16-cell stage when protein synthesis increases (Frei *et al.*, 1989). Further, glutamine and several of the non-essential amino acids have been shown to function in the capacity of organic osmolytes to protect the early embryo from osmotic stress in vitro (Beckmann and Day, 1993; Biggers *et al.*, 1993). In Chapter 5 it was revealed that, in the presence of the non-essential amino acids, glutamine may have a role as an osmolyte for the early cleavage stage cow embryo.

Through their conversion into TCA cycle intermediates, as well as their potential to allosterically regulate enzyme activity, amino acids undoubtedly have an important role in the regulation of energy metabolism in the embryo. A high rate of metabolism of glucose via glycolysis, in the early cleavage stage mammalian embryo, has been associated with impaired development (Gardner and Lane, 1993b). While the precise mechanisms are not fully understood, this is believed to be due to a Crabtree-like effect, resulting in a reduced oxidative capacity (Seshagiri and Bavister, 1991). Interestingly, Eagle's non-essential amino acids and glutamine have been shown to reduce glycolytic activity in the early mouse embryo (Gardner and Lane, 1993b; Vella *et al.*, 1997), resulting in an alleviation of the 2-cell block to development (Gardner and Lane, 1996). Lane and Gardner (1997a) found that culture of mouse embryos with different groups of amino acids affected the uptake of glucose and the production of

lactate by the blastocyst, with the uptake of glucose lowest in the absence of amino acids. In addition, culture of both in vivo produced mouse and rat blastocysts in the absence of amino acids for just six hours, induced metabolic perturbations which resulted in an increase in the production of lactate without a corresponding increase in the uptake of glucose (Lane and Gardner, 1998). Importantly, culture of in vivo produced blastocysts for just 6 h without amino acids decreased embryo viability following transfer (Lane and Gardner, 1998).

While it is believed that amino acids play an important role in the regulation of pHi in the mammalian embryo (Bavister and McKiernan, 1993; Bavister, 1995; Gardner and Lane, 1997a), there has been surprisingly little research into the regulation of pHi by amino acids. The only reported study to date found that amino acids helped to buffer the pHi of mouse zygotes when exposed to either an acid or an alkaline load (Edwards *et al.*, 1998b).

The purpose, therefore, of the present study was to determine the effect of culture with different amino acid groups on the relative activities of intracellular energy generating pathways within the embryo and the pHi of the embryo. In addition, the study aimed to examine the relationship between metabolism and development and pHi and development under different culture conditions.

6.2 MATERIALS AND METHODS

All embryos were cultured in modifications of SOF medium (see Appendix 6 for formulations of media) in groups of five in 30 µl drops of media, under 7 ml light mineral oil, in a 60 mm non-pyrogenic culture dish, at 39°C, at 7% O_2, 5% CO_2 and 88% N_2.

6.2.1 The Effect of Culture with Amino Acids on the Metabolism of the 8- to 16-cell Cow Embryo and the Relationship between Metabolism and Development.

Experiments were designed to determine the effect of amino acid groups on the relative activities of intracellular energy generating pathways, the EMP and the TCA cycle, in both the 8- to 16-cell stage cow embryo and the blastocyst.

Following in vitro maturation and fertilization, presumptive zygotes (18 h pi) were cultured for 72 h in one of five media: 1) SOF (control), 2) Gln (SOF with 1 mM glutamine), 3) NeGln (SOF with 1 mM glutamine and Eagle's non-essential amino acids), 4) EssGln (SOF with 1 mM glutamine and Eagle's essential amino acids), or 5) 20aa (SOF with 1 mM glutamine and Eagle's non-essential and Eagle's essential amino acids). The stage of embryo development in each medium was scored at 72 h culture and embryos at the 8- to 16-cell stage were transferred in their respective groups to the metabolic version of the medium in which they were cultured: 1) metSOF, 2) metGln, 3) metNeGln, 4) metEssGln, 5) met20aa (see Appendix 10). The metabolic versions of media were the same as culture media but did not contain pyruvate (pyruvate was subsequently added as radiolabelled substrate). Embryos were washed three times in the metabolic medium to ensure all pyruvate was removed

before being transferred to the respective metabolic medium containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [G-^{3}H] glutamine (at a concentration of 0.004 mM and specific activity of 0.25 μCi/μl). [G-^{3}H] glutamine, however, was not added to metSOF as this was the zero amino acid treatment medium. Thus, determinations of [2-^{14}C] pyruvate and [G-^{3}H] glutamine metabolism were made for the same embryos, except for those in metSOF where only [2-^{14}C] pyruvate metabolism was determined. The metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by individual embryos was correlated for each treatment to determine the effect of amino acid groups on the relationship between the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glutamine.

In a separate series of cultures, embryos were cultured under the same conditions as above but were transferred to the respective metabolic media containing 0.33 mM pyruvate (unlabelled) and [5-^{3}H] glucose (at a concentration of 0.015 mM and specific activity of 0.25 μCi/μl).

Determinations of the metabolism of [2-^{14}C] pyruvate, [G-^{3}H] glutamine and [5-^{3}H] glucose by embryos cultured with different amino acids, were carried out as described in section 2.8. Individual 8- to 16-cell embryos were incubated in 3 μl drops of metabolic medium containing the radioisotopes for 3 h, at 7% O_2, 5% CO_2 and 88% N_2, at 39^{0}C, in the lid of individual 2 ml tubes. Rates of nutrient metabolism by each embryo were calculated from the liberation of $^{3}H_2O$ and $^{14}CO_2$ (see section 2.8.2).

The development of embryos to the 8- to 16-cell stage in media containing different amino acid groups (percentage at the 8- to 16-cell stage from cleaved ova), was correlated with the metabolism of [2-^{14}C] pyruvate, [G-^{3}H] glutamine and [5-^{3}H]

glucose. Development and metabolic determinations were from the same cohort of embryos to minimize variation.

6.2.1.1 *Determination of the relationship between the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by 8- to 16-cell embryos.*

Due to results of the above experiments to determine the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by individual embryos and the metabolism of [5-^{3}H] glucose by individual embryos, the following experiment was designed to determine whether a Crabtree-like effect could be detected in the 8- to 16-cell embryo. The experiment examined the relationship between the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by individual embryos.

Following in vitro maturation and fertilization, presumptive zygotes (18 h pi) were cultured for 72 h in two media which had been shown to have different effects on the development of early cleavage stage embryos (Table 5.1) and the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by 8- to 16-cell embryos (SOF and NeGln). Eight- to 16-cell embryos were transferred in their respective groups to the metabolic version of the medium in which they were cultured: 1) metSOF, or 2) metNeGln. Embryos were washed three times in the metabolic medium to ensure all pyruvate was removed before being transferred to the respective metabolic medium containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [5-^{3}H] glucose (at a concentration of 0.015 mM and specific activity of 0.25 μCi/μl). Thus, determinations of [2-^{14}C] pyruvate and [5-^{3}H] glucose metabolism were made for the same embryos. The metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by individual embryos was then correlated for both treatment groups to determine the relationship between [2-^{14}C] pyruvate and [5-^{3}H] glucose metabolism.

6.2.2 The Effect of Culture with Amino Acids on the Metabolism of the Cow Blastocyst and the Relationship between Metabolism and Development.

Presumptive zygotes (18 h pi) were cultured in NeGln for 72 h to Day 4 pi. Embryos were then divided into five groups with each group containing equivalent numbers of embryos less than the 8-cell stage and greater than or equal to the 8-cell stage. Each group of embryos was randomly transferred to one of five media: 1) SOF, 2) Gln, 3) NeGln, 4) EssGln, or 5) 20aa and cultured for a further 72 h to the blastocyst stage. Development was scored and blastocysts were transferred in their respective groups to the metabolic version of the medium in which they were cultured: 1) metSOF, 2) metGln, 3) metNeGln, 4) metEssGln, or 5) met20aa (see Appendix 10). Note that the metabolic versions of media were the same as culture media but did not contain pyruvate (pyruvate was subsequently added as radiolabelled substrate). Embryos were washed three times in the metabolic medium to ensure all pyruvate was removed before being transferred to the respective metabolic medium containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [5-^{3}H] glucose (at a concentration of 0.015 mM and specific activity of 0.25 μCi/μl).

Determinations of the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by blastocysts cultured with different amino acids were thus taken from the same embryos and were carried out as described in section 6.2.1. Immediately following the metabolic incubations, blastocysts were stained with Hoechst (Number 33258) to determine the total cell number of each embryo (section 2.7.2). Thus, metabolic determinations were recorded as both pmoles/embryo/h and fmoles/cell/h.

The metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by individual blastocysts was correlated for each treatment to determine the effect of amino acid groups on the relationship between the metabolism [2-^{14}C] pyruvate and [5-^{3}H] glucose. Further, the metabolism of both [2-^{14}C] pyruvate and [5-^{3}H] glucose by individual blastocysts was correlated with the total cell number of corresponding blastocysts for each treatment to determine the effect of amino acid groups on the relationship between blastocyst cell number and the metabolism of both [2-^{14}C] pyruvate and [5-^{3}H] glucose.

The development of embryos to the blastocyst stage, in media containing different amino acid groups (percentage of blastocysts formed from cleaved ova) was correlated with the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose. Development and metabolic determinations were from the same cohort of embryos to minimize variation.

6.2.3 The Effect of Culture with Amino Acids on the Intracellular pH of the 8- to 16-Cell Cow Embryo and the Relationship between Intracellular pH and Development.

This experiment was designed to determine the effect of culture of the cow embryo for the first 72 h, on the pHi of the 8- to 16-cell embryo and to examine the relationship between pHi and development.

Zygotes (18 h pi) were cultured as in section 6.2.1, in (1) SOF, (2) Gln, (3) NeGln, (4) EssGln or (5) 20aa, for 72 h. Embryo development in each medium was scored and the pHi of the 8- to 16-cell embryos was determined as described in section 2.10. Embryos were loaded with the fluorescent pH indicator SNARF-1 and then transferred to a HEPES-buffered version of each culture medium: 1) H-SOF, 2) H-Gln, 3) H-NeGln, 4) H-EssGln, or 5) H-20aa, pH 7.4, at 39^{0}C (see Appendix 8).

Measurements of the pHi of individual embryos were taken using a confocal microscope. Intracellular pH was calibrated to measurements recorded for embryos from the same culture that had been treated with the potassium ionophore nigericin, in buffer pH 6.9, 7.0, 7.1, 7.2, 7.3 and 7.4.

The development of embryos to the 8- to 16-cell stage in media containing different amino acid groups (percentage blastocyst from cleaved ova) was then correlated with pHi. Development to the 8- to 16-cell stage and metabolic determinations were from the same cohort of embryos to minimize variation.

6.2.4 Lactate Production in the Cow Embryo at the 8- to 16-cell Stage and the Blastocyst.

This experiment was carried out to determine whether glutamine contributes to lactate production in the 8- to 16-cell cow embryo and the blastocyst. The experiment was also designed to determine whether the presence of glutamine affects the production of lactate from glucose and to determine the extent to which pyruvate contributes to lactate production in the embryo.

Presumptive zygotes (18 h pi) were cultured for 72 h in NeGln. In the first series of cultures the resultant 8- to 16-cell embryos were transferred to one of five media: 1) metnil (SOF with no substrates), 2) metpyr (metnil with 0.5 mM pyruvate), 3) metglc (metnil with 0.5 mM glucose), 4) metgln (metnil with 0.5 mM glutamine), or 5) metglc/gln (metnil with 0.5 mM glucose and 0.5 mM glutamine). Media are defined in Appendix 13). In the second series of cultures, embryos were cultured for a further 72 h in 20aa and resultant blastocysts were divided equally among five morphologically similar groups containing mid and expanded blastocysts. Blastocyst

groups were then transferred to one of five media: 1) metnil, 2) metpyr, 3) metglc, 4) metgln, or 5) metglc/gln.

Blastocysts and 8- to 16-cell embryos were washed three times in their respective media before individual embryos were transferred to either a 400 nl (blastocyst) or 160 nl (8- to 16-cell) drop of the metabolic medium under oil, in the lid of a 35 mm culture dish, at 7% O_2, 5% CO_2, 88% N_2 at 39^{0}C. Lactate production by individual embryos from endogenous energy stores, from pyruvate, from glucose and from glutamine was then determined via the non-invasive technique of microfluorescence (see section 2.9.1). Lactate concentration was determined by measuring the generation of fluorescent NADH, following the conversion of lactate to pyruvate by lactate dehydrogenase. In order to determine the production of lactate from individual energy substrates, lactate production from endogenous stores in each replicate was subtracted from the total lactate production determined in each treatment medium.

6.2.5 Statistical Analysis

For experiments with more than two treatment groups, differences among treatment means for substrate metabolism, pHi and lactate production, were determined by an ANOVA, followed by the Tukey-Kramer Multiple Comparison Test. Bartlett's test was used to check for homogeneity of variances. When variances were found to be heterogenous, data were analysed by the Kruskal-Wallis non parametric test and differences between individual treatments were determined using Dunn's multiple comparison test. When substrate metabolism was compared for two treatment groups only, an unpaired, two-tailed t-test was used to analyse the statistical

difference between treatment means. When the degree of association between two variables was determined, the correlation coefficient (r) was calculated. A probability of $P < 0.05$ was considered to be significant for all statistical tests.

6.3 RESULTS

6.3.1 The Effect of Culture with Amino Acids on the Metabolism of the 8- to 16-cell Cow Embryo.

6.3.1.1 [2-^{14}C] pyruvate and [G-^{3}H] glutamine metabolism by individual embryos.

Culture with any of the amino acid groups during the first 72 h significantly reduced the metabolism of [2-^{14}C] pyruvate by 8- to 16-cell embryos when compared to culture without amino acids (Gln: 0.20 pmoles/embryo/h, NeGln: 0.34 pmoles/embryo/h, EssGln: 0.33 pmoles/embryo/h and 20aa: 0.43 pmoles/embryo/h versus SOF: 0.92 pmoles/embryo/h, $P < 0.01$; Figure 6.1a). Culture with glutamine alone (Gln) significantly decreased pyruvate metabolism with respect to culture with glutamine in the presence of the non-essential amino acids (NeGln, $P < 0.01$) and/or the essential amino acids (20aa, $P < 0.01$; EssGln, $P < 0.05$). Data in Figure 6.1a were graphed as notch whisker box plots, illustrating the median of each treatment group because the variances among treatments were heterogenous.

The metabolism of [G-^{3}H] glutamine was not quite significantly different amongst embryos that had been cultured for the first 72 h with different groups of amino acids (Gln versus NeGln versus EssGln versus 20aa, $P = 0.055$; Figure 6.1b). The metabolism of [G-^{3}H] glutamine was not determined for embryos that had been cultured for the first 72 h in the absence of amino acids (SOF). Data in Figure 6.1b were graphed as notch whisker box plots to show the median of each treatment group because the variances among treatments were heterogenous.

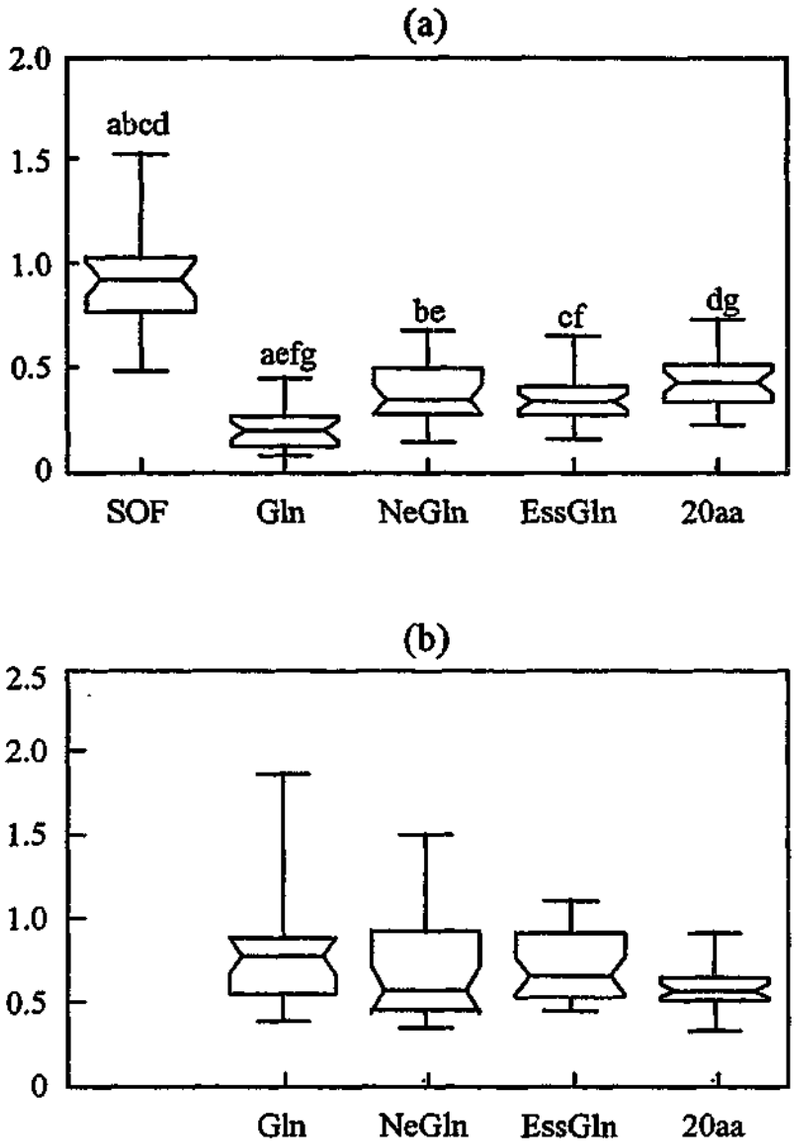


Figure 6.1 Metabolism of (a) [2-^{14}C] pyruvate and (b) [G-^{3}H] glutamine by 8- to 16-cell embryos following culture in SOF, Gln, NeGln, EssGln or 20a (a) and Gln, NeGln, EssGln or 20a (b) for 72 h from the zygote. Rates are in pmoles/embryo/h (pmoles/e/h). Media are defined in section 6.2.1. Metabolic determinations for [2-^{14}C] pyruvate and [G-^{3}H] glutamine metabolism were made concurrently from at least 24 embryos per treatment, over three replicates. Each box represents data between the 25th and the 75th percentile. Horizontal lines within each box indicate the median as variances were heterogenous. Vertical lines indicate the spread of each data set. $^{a\text{-}g}$Like pairs of letters are significantly different; $^{abcd}P < 0.01$, $^{efg}P < 0.05$.

There was no observed correlation between the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by individual 8- to 16-cell embryos in any of the treatment groups: Gln (r = 0.32, P > 0.05; Figure 6.2a), NeGln (r = 0.30, P > 0.05; Figure 6.2b), EssGln (r = 0.04, P > 0.05; Figure 6.2c), and 20aa (r = 0.11, P > 0.05; Figure 6.2d).

6.3.1.2 [5-^{3}H] glucose metabolism by individual embryos.

Culture with the non-essential amino acids and glutamine for the first 72 h significantly reduced [5-^{3}H] glucose metabolism by 8- to 16-cell embryos, with respect to culture without amino acids (0.8 ± 0.2 pmoles/embryo/h versus 1.5 ± 0.2 pmoles/embryo/h, respectively, P < 0.05; Figure 6.3). Culture with glutamine alone (Gln) or culture in the presence of the essential amino acids (EssGln and 20aa) reduced glycolytic activity compared with culture without amino acids, however, the reduction was not significant (1.1 ± 0.2, 1.1 ± 0.1 and 1.0 ± 0.1 pmoles/embryo/h versus 1.5 ± 0.2 pmoles/embryo/h, respectively, P > 0.05).

6.3.1.3 The relationship between the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate by individual embryos.

With respect to culture in the absence of amino acids (SOF), culture in the presence of the non-essential amino acids (NeGln) to Day 4 pi significantly reduced the metabolism of [5-^{3}H] glucose (1.7 ± 0.2 and 1.0 ± 0.2, respectively, P < 0.01; Figure 6.4a) and [2-^{14}C] pyruvate (0.35 ± 0.03 and 0.18 ± 0.02, respectively, P < 0.001; Figure 6.4a) by embryos at the 8- to 16-cell stage. While the relationship between the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate by embryos appeared to be positive for embryos cultured in SOF and negative for embryos cultured in

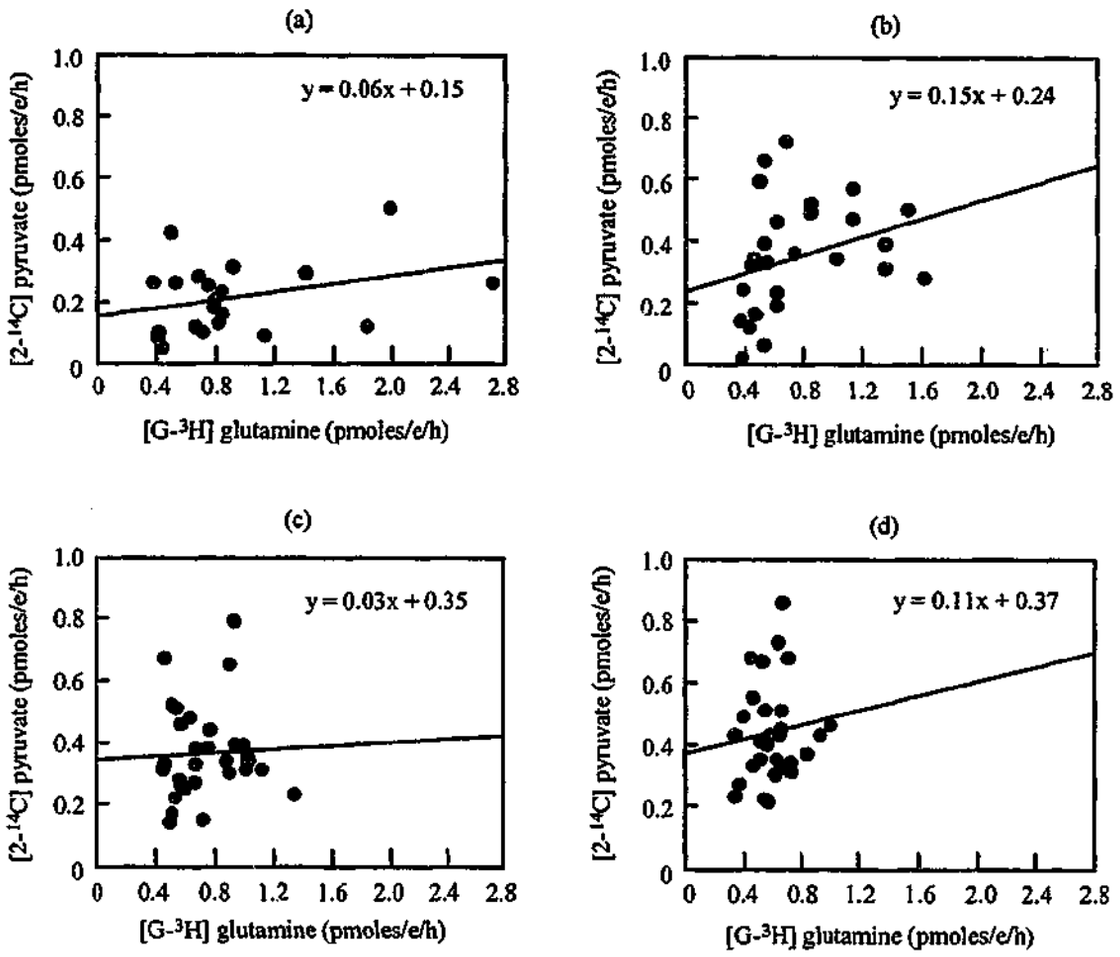


Figure 6.2 Correlations of the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by cow embryos at the 8- to 16-cell stage following culture to Day 4 pi in: a) Gln (r = 0.32, P > 0.05), b) NeGln (r = 0.30, P > 0.05), c) EssGln (r = 0.04, P > 0.05), and d) 20aa (r = 0.11, P > 0.05). Metabolic rates are pmoles/embryo/h (pmoles/e/h). Media are defined in section 6.2.1. Data are from three replicates.

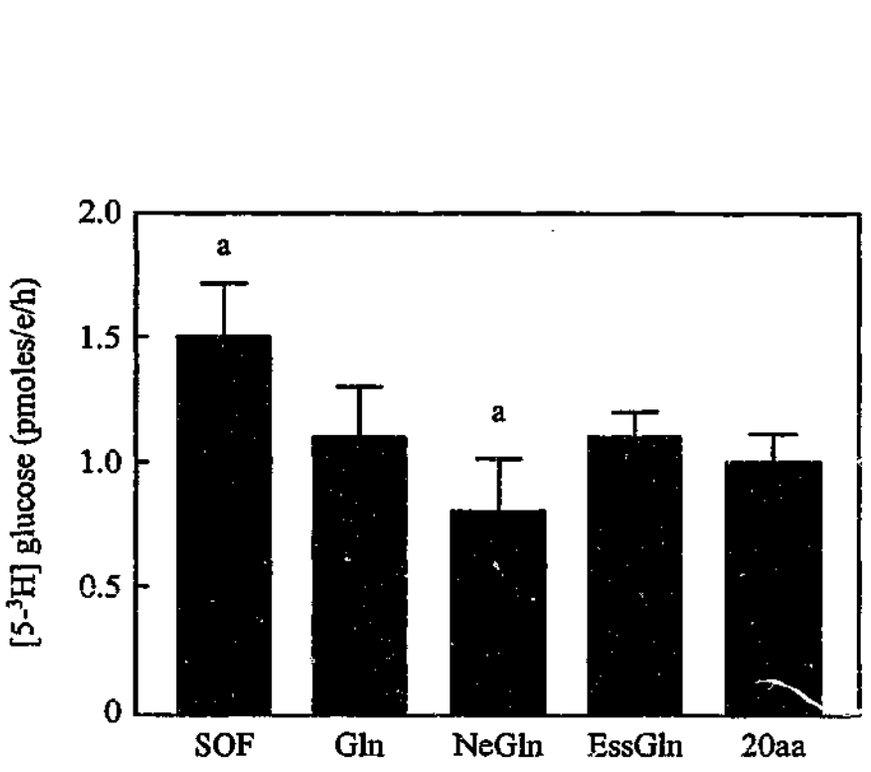


Figure 6.3 Metabolism of [5-^{3}H] glucose by 8- to 16-cell embryos following culture in SOF, Gln, NeGln, EssGln and 20aa for 72 h from the zygote. Media are defined in section 6.2.1. Metabolic rates are mean ± SEM in pmoles/embryo/h (pmoles/e/h). Data are from four replicates; n ≥ 25 embryos per treatment. [a]Like pairs of letters are significantly different; $P < 0.05$.

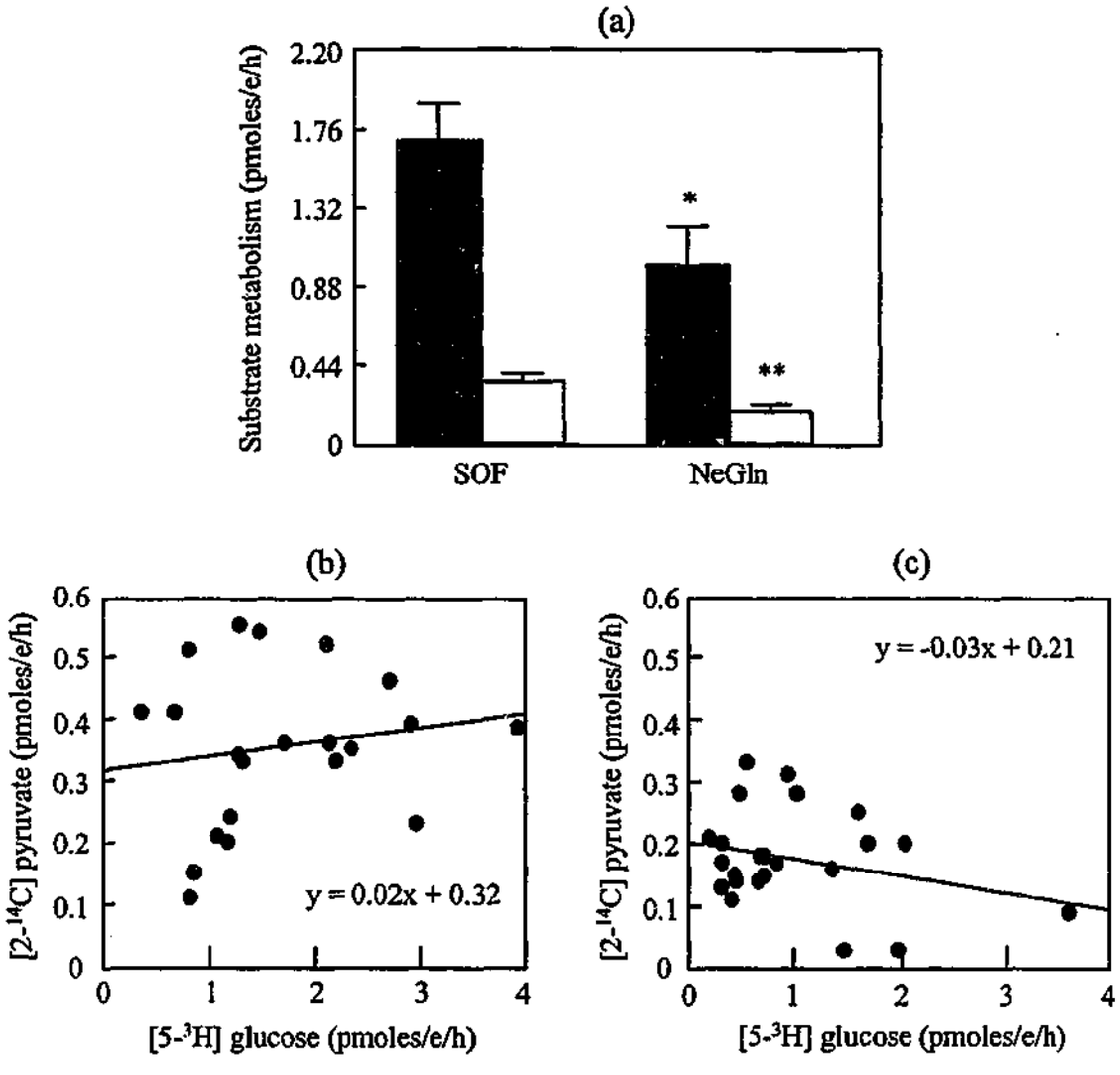


Figure 6.4 (a) metabolism of [5-^{3}H] glucose (solid bars) and [2-^{14}C] pyruvate (open bars) by 8- to 16-cell embryos following culture in SOF and NeGln for 72 h from the zygote. Metabolic rates are mean ± SEM in pmoles/embryo/h (pmoles/e/h). NeGln significantly different to SOF for same substrate (*$P < 0.01$, **$P < 0.001$). (b-c) correlations of the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate by individual 8- to 16-cell embryos following culture in (b) SOF ($r = 0.16$, $P > 0.05$) and (c) NeGln ($r = -0.29$, $P > 0.05$) for 72 h from the zygote. Each point represents one embryo. (a-c) data are from three replicates; $n \geq 21$ embryos per treatment.

NeGln, correlations of substrate metabolism were not significant following culture in either SOF (r = 0.16, P > 0.05; Figure 6.4b) or NeGln (r = -0.29, P > 0.05; Figure 6.4c).

6.3.1.4 The relationship between embryo metabolism and development.

There was a significant negative correlation between [5-^{3}H] glucose metabolism by 8- to 16-cell embryos and development to the 8- 16-cell stage, following culture for the first 72 h with different amino acid groups (r = -0.77, P < 0.01; Figure 6.5a). Similarly, there was a negative correlation with [2-^{14}C] pyruvate metabolism and development to the 8- 16-cell stage, following culture for the first 72 h with different amino acid groups (r = -0.71, P < 0.01; Figure 6.5b). The degree of association, however, between pyruvate metabolism and development was largely due to poor development and a high pyruvate metabolism by embryos cultured in the absence of any amino acids. When development and metabolism were correlated for only embryos cultured in the presence of amino acids, there was no significant relationship between the two parameters (r = 0.29, P > 0.05). Similarly, there was no relationship between the metabolism of [G-^{3}H] glutamine by 8- to 16-cell embryos and development to the 8- to 16-cell stage, following culture with different groups of amino acids (r = -0.25, P > 0.05; Figure 6.5c). The observed correlation between glucose metabolism and development however, was not dependent on the metabolism and development of embryos cultured in the absence of amino acids (r = -0.71, P < 0.01; excluding SOF treatment group).

The mean developments of embryos to the 8- to 16-cell stage in SOF, Gln,

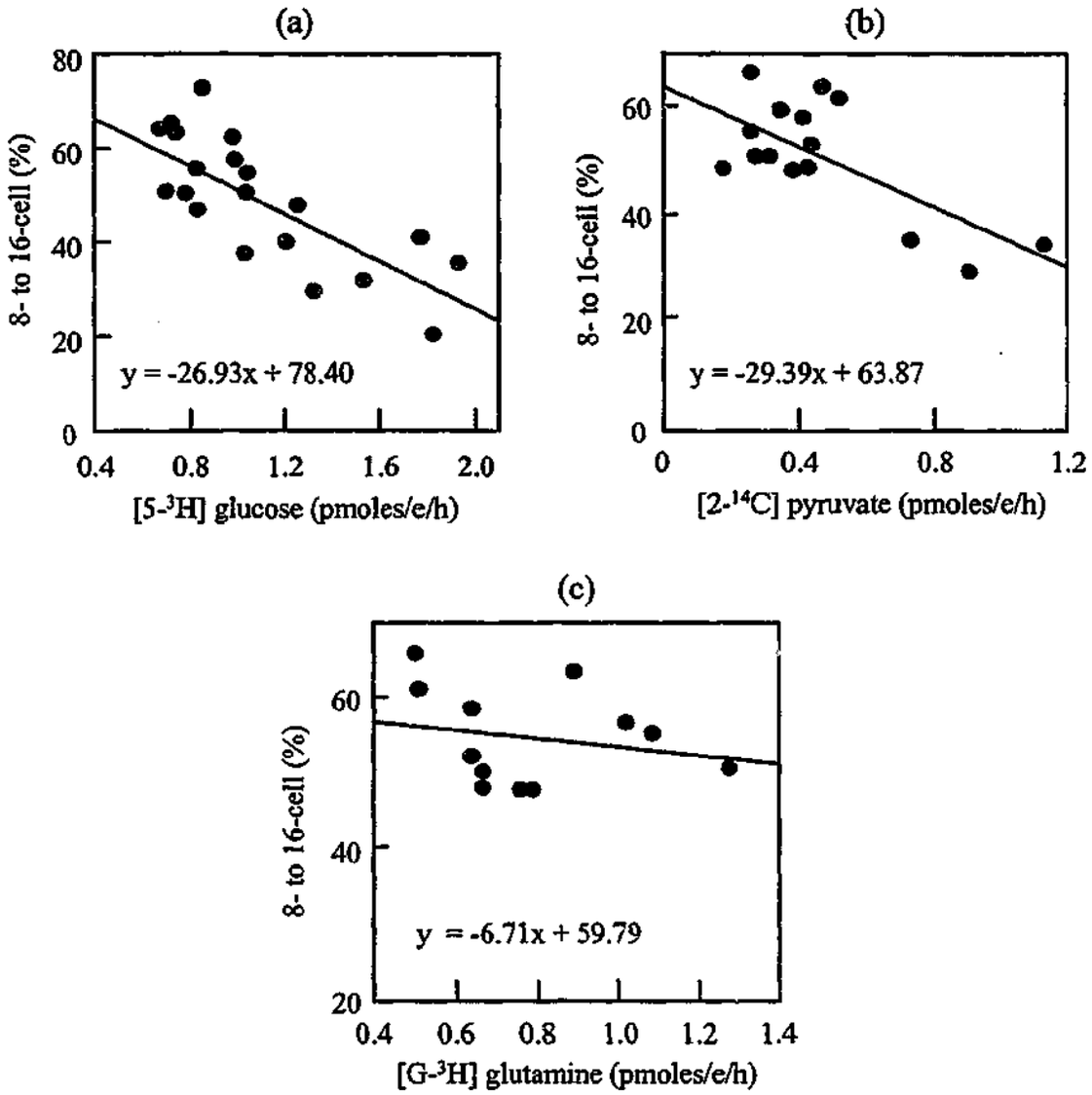


Figure 6.5 The correlation of percentage development to the 8- to 16-cell stage from cleaved ova and the mean metabolism of (a) [5-^{3}H] glucose ($r = -0.77$, $P < 0.01$); (b) [2-^{14}C] pyruvate ($r = -0.75$, $P < 0.01$); and (c) [G-^{3}H] glutamine ($r = -0.25$, $P > 0.05$), by 8- to 16-cell embryos following culture in SOF, Gln, NeGln, EssGln and 20aa (a and b) or culture in Gln, NeGln, EssGln and 20aa (c). Metabolic rates are pmoles/embryo/h (pmoles/e/h). Metabolic determinations and developmental data were from the same cohort of embryos. (a) data are from four replicates. (b) and (c) data are from three replicates.

NeGln, EssGln and 20aa for the determination of [5-^{3}H] glucose metabolism were 32.0%, 48.9%, 64.1%, 40.6% and 57.0%, respectively. The mean developments of embryos to the 8- to 16-cell stage in SOF, Gln, NeGln, EssGln and 20aa for the determination of [2-^{14}C] pyruvate metabolism and [G-^{3}H] glutamine metabolism were 35.8%, 51.0%. 61.6%, 48.4% and 57.1%, respectively.

6.3.2 The Effect of Culture with Amino Acids on the Metabolism of the Cow Blastocyst.

6.3.2.1 [5-^{3}H] glucose and [2-^{14}C] pyruvate metabolism by individual blastocysts.

The metabolism of [5-^{3}H] glucose by blastocysts was significantly higher following culture of embryos from Day 4 pi to Day 7 pi with 20aa, compared with embryos cultured in SOF, Gln, NeGln and EssGln (Table 6.1, $P < 0.05$). [5-^{3}H] glucose metabolism by blastocysts cultured in Gln, NeGln or EssGln was not significantly different to that of blastocysts cultured without amino acids (SOF, $P > 0.05$). Culture of embryos with the non-essential or the essential amino acids significantly increased glucose metabolism, compared with culture with glutamine alone (NeGln or EssGln versus Gln, $P < 0.05$). Due to the fact that culture with amino acids during the second 72 h affected the total cell number of resultant blastocysts (Figure 5.2), substrate metabolism was determined per cell as well as per embryo. On a per cell basis, there was no difference in [5-^{3}H] glucose metabolism by embryos cultured in SOF, NeGln, EssGln or 20aa ($P > 0.05$, Table 6.1). The metabolism of [5-^{3}H] glucose per cell, however, was significantly lower following culture with

Table 6.1 The metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate by cow blastocysts following culture with different amino acids during the final 72 h culture.

Media* 1st 72 h	Media** 2nd 72 h	[5-^{3}H] glucose (pmoles/embryo/h)†	[5-^{3}H] glucose (fmoles/cell/h)†	[2-^{14}C] pyruvate (pmoles/embryo/h)†	[2-^{14}C] pyruvate (fmoles/cell/h)†
NeGln	SOF	8.2 ± 0.7^{d}	149.3 ± 11.2^{a}	1.6 ± 0.1^{a}	27.9 ± 1.6^{ab}
NeGln	Gln	6.1 ± 0.6^{abc}	97.4 ± 5.8^{abcd}	1.2 ± 0.1^{b}	19.9 ± 1.2^{a}
NeGln	NeGln	9.4 ± 0.6^{b}	145.3 ± 11.0^{b}	0.6 ± 0.1^{abc}	8.2 ± 0.7^{ab}
NeGln	EssGln	9.7 ± 1.0^{c}	167.2 ± 17.6^{c}	1.1 ± 0.1^{a}	18.7 ± 1.9^{b}
NeGln	20aa	13.9 ± 0.9^{abcd}	158.9 ± 12.8^{d}	1.2 ± 0.1^{c}	13.1 ± 0.8^{ab}

*Media for culture 0-72 h (0 = 18-20 h pi). **Media for culture 72-144 h. Media are defined in section 6.2.1.

[a-d]Like pairs of letters are significantly different within a column, $P < 0.05$.

[†]Each value represents the mean ± SEM of data collected over three replicates, with at least 22 embryos per mean.

glutamine alone (Gln), compared with culture in SOF, NeGln, EssGln and 20aa ($P < 0.05$).

Culture in 20aa for the second 72 h did not affect the metabolism of [2-^{14}C] pyruvate by resultant blastocysts compared with culture without amino acids ($P > 0.05$, Table 6.1). [2-^{14}C] pyruvate metabolism was significantly lower following culture in NeGln compared with culture in SOF, Gln, EssGln and 20aa ($P > 0.05$). [2-^{14}C] pyruvate metabolism per cell was also significantly lower after culture in NeGln compared to culture with all other treatment groups ($P < 0.05$, Table 6.1). The addition of the non-essential amino acids to medium containing the essential amino acids and/or glutamine significantly lowered [2-^{14}C] pyruvate metabolism per cell (20aa versus EssGln and Gln ($P < 0.05$). [2-^{14}C] pyruvate metabolism per cell was significantly lower following culture in the absence of amino acids (SOF) compared with culture in the presence of amino acids (SOF versus Gln, NeGln, EssGln and 20aa, $P < 0.05$).

6.3.2.2 The relationship between [5-^{3}H] glucose and [2-^{14}C] pyruvate metabolism and blastocyst total cell number.

There was a significant correlation between [5-^{3}H] glucose metabolism and blastocyst total cell number following culture in SOF ($r = 0.53$, $P < 0.05$; Figure 6.6a), Gln ($r = 0.75$, $P < 0.01$; Figure 6.6b), EssGln ($r = 0.63$, $P < 0.01$; Figure 6.6d) and 20aa ($r = 0.47$, $P < 0.01$; Figure 6.6e). There was no correlation between [5-^{3}H] glucose metabolism and blastocyst total cell number, however, following culture in NeGln ($r = 0.33$, $P > 0.05$; Figure 6.6c). The addition of the non-essential amino acids

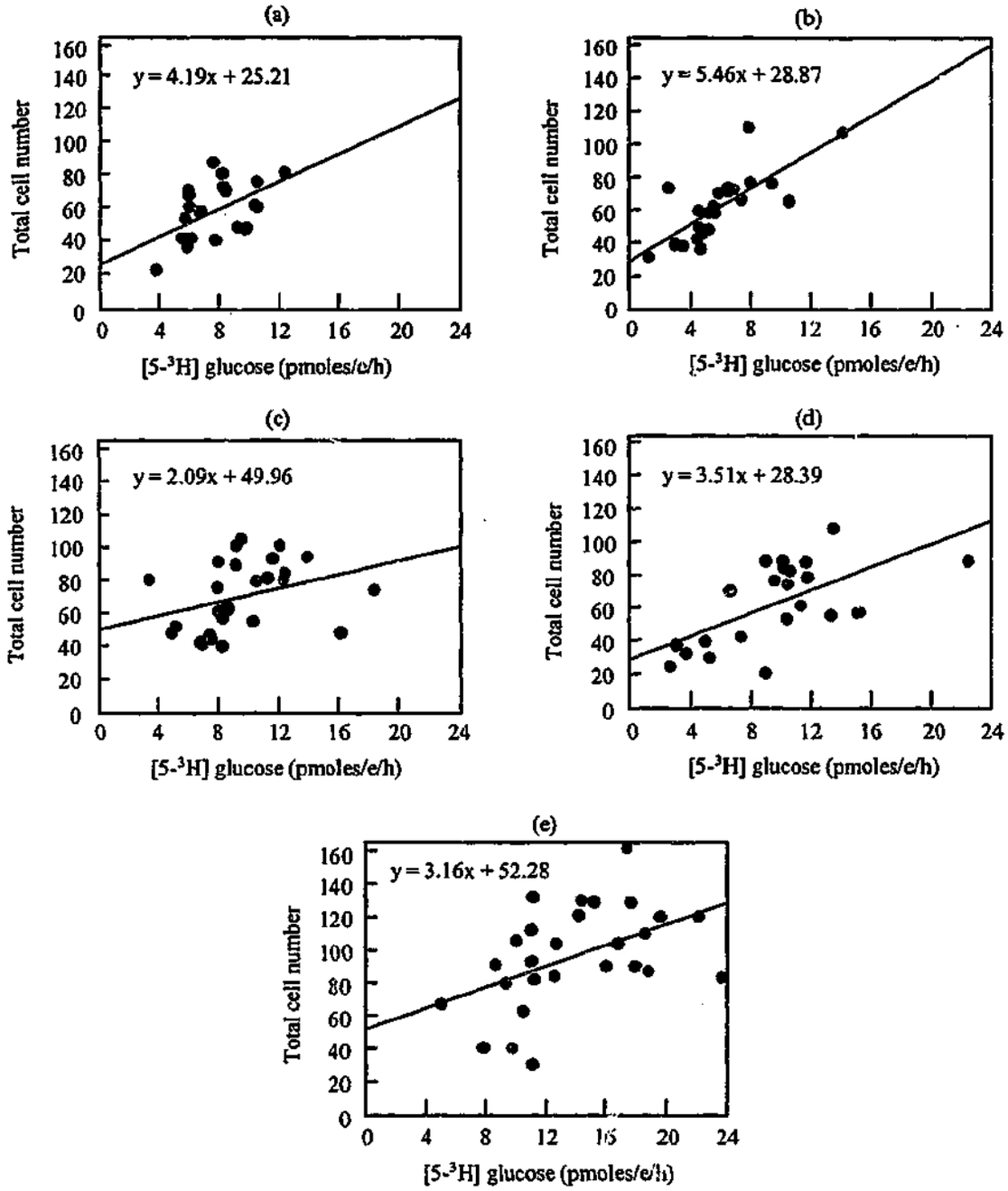


Figure 6.6 Correlations of blastocyst total cell number and the metabolism of [5-^{3}H] glucose by cow blastocysts following culture to Day 4 pi in NeGln followed by culture to Day 7 pi in (a) SOF ($r = 0.53$, $P < 0.05$); (b) Gln ($r = 0.75$, $P < 0.01$); (c) NeGln ($r = 0.33$, $P > 0.05$); (d) EssGln ($r = 0.63$, $P < 0.01$); and (e) 20aa ($r = 0.47$, $P < 0.01$). Media are defined in section 6.2.3. Metabolic rates are pmoles/embryo/h (pmoles/e/h). Data are from three replicates. Each point represents the metabolism of one embryo.

to media containing glutamine, decreased the degree of the relationship between [5-^{3}H] glucose metabolism and blastocyst total cell number, as evidenced by the level of significance of the correlation coefficients (Gln, $P < 0.01$ versus NeGln, $P > 0.05$). The greatest increase in [5-^{3}H] glucose metabolism with an increase in cell number, followed culture with glutamine alone (Gln), as evidenced by the slopes of the lines of best fit for each data set (4.19, 5.46, 3.51 and 3.16 for SOF, Gln, EssGln and 20aa, respectively; Figure 6.6a,b,d and e).

There was a significant correlation between [2-^{14}C] pyruvate metabolism and blastocyst total cell number following culture in SOF ($r = 0.57$, $P < 0.01$; Figure 6.7a), Gln ($r = 0.74$, $P < 0.01$; Figure 6.7b), EssGln ($r = 0.79$, $P < 0.01$; Figure 6.7d) and 20aa ($r = 0.67$, $P < 0.01$; Figure 6.7e), but not following culture in NeGln ($r = 0.35$, $P > 0.05$; Figure 6.7c). The increase in [2-^{14}C] pyruvate metabolism with an increase in blastocyst cell number was highest following culture in the absence of amino acids (SOF) or with glutamine alone (Gln) and lowest following culture in EssGln and 20aa, as evidenced by the slopes of the lines of best fit for each data set (21.6, 29.1, 43.8 and 59.4 for SOF, Gln, EssGln and 20aa, respectively; Figure 6.7a,b,d and e).

6.3.2.3 The relationship between [5-^{3}H] glucose and [2-^{14}C] pyruvate metabolism by individual blastocysts.

There was a significant correlation between the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate by blastocysts following culture in SOF ($r = 0.58$, $P < 0.01$; Figure 6.8a), Gln ($r = 0.83$, $P < 0.01$; Figure 6.8b), NeGln ($r = 0.45$, $P < 0.05$; Figure 6.8c), EssGln ($r = 0.63$, $P < 0.01$; Figure 6.8d) and 20aa ($r = 0.68$, $P < 0.01$; Figure

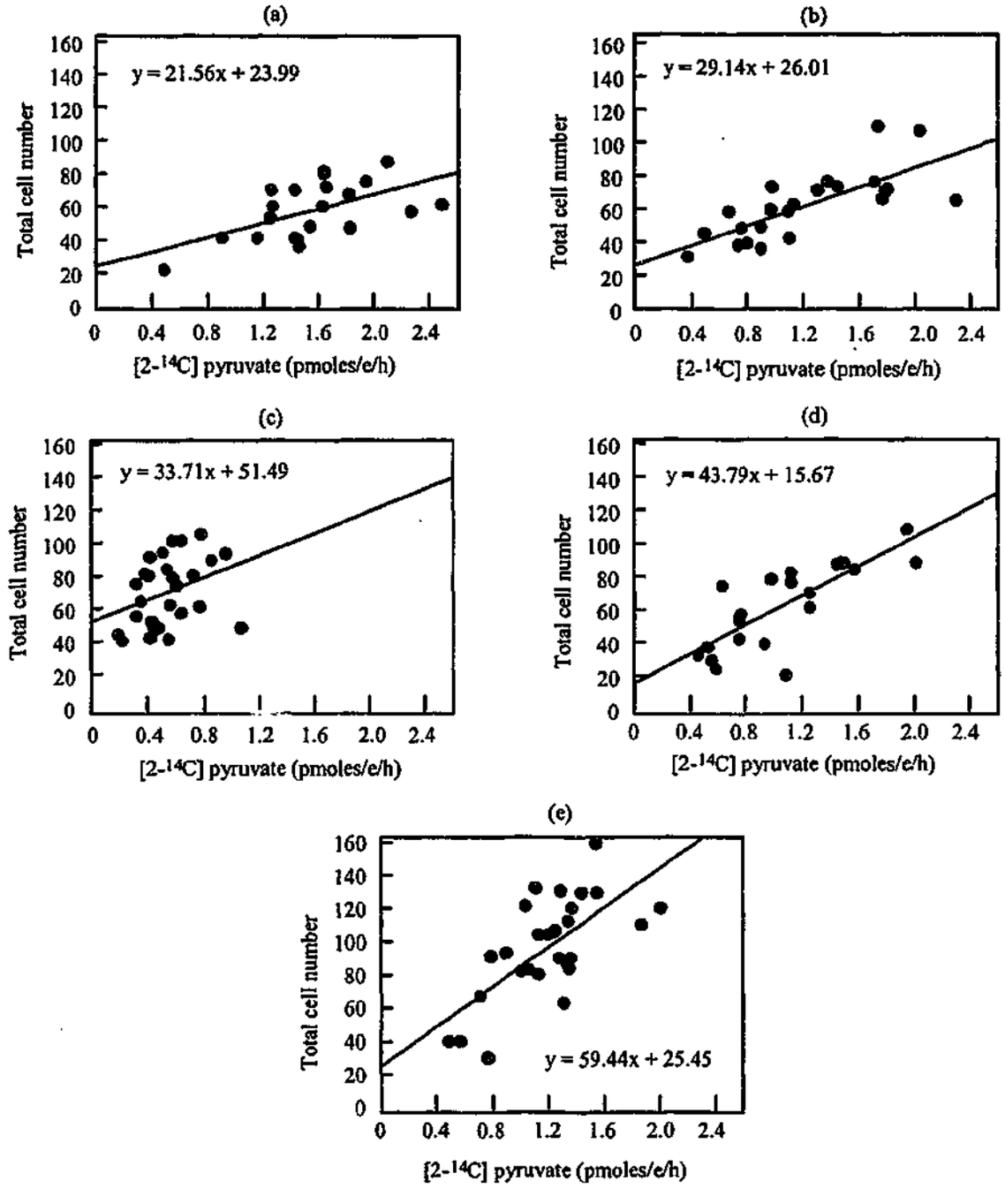


Figure 6.7 Correlations of blastocyst total cell number and the metabolism of [2-^{14}C] pyruvate by cow blastocysts following culture to Day 4 pi in NeGln followed by culture to Day 7 pi in (a) SOF ($r = 0.57$, $P < 0.01$); (b) Gln ($r = 0.74$, $P < 0.01$); (c) NeGln ($r = 0.35$, $P > 0.05$); (d) EssGln ($r = 0.79$, $P < 0.01$); and (e) 20aa ($r = 0.67$, $P < 0.01$). Media are defined in section 6.2.3. Metabolic rates are pmoles/embryo/h (pmoles/e/h). Data are from three replicates. Each point represents the metabolism of one embryo.

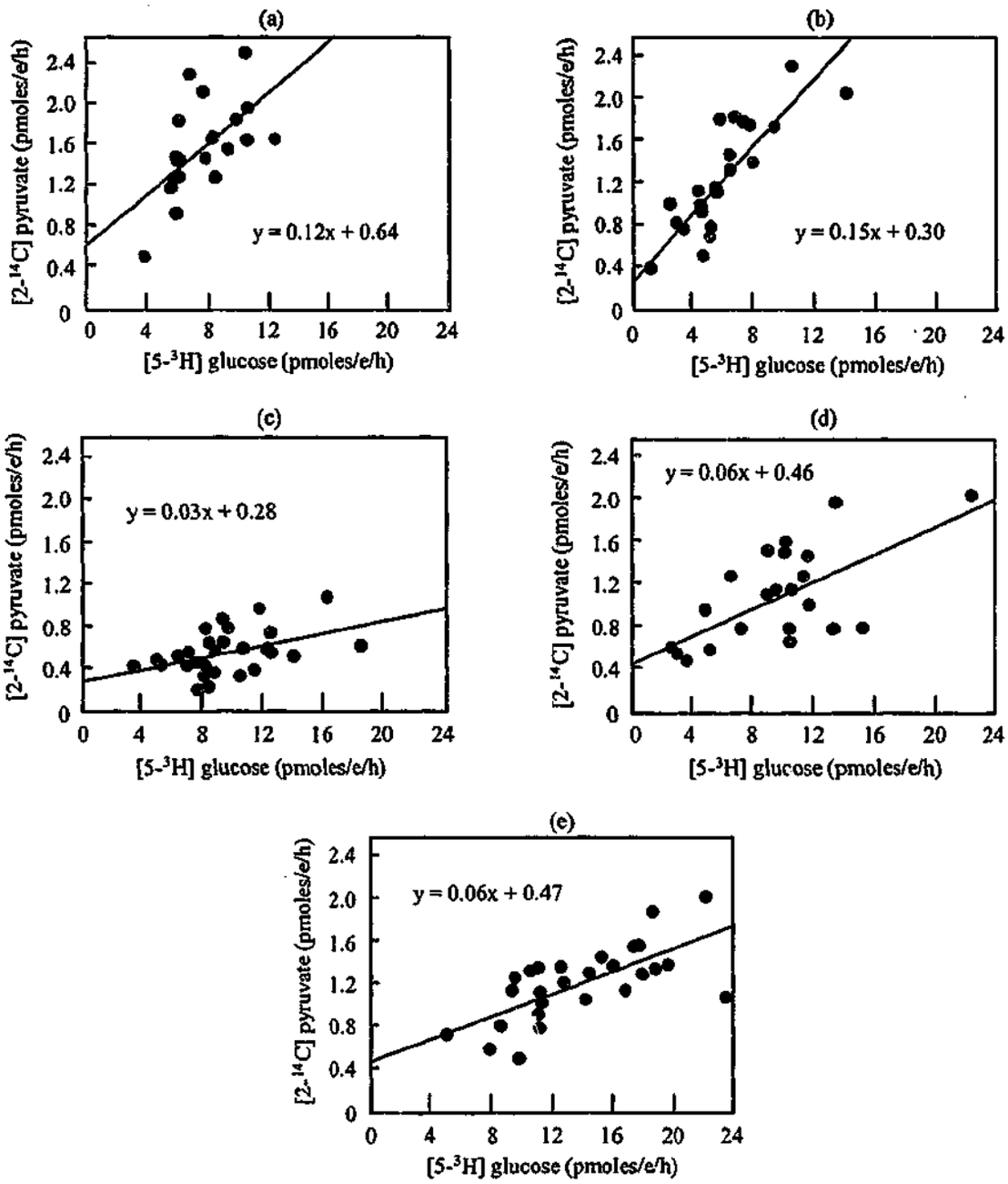


Figure 6.8 Correlations of the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by cow blastocysts following culture to Day 4 pi in NeGln followed by culture to Day 7 pi in (a) SOF ($r = 0.58$, $P = 0.01$); (b) Gln ($r = 0.83$, $P < 0.01$); (c) NeGln ($r = 0.45$, $P < 0.05$); (d) EssGln ($r = 0.63$, $P < 0.01$); and (e) 20aa ($r = 0.68$, $P < 0.01$). Media are defined in section 6.2.3. Metabolic rates are pmoles/embryo/h (pmoles/e/h). Data are from three replicates. Each point represents the metabolism of one embryo.

6.8e). The strongest relationships between the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate were for blastocysts cultured in SOF, Gln, EssGln and 20aa, as evidenced by the level of significance of the correlation coefficients above.

Culture in NeGln, EssGln and 20aa changed the relationship between the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate, with respect to culture in SOF or Gln, as evidenced by the slopes of the lines of best fit for each data set (0.12, 0.15, 0.03, 0.06 and 0.06, for SOF, Gln, NeGln, EssGln and 20aa, respectively; Figure 6.8a, b,c,d and e). Calculations of X and Y coordinates from each line of best fit revealed the ratios of the metabolism of [2-^{14}C] pyruvate:[5-^{3}H] glucose to be 0.24:1 (SOF), 0.27:1 (Gln), 0.09:1 (NeGln), 0.1:1 (EssGln) and 0.09:1 (20aa).

6.3.2.4 The relationship between embryo metabolism and development.

There was a significant correlation between the metabolism of [5-^{3}H] glucose by blastocysts and the percentage of embryos developing to the blastocyst stage following culture in SOF, Gln, NeGln, EssGln and 20aa (r = 0.55, $P < 0.05$; Figure 6.9a). The correlation between [2-^{14}C] pyruvate metabolism by blastocysts and blastocyst development, however, was not significant (r = -0.23, $P > 0.05$; Figure 6.9b).

Culture with different amino acid groups was found to affect blastocyst cell number (Figure 5.2), thus the cell number of each blastocyst was recorded following each metabolic determination. After correction of metabolic rates for blastocyst cell number, there was no observed relationship between [5-^{3}H] glucose metabolism and development, following culture in SOF, Gln, NeGln, EssGln or 20aa (r = -0.09, $P > 0.05$; Figure 6.9c). There was, however, a significant negative correlation between the

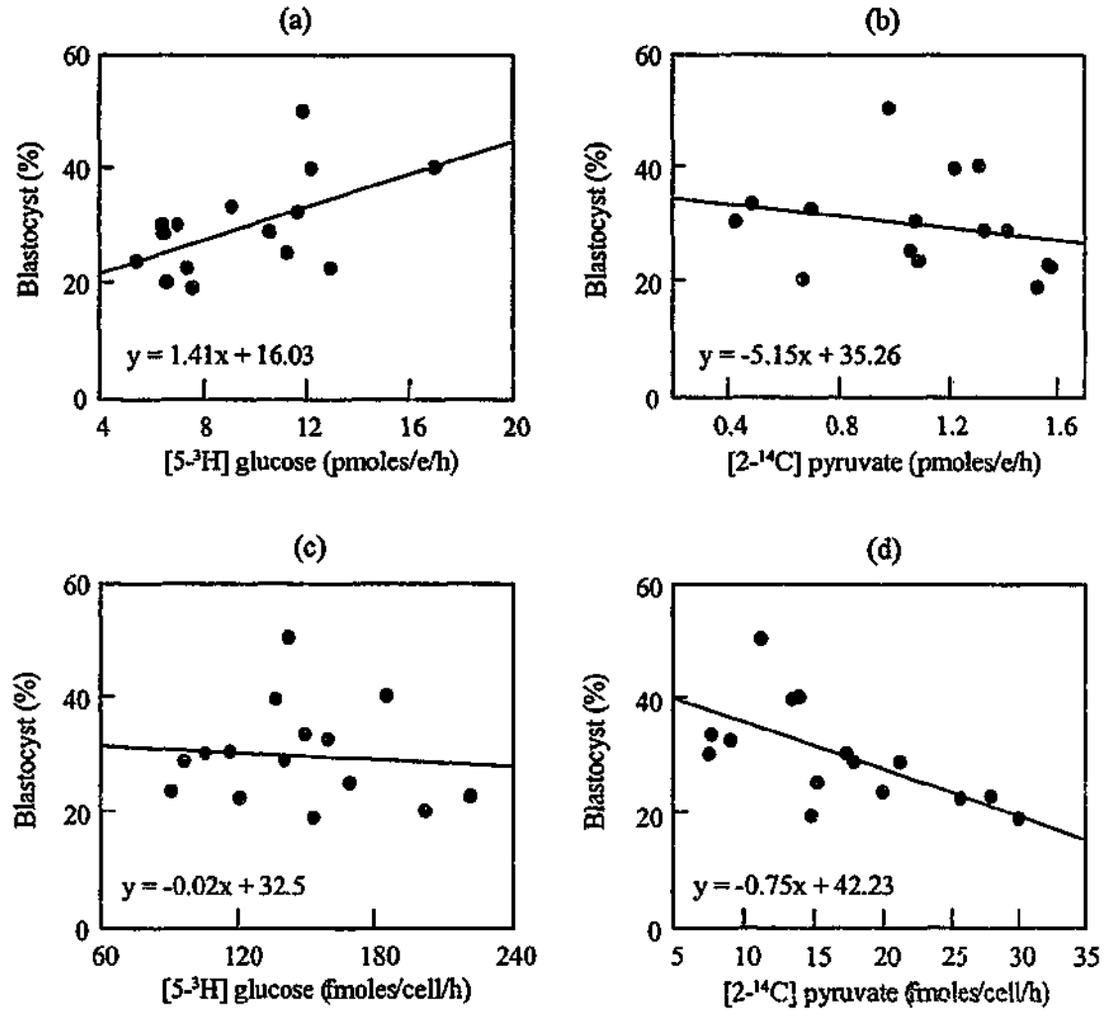


Figure 6.9 The correlation of percentage development to the blastocyst stage from cleaved ova and the mean metabolism of (a) [5-^{3}H] glucose per embryo (r = 0.55, P = 0.05); (b) [2-^{14}C] pyruvate per embryo (r = -0.23, P > 0.05); (c) [5-^{3}H] glucose per cell per hour (r = -0.09, P > 0.05); and (d) [2-^{14}C] pyruvate per cell per hour (r = -0.70, P < 0.01) following culture in SOF, Gln, NeGln, EssGln or 20aa. Metabolic rates for whole blastocysts were pmoles/embryo/h (pmoles/e/h). Metabolic determinations and developmental data were from the same cohort of embryos for each correlation. Metabolic determinations for [5-^{3}H] glucose and [2-^{14}C] pyruvate metabolism were made concurrently and were from three replicates.

metabolism of [2-^{14}C] pyruvate per cell and blastocyst development (r = -0.70, P < 0.01; Figure 6.9d).

The mean developments of embryos to the blastocyst stage in SOF, Gln, NeGln, EssGln and 20aa for the determination of [5-^{3}H] glucose metabolism and [2-^{14}C] pyruvate metabolism were 21.2%, 27.3%, 30.2%, 26.2% and 43.2%, respectively. The mean cell numbers of blastocysts used for metabolic determinations were 57.6 ± 3.6, 61.9 ± 4.2, 69.9 ± 4.0, 62.4 ± 5.3 and 96.3 ± 6.0, respectively for SOF, Gln, NeGln, EssGln and 20aa.

6.3.3 The Effect of Culture with Amino Acids on the Intracellular pH of the 8- to 16-Cell Cow Embryo and the Relationship between Intracellular pH and Development.

6.3.3.1 Intracellular pH.

The pHi of 8- to 16-cell embryos was significantly lower following culture to Day 4 pi in Gln or NeGln, compared with culture in the absence of amino acids (Gln: 7.27 ± 0.03 versus SOF: 7.37 ± 0.02, P < 0.05 and NeGln: 7.20 ± 0.02 versus SOF, P < 0.01; Figure 6.10). Culture in EssGln did not affect the pHi of the 8- to 16-cell embryo (EssGln: 7.29 ± 0.02 versus SOF, P > 0.05). The presence of the essential amino acids negated the effect of the non-essential amino acids on pHi (20aa: 7.30 ± 0.02 versus NeGln, P < 0.05; and 20aa versus SOF, P > 0.05).

6.3.3.2 The relationship between intracellular pH and development.

There was a significant negative correlation between the pHi of 8- to 16-cell embryos and development to the 8- to 16-cell stage, following culture in SOF, Gln,

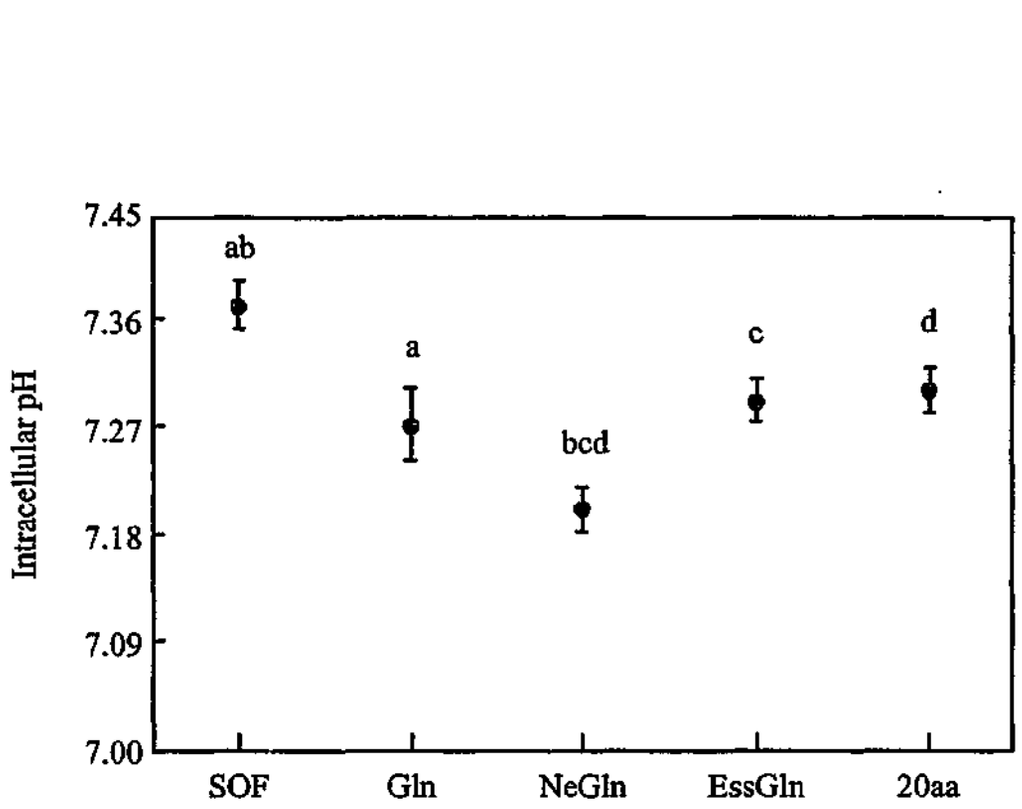


Figure 6.10 Intracellular pH of 8- to 16-cell cow embryos, following culture in SOF, Gln, NeGln, EssGln or 20aa for 72 h from the zygote. Like pairs of letters are significantly different: a,c, $P < 0.05$; b,d, $P < 0.01$. $n \geq 21$ embryos per treatment group. Values are mean ± SEM from three replicates.

NeGln, EssGln and 20aa (r = -0.61, P < 0.01; Figure 6.11). The mean developments of embryos to the 8- to 16-cell stage (from cleaved ova) for the determination of pHi were as follows: SOF 29.8%, Gln 40.9%, NeGln 63.9%, EssGln 40.6% and 20aa 56.5%.

6.3.4 Lactate Production in the Cow Embryo, at the 8- to 16-Cell Stage and the Blastocyst.

6.3.4.1 The 8- to 16-cell stage.

At the 8- to 16-cell stage, lactate production from glutamine was low (Table 6.2; metgln: 1.7 ± 0.2 pmoles/embryo/h) and was not significantly different to the amount of lactate produced from endogenous energy stores (metnil: 1.2 ± 0.1 pmoles/embryo/h, P > 0.05). The production of lactate in medium containing glucose or pyruvate, however, was significantly higher than in medium containing no energy substrates (metpyr and met glc versus netnil, P < 0.05). Lactate production by the 8- to 16-cell embryo was significantly greater from pyruvate than from glucose (6.7 ± 0.3 versus 2.9 ± 0.4 pmoles/embryo/h, respectively, P < 0.05; Table 6.2). The presence of glutamine did not affect the production of lactate from glucose (metglc/gln: 2.8 ± 0.3 pmoles/embryo/h versus metglc: 2.9 ± 0.4 pmoles/embryo/h, P > 0.05).

6.3.4.2 The blastocyst.

The production of lactate from glutamine by the blastocyst was low (metgln: 2.2 ± 0.3 pmoles/embryo/h; Table 6.2) and was not significantly different to the amount

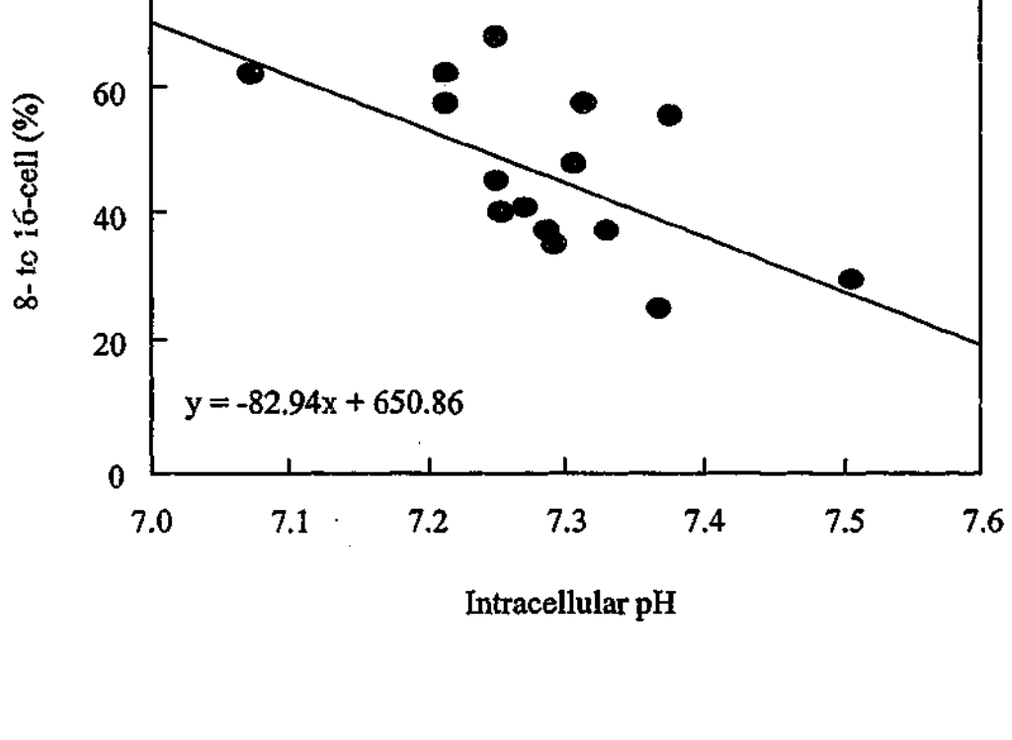


Figure 6.11 Correlation of the intracellular pH of 8- to 16-cell cow embryos cultured with different amino acids (SOF, Gln, NeGln, EssGln or 20aa) for 72 h and development to the 8- to 16-cell stage ($r = -0.61$, $P < 0.01$). Values represent means from three replicates, with determinations of pHi and development taken from the same cohort of embryos.

Table 6.2 The production of lactate from endogenous and exogenous energy substrates in the 8- to 16-cell cow embryo and the blastocyst.

	8- to 16-cell		Blastocyst	
Media[†]	Lactate production[A] (pmoles/embryo/h)	Lactate production[B] (pmoles/embryo/h)	Lactate production[A] (pmoles/embryo/h)	Lactate production[B] (pmoles/embryo/h)
metnil	1.2 ± 0.1^{ab}	-	4.9 ± 0.5^{ab}	-
metpyr	6.7 ± 0.3^{abc}	5.5 ± 0.3^{abc}	12.1 ± 0.9^{cd}	7.2 ± 0.9^{ab}
metglc	2.9 ± 0.4^{a}	1.7 ± 0.3^{a}	42.8 ± 4.2^{ace}	37.9 ± 4.2^{ac}
metgln	1.7 ± 0.2^{c}	0.6 ± 0.2^{b}	2.2 ± 0.3^{ef}	-2.8 ± 0.3^{cd}
metglc/gln	2.8 ± 0.3^{b}	1.6 ± 0.3^{c}	42.9 ± 5.9^{hdf}	38.0 ± 5.9^{bd}

[†]Media for determination of lactate production from specific energy substrates (see Appendix 13).

[A]Total lactate production. Values are mean ± SEM of at least 12 embryos from three replicates.

[B]Lactate production minus the lactate produced from endogenous substrates (metnil). Values are mean ± SEM of at least 12 embryos from three replicates.

[a-f]Like pairs of letters are significantly different, $P < 0.05$.

of lactate produced from endogenous stores (metnil: 4.9 ± 0.5 pmoles/embryo/h, $P > 0.05$).

In contrast to the 8- to 16-cell stage, the majority of lactate produced by the blastocyst was from glucose and not pyruvate (42.8 ± 4.2 pmoles/embryo/h versus 12.1 ± 0.9 pmoles/embryo/h, respectively, $P < 0.05$). The production of lactate in medium containing pyruvate was in fact not significantly different to that produced in medium without energy substrates (metpyr: 12.1 ± 0.9 pmoles/embryo/h versus metnil: 4.9 ± 0.5 pmoles/embryo/h, $P > 0.05$). Lactate production in metglc and metglc/gln was equivalent (42.8 ± 4.2 pmoles/embryo/h and 42.9 ± 5.9 pmoles/embryo/h, respectively, $P > 0.05$), indicating that the presence of glutamine did not affect the production of lactate from glucose.

6.4 DISCUSSION

Amino acids are known to have a beneficial effect on development of the cow embryo in vitro, however, the cellular mechanisms behind their action have not previously been determined. The present experiments revealed that amino acids had a significant effect on the activities of metabolic pathways and pHi within the embryo and that such changes were associated with embryo development.

Embryos cultured in the presence of the non-essential amino acids and glutamine had a reduced glycolytic activity at the 8- to 16-cell stage, compared with embryos cultured in the absence of amino acids. In contrast, culture with glutamine alone or the essential amino acids and glutamine, did not significantly alter glycolytic activity. The effect of Eagle's non-essential amino acids and glutamine on the glycolytic activity of the early cleavage stage embryo, has also been reported in CF1 mice at the 2-cell stage (Gardner and Lane, 1993b) and F1 mice at the 4- to 8-cell stages (Vella *et al.*, 1997). Importantly, the glycolytic activity of cow embryos cultured to the 8- to 16-cell stage in the absence of amino acids and with different amino acid groups was negatively correlated with development to the 8- to 16-cell stage. This indicates that a lowered glycolytic activity in the cleavage stage cow embryo, is conducive to an improved developmental capacity. This phenomenon has also been reported in the mouse (Gardner and Lane, 1993b) and cow (Gardner *et al.*, 2000a) following culture of early cleavage stage embryos with EDTA.

While the early cleavage stage cow embryo takes up glucose and metabolizes glucose, levels are extremely low compared with rates reported for the embryo post-compaction (Rieger *et al.*, 1992a, 1992b; Gardner *et al.*, 1993; Thompson *et al.*, 1996). Unlike lactate or pyruvate, glucose as the sole energy source cannot support

development of the cleavage stage cow embryo (Pinyopummintr and Bavister, 1996b). Barbehenn *et al.* (1974, 1978) reported that the inability of the cleavage stage mouse embryo to utilize glucose was due to an inhibition of PFK. The allosteric inhibition of PFK was subsequently shown to result from a high ATP:ADP ratio in the early cleavage stage embryo (Leese *et al.*, 1984; Rozell *et al.*, 1992). Further, a high ATP:ADP ratio has been associated with culture conditions which facilitate development of the mouse embryo through the 2-cell block (Gardner and Lane, 1997b). While there is an apparent block to glycolysis in the cleavage stage embryo, it is evident that high concentrations of glucose in culture media can increase the activity of glycolytic enzymes (Edwards and Gardner, 1995) and stimulate glycolytic flux (Vella *et al.*, 1997). Culture of ruminant embryos with a high concentration of glucose (greater than 1.5 mM) was in fact found to inhibit development (Thompson *et al.*, 1989; Wang *et al.*, 1990; Takahashi and First, 1992; Kim *et al.*, 1993a; Matsuyama *et al.*, 1993).

There have been several suggestions as to the mechanisms by which Eagle's non-essential amino acids and glutamine could potentially regulate glycolytic activity in the embryo (Bavister, 1995; Gardner and Lane, 1997). These amino acids all have entry points into the TCA cycle. Thus, an increase in the activity of the TCA cycle would result in an increase in ATP production, potentially inhibiting the activity of PFK. In support of this, glutamine, glutamate and asparagine were found to decrease glycolytic activity in ascites tumour cells through the allosteric regulation of PFK by ATP (Gonzales-Mateos *et al.*, 1993). Further, the non-essential amino acid proline, was found to decrease the level of the glycolytic intermediate fructose 6-phosphate (Gonzales-Mateos *et al.*, 1993). Ascites tumour cells are similar to the mammalian

embryo in that they undergo rapid proliferation and have a high level of aerobic glycolysis, with only 1.5% of glucose metabolized through the TCA cycle (Lazo, 1981). In addition, the non-essential amino acids and glutamine may affect glycolytic activity and/or development, by the chelation of heavy metal contaminants (Lindenbaum, 1973; Fissore *et al.*, 1989) which have been found to affect embryo development (Van Winkle and Campione, 1982; Nasr-Esfahani *et al.*, 1990b; Goto *et al.*, 1993). It has been suggested that the non-essential amino acid alanine may reduce glycolytic activity of the embryo via the allosteric inhibition of pyruvate kinase (Lane and Gardner, 1998). Alanine is in fact a known inhibitor of pyruvate kinase (Feliu and Sols, 1976; Giles *et al.*, 1977; Gosalvez *et al.*, 1978). The rate of glycolytic activity in the present study, however, was determined by the production of $^3H_20$ in the dehydration of 2-phosphoglycerate to PEP, just prior to the conversion of PEP to pyruvate by pyruvate kinase.

While the early cleavage stage embryo appears to require specific regulators of glycolysis in vitro, the reasons for the inhibitory effect of an increased glycolytic activity on embryo development are poorly understood. It has been suggested that an increase in glycolytic activity could upset the balance of energy production in the embryo via a Crabtree-like effect, whereby an increase in the use of phosphate in the presence of glucose suppresses oxidative phosphorylation (Seshagiri and Bavister, 1991). An inhibition to development of the cleavage stage hamster embryo by glucose and phosphate was in fact alleviated by the addition of amino acids (Barnett and Bavister, 1996a). It has been suggested that culture with the non-essential amino acids and glutamine may function to alleviate a potential Crabtree-like effect in the early cleavage stage mouse embryo (Gardner, 1998b). Gardner and Lane (1993b) found that

a decrease in glycolytic activity in the presence of the non-essential amino acids and glutamine was accompanied by a significant increase in pyruvate metabolism through the TCA cycle. Furthermore, a decrease in glycolytic activity and a corresponding increase in pyruvate oxidation in early cleavage stage mouse and cow embryos, following culture with EDTA, was hypothesised to be due to a reduction in the influence of a Crabtree-like effect (Gardner and Lane, 1997a; Gardner and Lane, 1996).

Results from initial experiments in the present study indicated that there was not a reciprocal relationship between glycolytic activity and pyruvate oxidation following culture with and without amino acids. Determinations of glucose and pyruvate metabolism, however, were not from the same embryos. A further experiment was, therefore, carried out to determine whether the activity of the TCA cycle did in fact increase in individual embryos that were cultured with amino acids known to reduce glycolytic activity. In contrast to mouse 2-cell embryos (Gardner and Lane, 1993b), both glycolytic activity and pyruvate oxidation were significantly reduced in individual 8- to 16-cell cow embryos following culture in NeGln, compared with those cultured in the absence of amino acids. The reduction in pyruvate oxidation was more significant than the reduction in glycolytic activity. There was in fact no correlation between glucose and pyruvate metabolism in 8- to 16-cell embryos cultured in either SOF or NeGln. Despite the apparent differences in the effect of the non-essential amino acids and glutamine on the metabolism of early cleavage stage embryos of the mouse and cow, the results of the present study do not rule out the potential existence of a Crabtree-like effect in embryos with a compromised developmental capacity. The reduction of pyruvate oxidation in the presence of the

non-essential amino acids and glutamine may not be reflective of the overall activity of the TCA cycle in the cow embryo. This is discussed below with respect to the competition of pyruvate and amino acids for entry into the TCA cycle.

In contrast to the 8- to 16-cell stage, there was a significant correlation between glucose and pyruvate metabolism at the blastocyst stage. Culture with different amino acid groups altered the ratio of pyruvate:glucose metabolism in the blastocyst. The ratio of pyruvate:glucose metabolism was approximately 2.5 to 3 times higher in media without amino acids or with glutamine as the sole amino acid, compared with media containing non-essential and/or essential amino acids. Furthermore, culture in NeGln reduced the ratio of pyruvate:glucose metabolism by half compared with culture in EssGln. Pyruvate metabolism was in fact significantly reduced both per blastocyst and per cell, following culture in NeGln. The observed reduction in pyruvate metabolism was most likely due to an increase in endogenous pools of pyruvate via the conversion of specific non-essential amino acids, such as alanine, glycine and serine to pyruvate. In addition, the observed decrease in pyruvate metabolism could have resulted from the successful competition of specific non-essential amino acids for entry into the TCA cycle. The presence of alternative energy substrates in media has previously been shown to decrease the uptake of pyruvate by cow (Dorland *et al.*, 1991) and mouse blastocysts (Lane and Gardner, 2000a) and the rate of pyruvate metabolism by the sheep blastocyst (Thompson *et al.*, 1993). The fact that pyruvate metabolism per cell was significantly higher in 20aa than in NeGln, combined with the fact that the ratio of pyruvate:glucose metabolism was equivalent in EssGln and 20aa, indicates that the essential amino acids successfully competed with the non-essential amino acids for transporters at the blastocyst stage.

Alternatively, if the observed decrease in pyruvate metabolism in NeGln was in fact due to competition for entry of substrates into the TCA cycle, then the present results would indicate that the non-essential amino acids compete more successfully with pyruvate than do the essential amino acids.

Culture of embryos with and without different amino acid groups affected the positive relationship between blastocyst total cell number and the metabolism of pyruvate. Culture conditions have also been shown to affect the relationship between metabolism and blastocyst cell number in the mouse (Leppens-Luisier and Sakkas, 1997). The slope of the line of best fit for the correlation between cell number and pyruvate metabolism in the present study was 2.5 to 3 times higher for 20aa than for SOF or Gln. This indicates that as embryos increase in cell number and energy demands increase, embryos will utilize the available amino acids in preference to exogenous pyruvate.

The observed increase in the glycolytic activity of blastocysts cultured in 20aa, compared with those cultured in SOF, NeGln and 20aa, was simply a function of blastocyst cell number. Embryos cultured in Gln, however, had a significantly lower glycolytic activity than those cultured in all other treatments, irrespective of blastocyst cell number. Unlike the early cleavage stage embryo, glycolytic activity was not reduced in blastocysts cultured in NeGln, compared with culture in the absence of amino acids. Further analysis, however, revealed that the addition of the non-essential amino acids to medium containing glutamine, nullified the strong positive correlation between blastocyst cell number and glycolytic activity. Cell number and glycolytic activity were in fact significantly correlated in all treatment groups except NeGln.

Thus, the non-essential amino acids and glutamine did influence glycolytic activity at the blastocyst stage.

There was a positive correlation between the number of embryos developing to the blastocyst stage and the resultant glycolytic activity of blastocysts, following culture with and without amino acids. This relationship, however, was a function of a higher blastocyst cell number, following culture in media that supported a higher rate of embryo development. While there was no correlation between pyruvate metabolism by blastocysts and blastocyst development, following culture with and without different amino acid groups, there was a significant negative correlation between pyruvate metabolism per cell and blastocyst development. The observed rates of pyruvate metabolism under favourable and unfavourable conditions in both the early cleavage stage cow embryo and the blastocyst (on a per cell basis) appear to contradict reports of a decreased respiratory capacity in mouse and rat embryos under sub-optimal culture conditions (Menke and McLaren, 1970; Gardner and Lane, 1993b; Lane and Gardner, 1998). It has been suggested that sub-optimal culture conditions could affect respiration as a result of damage to mitochondria (Barnett and Bavister, 1996a, 1996b). In the present study, however, the observed decrease in pyruvate metabolism in media supporting a high rate of embryo development, was likely due to amino acids competing with pyruvate as an exogenous energy source for the embryo (discussed above). It is interesting that Gardner and Lane (1993b) and Lane and Gardner (1998) used identical conditions to the present study to determine pyruvate oxidation in mouse and rat embryos and did not find a decrease in pyruvate oxidation in the presence of amino acids. This suggests that unlike the mouse and rat, cow embryos utilize amino acids in preference to pyruvate. Alternatively, the conflicting

results could be indicative of differences in amino acid transporters in mouse and rat blastocysts compared with cow blastocysts. This is supported by the fact that differences in the uptake and output of individual amino acids have been documented for the mouse (Lamb and Leese, 1994) and cow (Partridge and Leese, 1996). Interestingly, a significant amount of threonine, which transaminates to pyruvate (Figure 1.2), was shown to be taken up by the cow blastocyst (Partridge and Leese, 1996) but not by the mouse blastocyst (Lamb and Leese, 1994). Thus, pyruvate metabolism could be decreased in the cow blastocyst in the presence of threonine, as a result of an increase in the endogenous pool of pyruvate.

It has been suggested that culture of embryos in vitro leads to an elevated pHi which adversely effects development. Culture of the hamster embryo from either the 2-cell stage or the 8-cell stage with an increased concentration of carbon dioxide (10%) significantly increased blastocyst development (Carney and Bavister, 1987b; McKiernan and Bavister, 1990). In addition, culture from the 8-cell stage with DMO had a similar effect on blastocyst development (Carney and Bavister, 1987b). Subsequently, DMO was shown to reduce the pHi of the in vivo produced mouse zygote, 2-cell, 4-cell and 8- to 16-cell embryos (Edwards *et al.*, 1998b). To date, however, there have been no direct measurements of the effect of extended culture under different culture conditions on the pHi of mammalian embryos. The present study revealed that embryos cultured to the 8- to 16-cell stage in the absence of amino acids had a pHi of 7.37. Culture with the non-essential amino acids and glutamine (NeGln) or glutamine alone (Gln) resulted in a lower pHi of 7.20 and 7.27, respectively. Lane and Bavister (2000) recently showed that the pHi of hamster 2-cell embryos increased from 7.2 to 7.35, following a decrease in the activity of the HCO_3^-

/Cl^- exchanger and the Na^+/H^+ antiporter as a result of cryopreservation. Interestingly, Lane and Bavister (1999) reported that, despite the presence of the HCO_3^-/Cl^- exchanger, cleavage stage cow embryos were unable to recover from an alkaline load, suggesting that a prolonged culture period may reduce the ability of the exchanger to function properly. The non-essential amino acids were not present in the medium when cow embryos were exposed to ammonium (Lane and Bavister, 1999). Glutamine was present, however, but at 0.2 mM compared to 1.0 mM in the present study. It is possible that the ability of glutamine to regulate pHi is concentration dependent. Furthermore, the embryos exposed to ammonium in the study by Lane and Bavister (1999) had a mean pHi of 7.9, which was substantially higher than that determined for embryos cultured in the absence of amino acids in the present study. Glutamine may thus be able to regulate pHi only within a certain range.

Lane and Bavister (1999) reported that even a slight increase in pHi of 0.13 units (from 7.2), following culture of cleavage stage embryos with 10 mM trimethylamine (TMA), totally inhibited development of embryos to the morula and blastocyst stages. Interestingly, development was inhibited even though the non-essential amino acids and glutamine were present in the culture medium. This result appears to contradict the findings of the present study in that, despite the fact that embryos cultured in the absence of amino acids had a mean pHi of 7.37, development in SOF was impaired but not totally inhibited. The development of embryos, however, may not have been arrested because of a slight rise in pHi following culture with TMA, as suggested by Lane and Bavister (1999). Guest and Varma (1992) reported that the partial inhibition of development of mouse embryos to the blastocyst stage following culture with 0.75 mM TMA was not due to changes in the osmolarity or pH

of the culture medium (pHi was not determined), but rather as a result of the teratogenic effects of TMA. Even at 0.75 mM TMA, DNA, RNA and the protein content of TMA-treated embryos were approximately 50% of embryos cultured for 42 h in the absence of TMA (Guest and Varma, 1992). Culture of cow embryos with 10 mM TMA would likely have a severe effect on the DNA, RNA and protein content of cow embryos and hence a significant effect on developmental capacity.

The present study suggests that the 8- to 16-cell stage cow embryo has difficulties in regulating a slight increase in pHi in the absence of amino acids. Similarly, despite the presence of the HCO_3^-/Cl^- exchanger in early cleavage stage in vivo produced mouse embryos, amino acids were found to act as buffers in response to an increasing alkaline load (Edwards *et al.*, 1998b). The effect of culture with amino acids beyond Day 4 pi on pHi was not determined in the present study as previous studies have reported that the embryo acquires the ability to regulate pHi upon compaction (Edwards *et al.*, 1998a, 1998b). Edwards *et al.* (1998b) found that amino acids had in fact lost their pHi buffering capacity in the mouse embryo at the 8- to 16-cell stage and hypothesised that this was due to a change in the predominant amino acid transport systems. The fact that amino acids were shown to buffer pHi in the present study suggests that the transport mechanisms for amino acids are different in cow and mouse embryos at the 8- to 16-cell stage. As discussed above, different profiles of amino acid uptakes and outputs have been documented for mouse (Lamb and Leese, 1994) and cow embryos (Partridge and Leese, 1996) at the blastocyst stage. Direct comparisons of the uptake and output of non-essential amino acids and glutamine by mouse and cow embryos cannot be made, however, as determinations were not carried out on embryos at the 8- to 16-cell stage in either study.

Interestingly, the pHi of the 8- to 16-cell embryo was negatively correlated with development to the 8- to 16-cell stage in the present study. Thus, culture of the early cleavage stage cow embryo under conditions which elevated pHi, resulted in a reduced developmental capacity.

In the present study, the addition of the essential amino acids to medium containing the non-essential amino acids and glutamine (20aa) reduced the ability of the non-essential amino acids and glutamine to buffer pHi, possibly through the successful competition of the essential amino acids for transporters. Despite this, culture in 20aa supported a high level of development to the 8-16 cell stage (Chapter 5). In addition, culture in Gln for the first 72 h resulted in a lower pHi than culture in 20aa, however, culture in Gln could not support as high a rate of development to the 8- to 16-cell stage as 20aa. Therefore, although a less alkaline pH appears to be important for development of the early cleavage stage embryo, it is not the sole requirement. This supports the notion that amino acids have more than one function in the development of the mammalian embryo.

There are several possibilities as to the mechanisms by which amino acids could regulate pHi in the embryo. It has been suggested that amino acids may play an important role in the suppression of ROS in the embryo, possibly via the chelation of free metal ions (Bavister, 1995). ROS, which are believed to impair embryo development (Legge and Sellens, 1991; Johnson and Nasr-Esfahani, 1994; Luvoni *et al.*, 1996), have been shown to increase pHi in somatic cells (Shibanuma *et al.*, 1988; Ikebuchi *et al.*, 1991). Alternatively, culture of embryos in the absence of amino acids could result in a mass efflux of amino acids and protons from the embryo, resulting in an increase in pHi (Bavister and McKiernan, 1993; Bavister, 1995). Due to their

zwitterionic properties, it has been hypothesised that amino acids could function as intracellular zwitterionic buffers during embryo culture, alleviating fluctuations in pHi (Bavister and McKiernan, 1993).

There is undoubtedly a link between the observed decrease in both pHi and glycolytic activity in the 8- to 16-cell cow embryo following culture with the non-essential amino acids and glutamine. Edwards *et al.* (1998a) reported a decrease in the glycolytic activity of the mouse zygote following exposure to a weak acid, DMO. This indicates that glycolysis decreased as a result of an acidification of pHi. A decrease in pHi could reduce the activity of metabolic enzymes such as PFK which is sensitive to changes in pH (Spriet, 1991). In addition, a decrease in pHi has been shown to release mitochondrial bound hexokinase, resulting in a decrease in glucose phosphorylation (Miccoli *et al.*, 1998). Alternatively, the observed decrease in pHi may have resulted from the regulation of metabolism by amino acids. Eisner *et al.* (1989) found that an inhibition of glycolysis and oxidative phosphorylation led to an increase in intracellular calcium concentration which in turn resulted in intracellular acidosis.

Recently, Lane and Bavister (2000) reported that an elevated pHi of 7.35, following a decrease in the activity of the HCO_3^-/Cl^- exchanger following cryopreservation, reduced oxidative metabolism in hamster 2-cell embryos. Although determinations of pHi and metabolism were not from the same cohort of embryos, in the present study an elevated pHi of 7.37 following culture of cow 8- to 16-cell embryos in the absence of amino acids, was associated with an increase in pyruvate metabolism. As already discussed, however, the determination of pyruvate metabolism by cow embryos in the presence of amino acids, may not be reflective of

actual oxidative metabolism due to competition of amino acids with pyruvate for entry into the TCA cycle.

The present study revealed that glycolytic activity was slightly depressed in the 8- to 16-cell embryo and significantly depressed in the blastocyst, following culture in media with glutamine as the sole amino acid. Further, the observed relationship between blastocyst cell number and glycolytic activity in the present study was strongest following culture with glutamine as the sole amino acid. Thus, there was evidently a relationship between glutamine and glucose metabolism in the cow embryo. Glutamine metabolism is equivalent to glucose metabolism at the 8- to 16-cell stage but is substantially lower at the blastocyst stage (Rieger *et al.*, 1992a, 1992b). Pig embryos can in fact utilize glutamine as an energy source in the absence of glucose (Petters *et al.*, 1990). This has also been shown for somatic cells (Zielke *et al.*, 1978). Although glucose as the sole energy substrate cannot support development of the early cow embryo (Pinyopummintr and Bavister, 1996b) it is believed that glucose is important for the generation of ribose moieties and NADPH through the PPP (Gardner, 1998a). Thompson *et al.* (1993) suggested that the metabolism of glucose to lactate could be important for the generation of pyruvate and thus NADH for the maintenance of an optimal redox state in the sheep embryo (Thompson *et al.*, 1993). There has, however, been some question as to the importance of an appropriate redox state in the cow embryo (Rosenkrans *et al.*, 1993; Edwards *et al.*, 1997). Irrespective, the metabolism of glucose and pyruvate to lactate could likely have a significant role in the production of NAD^+ for glycolysis.

It is not presently known whether glutamine is able to function in such a capacity, through its conversion to lactate. Pinyopummintr and Bavister (1996b)

found that, in the absence of other energy substrates, pyruvate was able to support development of the early cleavage stage cow embryo, however, glutamine could not. The present study revealed that significant amounts of lactate were produced from both glucose and pyruvate in the 8- to 16-cell cow embryo, however, lactate was not produced from glutamine. Likewise at the blastocyst stage, significant levels of lactate were produced from glucose, but not from glutamine. This indicates that glutamine was not able to be used by the embryo for lactate production, at either the 8- to 16-cell stage or the blastocyst. Of interest is the fact that the presence of glutamine at both stages did not affect the production of lactate from glucose. This indicates that the observed effects of glutamine in reducing glycolytic activity at the 8- to 16-cell stage and the blastocyst, were the result of an extended period of culture with glutamine as the sole amino acid. Alternatively, the inhibitory effects of glutamine on glycolytic activity may be dependent on the presence of other energy substrates, such as pyruvate and lactate.

The production of lactate from pyruvate was significantly higher than production from glucose at the 8- to 16-cell stage, however, but this was reversed at the blastocyst stage. Pyruvate uptake has been shown to be higher than glucose uptake at the 8- to 16-cell stage but is not markedly different at the blastocyst stage (Thompson *et al.*, 1996; Table 3.2). The fact that the amount of lactate produced from glucose is equivalent to twice the total amount of glucose found to be taken up by the blastocyst (Thompson *et al.*, 1996, Table 3.2) indicates that nearly 100% of glucose is converted to lactate via aerobic glycolysis (1 mol glucose is converted to 2 mol lactate). Thompson *et al.* (1996) came to a similar conclusion regarding the fate of glucose during development of the cow embryo, however, pyruvate was also present

in the culture medium making it difficult to conclude that all of the lactate produced actually came from glucose. Thus, the present study provides evidence that that there is not in fact a block to glycolysis at pyruvate kinase, as suggested by Rieger and Guay (1988) and Rieger *et al.* (1992b). Despite the oxidation of pyruvate via the TCA cycle, oxidation of glucose has been reported to be barely detectable (Rieger *et al.*, 1992b). This suggests that the embryo distinguishes between the metabolic fate of endogenous and exogenous pyruvate pools. While this concept would seem implausible, there is evidence to suggest that this in fact occurs in glioma cells which, as mentioned above, also have a high level of aerobic glycolysis. Bouzier *et al.* (1998) reported that there appeared to be two distinct pools of lactate and pyruvate in glioma cells. The endogenous pool was associated with aerobic glycolysis and the exogenous pool was directed to oxidative metabolism. Thompson *et al.* (1991) found that, in the presence of the metabolic uncoupler DNP and the absence of pyruvate and lactate, glucose oxidation increased in the sheep embryo. This suggests that, in the absence of oxidizable substrates, the embryo can alter the metabolic fate of endogenous pyruvate in order to meet demands for production of energy and metabolic intermediates through the TCA cycle.

In conclusion, this chapter revealed that amino acids have a key role in the regulation of intracellular processes in the cow embryo. Culture with amino acids affected the activities of metabolic pathways within the 8- to 16-cell embryo and blastocyst. Such effects were correlated with embryo development and cell number. Eagle's non-essential amino acids and glutamine appear to be key regulators of glycolytic activity in the early cleavage stage embryo. Furthermore, the data indicate that the cow embryo metabolizes exogenous amino acids in preference to exogenous

pyruvate. Amino acids were also found to buffer pHi in the early cleavage stage embryo. There was a significant correlation between pHi and development. Importantly, amino acids were found to have multiple roles in the development of the cow embryo in vitro.

CHAPTER 7

THE ROLE OF VITAMINS IN THE DEVELOPMENT AND REGULATION OF METABOLISM OF THE COW EMBRYO DURING CULTURE

7.1 INTRODUCTION

Vitamins play a key role in normal cellular growth and function. Many vitamins, particularly the B-complex vitamins, are components of coenzymes and thus are important factors in the regulation of intracellular metabolism. The B-complex vitamins, however, are water soluble and cannot be stored. Cells therefore, are largely dependent on an exogenous supply of B-complex vitamins.

The first studies to report a stimulatory effect of vitamins during embryo culture were in the hamster and rabbit. Culture with a group of B-complex vitamins stimulated hatching of the hamster blastocyst (Kane *et al.*, 1986; Kane and Bavister, 1988a) but had no effect on development to the blastocyst stage (Bavister *et al.*, 1983). Culture of the rabbit embryo with B-complex vitamins was found to be a requirement for blastocyst expansion (Kane, 1988). Subsequently, it was shown that the vitamins inositol, pantothenate and choline were responsible for the stimulation of hatching in the hamster (Kane and Bavister, 1988b), while inositol, pyridoxine, nicotinamide and riboflavin stimulated expansion of the rabbit blastocyst (Kane, 1988). Recently, pantothenate was found to stimulate development of hamster embryos to the blastocyst stage and have a beneficial effect on embryo viability following transfer to recipient hamsters (McKiernan and Bavister, 2000).

Studies on the effect of vitamins during the culture of ruminant embryos have been few, possibly due to the fact that culture with MEM vitamins (B-group complex) were reported to not have a stimulatory effect on blastocyst development (Takahashi and First, 1992; Rosenkrans and First, 1994). In fact, although not significant, culture with vitamins appeared to suppress blastocyst formation in the cow (Rosenkrans and First, 1994). In the study by Takahashi and First (1992), the null effect of MEM

vitamins on blastocyst development was likely due to the fact that embryos were cultured in the absence of glucose. In that study, blastocyst development was not even stimulated by the addition of amino acids to SOF. Of further concern has been the report that MEM vitamins decreased blastocyst cell number in the mouse when embryos were cultured in a medium lacking amino acids (Tsai and Gardner, 1994). It was established that nicotinamide was responsible for the reduction in cell number and a significant decrease in embryo viability following transfer (Tsai and Gardner, 1994). Reports of the stimulatory effects of vitamins have been in studies where embryos were cultured beyond the 8-cell stage. Tsai and Gardner (1994) hypothesised that the inhibitory effects of the MEM vitamins could be during development of the early cleavage stages. MEM vitamins, however, were not in fact inhibitory to development of the early cleavage stage mouse embryo when amino acids were present in the culture medium (Gardner and Sakkas, 1993). Even in the presence of amino acids, though, culture with vitamins beyond 88 h post-HCG suppressed hatching in mouse blastocysts (Gardner and Sakkas, 1993).

The effect of vitamins during culture of the in vitro produced cow embryo warrants further investigation. Of significant interest, are reports of the effects of vitamins on embryo metabolism. Culture with vitamins has been shown to increase both the uptake of glucose and production of lactate by sheep blastocysts (Gardner *et al.*, 1994b). Furthermore, the combination of MEM vitamins and amino acids was found to influence glycolytic activity and metabolism of pyruvate in the in vivo produced mouse blastocyst following 6 h culture, to levels more similar to in vivo produced embryos (Lane and Gardner, 1998). Lane and Gardner (1998) in fact found an interaction between amino acids and vitamins in reducing the proportion of glucose

converted to lactate by the mouse blastocyst. Importantly, culture of in vivo produced mouse embryos for 6 h in the absence of vitamins and amino acids significantly reduced embryo viability following transfer (Lane and Gardner, 1998). This indicates that a supply of exogenous vitamins could be important for mammalian blastocyst metabolism and viability. Interestingly, MEM vitamins were necessary for development of the cow blastocyst from Day 7 to Day 14 pi (Giliam and Gardner, unpublished data). There have been many reports of the positive effects of folic acid, pantothenic acid, riboflavin, nicotinamide and inositol on rodent post-implantation development and viability (Cockroft, 1979; Habibzadeh *et al.*, 1986; Cockroft, 1988; Cockroft, 1992; Mooij *et al.*, 1993).

The aims of the present study were, therefore, to assess the role of B-complex vitamins in development of both the early cleavage stage cow embryo and the later stages of development, with respect to cleavage and blastocyst expansion. In addition, the study aimed to determine the effect of vitamins on both glycolytic activity and activity of the TCA cycle in the cow blastocyst. Furthermore, the effects of several individual vitamins, known to either inhibit or stimulate embryos from other species, were examined.

7.2 MATERIALS AND METHODS

The exact composition of culture media for the following experiments is shown in Appendix 7. Embryos were cultured in groups of five in 30 µl drops of media, under 7 ml light mineral oil, in a 60 mm non-pyrogenic culture dish, at 39^{0}C, in 7% O_2, 5% CO_2 and 88% N_2.

7.2.1 The Temporal Effect of MEM Vitamins on Embryo Development.

The first set of experiments was designed to determine the effect of MEM vitamins on embryo development, blastocyst cell number and blastocyst expansion, during the first 72 h culture to Day 4 pi and culture beyond Day 4 pi to both Day 7 pi and Day 8 pi.

7.2.1.1 Zygote to Day 4 post-insemination.

Presumptive zygotes (18 h pi) were cultured for 72 h to Day 4 pi in either NeGln (SOF with 1 mM glutamine and Eagle's non-essential amino acids) or NeGlnVit (NeGln with MEM vitamins). Embryo development was scored after 22 h, 51 h and 72 h culture to determine the percentage of embryos at the 4-cell stage or greater at 22 h and the percentage of embryos at the 8-cell stage or greater at 51 h and 72 h culture. Following each developmental assessment, embryos were immediately returned to the incubator for continued culture. After the 72 h assessment, embryos from both NeGln and NeGlnVit were transferred in their respective groups to 20aa for a further 72 h culture to Day 7 pi. The percentages of embryos at the morula and blastocyst stages were determined and blastocysts were scored as either early, mid or expanded blastocysts. All blastocysts were then stained with Hoechst (Number 33258) to determine total blastocyst cell number (see section 2.7.2).

7.2.1.2 Day 4 to Day 7 post-insemination.

Presumptive zygotes (18 h pi) were cultured in NeGln for 72 h to Day 4 pi. Embryo development was scored and embryos were divided equally between two groups containing equivalent numbers of embryos less than 8-cell and greater than or equal to 8-cell. Embryos were then transferred to either 20aa (SOF with 1 mM glutamine and Eagle's non-essential and Eagle's essential amino acids) or 20aaVit (20aa with MEM vitamins) for a further 72 h culture to Day 7 pi. The percentages of embryos at the morula and blastocyst stages were determined and blastocysts were scored as either early, mid or expanded blastocysts. All blastocysts were then stained with Hoechst (Number 33258) to determine total blastocyst cell number (see section 2.7.2).

7.2.1.3 Day 4 to Day 8 post-insemination.

Embryos were cultured as above to Day 4 pi and divided equally between two groups (section 7.2.1.2). Embryos were then cultured for 72 h to Day 7 pi in either 20aa or 20aaVit. On Day 7 pi, embryo development was scored and embryos from each treatment were transferred to fresh drops of the same medium. Blastocysts were placed in separate drops to embryos that were not at the blastocyst stage and drops were numbered so that embryos that reached the blastocyst stage by Day 7 pi could be identified. Embryos were then cultured for a further 24 h to Day 8 pi. The percentages of embryos at the morula and blastocyst stages were determined for Day 8 pi and blastocysts were scored as either early, mid, expanded, hatching or hatched blastocysts. Blastocysts were labelled as those that had reached the blastocyst stage by the changeover on Day 7 pi and those that had developed to the blastocyst stage between 144 and 168 h culture.

Embryos that had reached the blastocyst stage by the changeover on Day 7 pi were viewed under an inverted microscope with an eyepiece micrometer. The diameter of each blastocyst was recorded. The zona pellucida was not included in the measurement of blastocyst diameter. Blastocysts were then stained with Hoechst (Number 33258) to determine total blastocyst cell number (see section 2.7.2). Embryos that had developed to the blastocyst stage between 144 and 168 h culture were not measured for the determination of diameter or stained for the determination of cell number.

7.2.2 The Effect of MEM Vitamins and Serum on Development from Day 6 Post-Insemination.

This experiment was carried out to determine whether there was a synergistic effect between MEM vitamins and serum on embryo development, following culture of embryos from Day 6 pi to Day 8 pi, in the presence and absence of vitamins and serum.

Presumptive zygotes (18 h pi) were cultured in NeGln for 72 h to Day 4 pi. Embryo development was scored and embryos were divided equally among four groups, containing equivalent numbers of embryos less than 8-cell and greater than or equal to 8-cell. Two groups of embryos were then transferred to 20aa and two groups were transferred to 20aaVit, for a further 48 h culture to Day 6 pi. On Day 6 pi, One group of embryos in 20aa was transferred to fresh drops of 20aa and the other group was transferred to drops of a modified version of 20aa, where BSA was replaced with 10% heat inactivated FCS (20aa/serum). For the embryos in 20aaVit, one group was transferred to fresh drops of 20aaVit and the other group was transferred to drops of a

modified version of 20aaVit, where BSA was replaced with 10% heat inactivated FCS (20aaVit/serum). Embryos were then cultured for 24 h to Day 7 pi. The percentages of embryos at the morula and blastocyst stages were determined and blastocysts were grouped separately to embryos that were not at the blastocyst stage. Drops were numbered so that embryos that reached the blastocyst stage by Day 7 pi could be identified. Embryos were not changed to fresh medium, but remained in the same drops of medium for a further 24 h culture to Day 8 pi. The percentages of embryos at the morula and blastocyst stages were determined for Day 8 pi and blastocysts were scored as either early, mid, expanded, hatching or hatched blastocysts. Blastocysts were labelled as those that had reached the blastocyst stage by the changeover on Day 7 pi and those that had developed to the blastocyst stage between 144 and 168 h culture.

Blastocysts (Day 8 pi) that had reached the blastocyst stage by the changeover on Day 7 pi were viewed under an inverted microscope with an eyepiece micrometer. The diameter of each blastocyst was recorded. The zona pellucida was not included in the measurement of blastocyst diameter. Blastocysts were then stained with Hoechst (Number 33258) to determine total blastocyst cell number (see section 2.7.2). Embryos that had developed to the blastocyst stage between 144 and 168 h culture were not measured for the determination of diameter or stained for the determination of cell number.

7.2.3 The Effect of Culture with MEM Vitamins on the Metabolism of the Cow Blastocyst.

Due to the results of experiment 7.2.1.1, embryos were cultured for 72 h from the zygote (18 h pi) to Day 4 pi, in NeGln. Embryos were then divided equally between two groups and transferred to either 20aa or 20aaVit for a further 72 h culture to Day 7 pi. Blastocysts were removed from each medium and transferred in their respective groups to the metabolic version of the medium in which they were cultured: met20aa or met20aaVit (see Appendix 11). Note that the metabolic versions of each medium were the same as the culture medium but did not contain pyruvate. Embryos were washed three times in the metabolic medium to ensure all pyruvate was removed before being transferred to the respective metabolic media containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [5-^{3}H] glucose (at a concentration of 0.015 mM and specific activity of 0.25 μCi/μl). Thus, determinations of [2-^{14}C] pyruvate and [5-^{3}H] glucose metabolism were made for the same embryos.

In a separate series of cultures, embryos were cultured under the same conditions as above, but were transferred to the respective metabolic media containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [G-^{3}H] glutamine (at a concentration of 0.004 mM and specific activity of 0.25 μCi/μl). Thus, determinations of [2-^{14}C] pyruvate and [G-^{3}H] glutamine metabolism were made for the same embryos.

Determinations of the metabolism of [2-^{14}C] pyruvate, [G-^{3}H] glutamine and [5-^{3}H] glucose by blastocysts were carried out as described in section 2.8. Blastocysts were incubated in 3 μl drops of metabolic medium containing the radioisotopes for 3

h, at 7% O_2, 5% CO_2 and 88% N_2, at 39°C, in the lid of individual 2 ml tubes. Rates of nutrient metabolism by each blastocyst were calculated from the liberation of ^3H_2O and $^{14}CO_2$ (see section 2.8.2).

At the end of the metabolic incubation period, each blastocyst was ranked as either an early, mid, expanded, hatching or hatched blastocyst. Each blastocyst was then stained with Hoechst (Number 33258) to determine total cell number (section 2.7.2).

The metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by individual blastocysts and the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by individual blastocysts were correlated for each treatment to determine the effect of MEM vitamins on the relationship between substrate metabolism by the blastocyst.

Further, the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose and [G-^{3}H] glutamine by individual blastocysts was correlated with the total cell number of corresponding blastocysts for each treatment, to determine the effect of MEM vitamins on the relationship between blastocyst cell number and substrate metabolism.

7.2.4 The Effect of Culture with Specific Vitamins on the Development and Metabolism of the Cow Blastocyst.

The aim of the following experiments was to determine the effect of culture with specific vitamins on blastocyst development and metabolism, compared with culture in medium without vitamins. Due to the results of the experiments in section 2.1.3, the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by individual blastocysts cultured with specific vitamins was determined. The selection of the specific vitamins was based on previous reports of the stimulatory or inhibitory effects

of the vitamins on mammalian embryos. The concentrations of individual vitamins were those of vitamins in MEM and BME (Eagle, 1959) solutions. The study of the effects of specific vitamins was performed in two separate experiments. This was due to the limitation of the number of treatment groups that could be processed for metabolic determinations at one time.

7.2.4.1 Folic acid, nicotinamide, riboflavin and biotin.

Embryos were cultured for 72 h from the zygote (18 h pi) to Day 4 pi in NeGln. Embryos were then divided equally among five groups and transferred to either: 1) 20aa, 2) Folic (20aa with 2.3 μM folic acid), 3) Nicotin (20aa with 8.2 μM nicotinamide), 4) Ribo (20aa with 0.27 μM riboflavin), or 5) Biotin (20aa with 4.2 μM biotin) for a further 72 h culture to Day 7 pi. Development to the morula and blastocyst stages was scored and blastocysts were removed from each medium and transferred in their respective groups to the metabolic version of the medium in which they were cultured: 1) met20aa, 2) metFolic, 3) metNicotin, 4) metRibo, and 5) metBiotin (see Appendix 11). The metabolic versions of media were the same as culture media but did not contain pyruvate (pyruvate was subsequently added as labelled pyruvate). Embryos were washed three times in metabolic medium to ensure that all pyruvate was removed before being transferred to respective metabolic media containing [2-^{14}C] pyruvate (at a concentration of 0.33 mM and specific activity of 0.005 μCi/μl) and [G-^{3}H] glutamine (at a concentration of 0.004 mM and specific activity of 0.25 μCi/μl). Thus, determinations of [2-^{14}C] pyruvate and [G-^{3}H] glutamine metabolism were made for the same embryos.

Determinations of the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by blastocysts were carried out as described in section 2.8. Individual blastocysts were incubated in 3 µl drops of metabolic medium containing the radioisotopes for 3 h, at 7% O_2, 5% CO_2 and 88% N_2, at 39^{0}C, in the lid of individual 2 ml tubes. Rates of nutrient metabolism by each blastocyst were calculated from the liberation of $^{3}H_2O$ and $^{14}CO_2$ (see section 2.8.2).

At the end of the metabolic incubation period, each blastocyst was ranked as either an early, mid, expanded, hatching or hatched blastocyst. Blastocysts were then stained with Hoechst (Number 33258) to determine total cell number (section 2.7.2).

7.2.4.2 Pantothenate, myo-inositol and the combination of folic acid, nicotinamide and riboflavin.

The procedure for the following experiment was identical to the above experiment (section 7.2.4.1), however, the treatment groups were different. Embryos were cultured for 72 h from the zygote (18 h pi) to Day 4 pi, in NeGln. Embryos were then divided equally among four groups and transferred to either: 1) 20aa, 2) Myo (20aa with 11 µM myo-inositol), 3) Panto (20aa with 4.2 µM pantothenate), or 4) Folic/Nicotin/Ribo (20aa with 2.3 µM folic acid, 8.2 µM nicotinamide and 0.27 µM riboflavin) for a further 72 h culture to Day 7 pi. Development to the morula and blastocyst stages was scored and blastocysts were removed from each medium and transferred in their respective groups to the metabolic version of the medium in which they were cultured: 1) met20aa, 2) metMyo, 3) metPanto, 4) metFolic/Nicotin/Ribo (see Appendix 11).

Determinations of the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by blastocysts were carried out as described in section 7.2.4.1. At the end of the

metabolic incubation period, each blastocyst was ranked as either an early, mid, expanded, hatching or hatched blastocyst. Blastocysts were then stained with Hoechst (Number 33258) to determine total cell number (section 2.7.2).

7.2.5 Statistical Analysis

For experiments assessing the equality between two treatment groups, an unpaired, two-tailed t-test was used to analyse the statistical difference between treatment means. For experiments determining the equality among several treatment groups, an ANOVA was used to analyse the differences among treatment means. The Tukey-Kramer Multiple Comparison Test was used following the ANOVA to determine the statistical significance of the differences among treatment means. Dunnett's test was used to determine the difference between individual treatment means and the mean of the experimental control when media containing individual vitamins were compared to media without vitamins. Bartlett's test was used to check for homogeneity of variances. When the degree of association between two variables was determined, the correlation coefficient (r) was calculated. A probability of $P < 0.05$ was considered to be significant for all statistical tests.

7.3 RESULTS

7.3.1 The Temporal Effect of MEM Vitamins on Embryo Development.

7.3.1.1 Zygote to Day 4 post-insemination.

Culture with MEM vitamins for the initial 72 h inhibited development of the cow embryo. The inhibitory effect of culture in NeGlnVit was not evident until 72 h culture. Development to the 4-cell stage and greater than the 4-cell stage after 22 h culture and development to the 8-cell stage and greater than the 8-cell stage after 51 h culture, were not significantly different for embryos cultured in NeGln and NeGlnVit ($P > 0.05$, Table 7.1). After 72 h culture, 63.4% of embryos cultured in NeGln and 64.0% of embryos cultured in NeGlnVit, had developed to at least the 8-cell stage (Table 7.1). Significantly fewer embryos, however, had developed beyond the 8-cell stage following culture in NeGlnVit, compared with culture in NeGln ($P < 0.05$, Table 7.1).

Subsequent development to the morula and/or blastocyst stages and the total number of cells in blastocysts, were not significantly different following culture in NeGln and NeGlnVit for the initial 72 h ($P > 0.05$, Table 7.1). Further, the subsequent development of blastocysts to the early, mid and expanded stages, was not significantly different following culture in NeGln and NeGlnVit for the initial 72 h ($P > 0.05$, Figure 7.1).

Table 7.1 Effect of culture of the early cow embryo with MEM vitamins for the first 72 h culture on development and subsequent developmental competence and blastocyst cell number (Day 7 post-insemination).

			Development[A] to various stages after								
			22 h culture		51 h culture		72 h culture		144 h culture		
Media* 1st 72 h	Media** 2nd 72 h	Cleaved ova	4-cell	> 4-cell	8-cell	> 8-cell	8-cell	> 8-cell	Morula and blastocyst	Blastocyst	Total cell number[B]
NeGln	20aa	96	29.5	18.3	49.1	12.7	31.7	31.7[a]	63.0	48.8	86.4 ± 6.0
NeGlnVit	20aa	93	46.6	18.8	45.4	2.5	43.3	20.7[a]	53.8	47.2	85.7 ± 5.2

[A]Percentage development from cleaved ova. Data are from four replicates

*Media for culture 0-72 h (0 = 18-20 h pi). **Media for culture 72-144 h. Media are defined in section 7.2.1.1.

[a]Like pairs of letters are significantly different within a column ($P < 0.05$).

[B]Blastocyst total cell number (mean ± SEM).

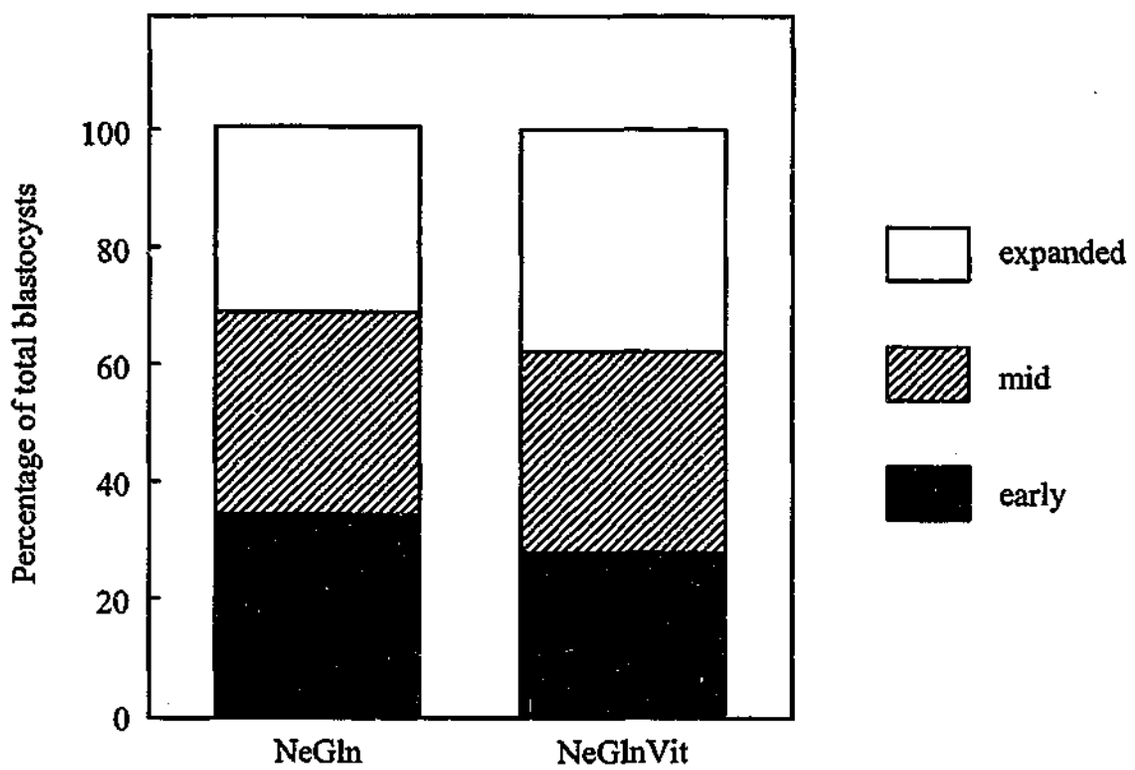


Figure 7.1 The effect of culture with NeGln and NeGlnVit (media are defined in section 7.2.1.1) for the first 72 h, on the percentage of early, mid and expanded blastocysts (of total blastocysts) on Day 7 post-insemination, following culture for the second 72 h in 20aa. Data are from four replicates. No significant differences in blastocyst expansion between treatment groups, $P > 0.05$.

7.3.1.2 Day 4 to Day 7 post-insemination.

Culture with MEM vitamins from Day 4 pi to Day 7 pi, did not affect development to the morula and/or blastocyst stages or total blastocyst cell number (20aa versus 20aaVit, $P > 0.05$; Table 7.2). Further, the development of blastocysts to the early, mid, expanded and hatching stages on Day 7 pi, was not affected by culture with MEM vitamins (20aa versus 20aaVit, $P > 0.05$; Figure 7.2a).

7.3.1.3 Day 4 to Day 8 post-insemination.

Culture with MEM vitamins from Day 4 pi to Day 8 pi, did not affect development to the morula and/or blastocyst stages (20aa versus 20aaVit, $P > 0.05$; Table 7.2). Further, the development of blastocysts to the early, mid, expanded, hatching and hatched stages on Day 8 pi, was not affected by culture with MEM vitamins (20aa versus 20aaVit, $P > 0.05$; Figure 7.2b).

For embryos that had reached the blastocyst stage by Day 7 pi, there was no significant difference in development of blastocysts to the early, mid, expanded, hatching or hatched stages (Figure 7.2c) or the total cell number of blastocysts (Table 7.2) on Day 8 pi, following culture in 20aa and 20aaVit ($P > 0.05$). For embryos that had reached the blastocyst stage by Day 7 pi, blastocyst diameter was significantly greater on Day 8 pi following culture in 20aaVit than after culture in 20aa ($P < 0.05$; Table 7.2).

Table 7.2 Effect of culture of the cow embryo with MEM vitamins for 72 h and 96 h from Day 4 post-insemination, on development and total blastocyst cell number.

		Development[†] to various stages, blastocyst cell number and diameter after						
		144 h culture[A]			168 h culture[B]			
Media* 0-72 h	Media** 72-168 h	Morula and blastocyst	Blastocyst	Total cell number	Morula and blastocyst	Blastocyst	Total cell number[C]	Diameter[C] µm
NeGln	20aa	56.4	46.3	89.4 ± 5.1	53.5	45.0	124.0 ± 5.3	179.8 ± 5.0[a]
NeGln	20aaVit	49.4	43.6	93.8 ± 5.6	54.8	46.7	121.1 ± 6.0	205.7 ± 7.5[a]

[†]Percentage development from cleaved ova. [A]Developmental data are from eight replicates, n = 262 cleaved ova per treatment; cell number data (mean ± SEM) from four replicates, n = 105 cleaved ova per treatment. [B]Data from four replicates, n = 93 cleaved ova per treatment. *Media for culture 0-72 h (0 = 18-20 h pi). **Media for culture 72-168 h. Media defined in section 7.2.1. [C]Data from blastocysts that had reached the blastocyst stage by 144 h culture and were cultured for a further 24 h. [a]Like pairs of letters are significantly different, $P < 0.05$.

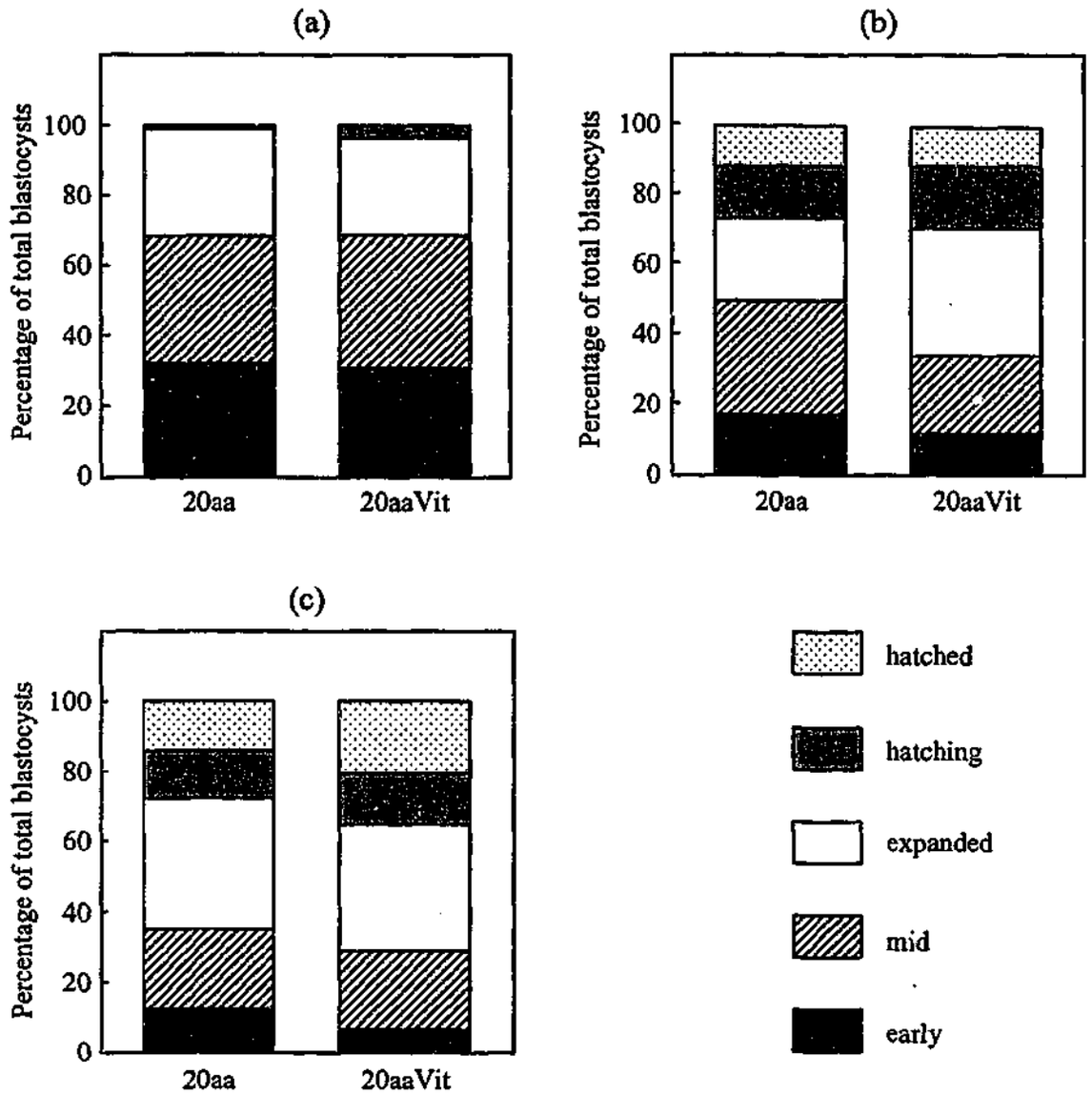


Figure 7.2 The effect of culture with 20aa and 20aaVit (media are defined in section 7.2.1) from Day 4 post-insemination on the percentage of early, mid, expanded, hatching and hatched blastocysts (of total blastocysts) on (a) Day 7 post-insemination; (b) Day 8 post-insemination; and (c) Day 8 post-insemination for embryos that reached the blastocyst stage on Day 7 post-insemination. Embryos cultured in NeGln for the first 72 h to Day 4 post-insemination. Data are from four replicates; n = 120 cleaved ova per treatment. No significant differences in blastocyst expansion between treatment groups, $P > 0.05$.

7.3.2 The Effect of MEM Vitamins and Serum on Development from Day 6 Post-Insemination.

Culture of embryos from Day 6 pi with serum, compared with culture of embryos with BSA, did not affect development to the morula and/or blastocyst stages on Day 7 pi or Day 8 pi (20aa/serum versus 20aaVit/serum, $P > 0.05$; Table 7.3) or the development of blastocysts to early, mid, expanded, hatching or hatched stages on either Day 7 pi (20aa/serum versus 20aaVit/serum, $P > 0.05$; Figure 7.3a) or Day 8 pi (20aa/serum versus 20aaVit/serum, $P > 0.05$; Figure 7.3b). Furthermore, there was not a synergistic effect between serum and vitamins, during culture from Day 6 pi to either Day 7 pi or Day 8 pi, on development to the morula and/or blastocyst stages (20aa/serum versus 20aaVit/serum, $P > 0.05$; Table 7.3) or the development of blastocysts to early, mid, expanded, hatching or hatched stages (20aa versus 20aa/serum, $P > 0.05$; Figure 7.3a for Day 7 pi and Figure 7.3b for Day 8 pi). In fact, development to the morula and/or blastocyst stages (Table 7.3) and development of blastocysts to the early, mid, expanded, hatching and hatched stages (Figure 7.3a for Day 7 pi and Figure 7.3b for Day 8 pi) was not different following culture to either Day 7 pi or Day 8 pi in 20aa, 20aaVit, 20aa/serum and 20aaVit/serum, $P > 0.05$).

For embryos that had reached the blastocyst stage by Day 7 pi, there was no difference in development of blastocysts to the early, mid, expanded, hatching or hatched stages (Figure 7.3c) or the total cell number of blastocysts on Day 8 pi (Table 7.3), following culture in 20aa, 20aaVit, 20aa/serum and 20aaVit/serum ($P > 0.05$). Blastocyst diameter, however, was significantly bigger following culture in 20aaVit, 20aa/serum and 20aaVit/serum than after culture in 20aa ($P < 0.05$, Table 7.3).

Table 7.3 Effect of culture of the cow embryo from Day 6 post-insemination (120 h culture) with BSA with or without vitamins and serum with or without vitamins, on development, blastocyst cell number and diameter.

			Development[A] to various stages after					
			144 h culture		168 h culture			
Media* 0-72 h	Media* 72-120 h	Media* 120-168 h	Morula and blastocyst	Blastocyst	Morula and blastocyst	Blastocyst	Total cell number[B]	Diameter[B] µm
NeGln	20aa	20aa	56.4	46.2	57.3	50.8	141.4 ± 9.2	189.9 ± 4.9 [abc]
NeGln	20aaVit	20aaVit	59.5	53.1	59.8	54.7	142.2 ± 8.4	217.5 ± 5.8 [a]
NeGln	20aa	20aa/serum	59.3	51.9	61.4	56.6	157.4 ± 11.5	219.7 ± 7.5 [b]
NeGln	20aaVit	20aaVit/serum	58.6	46.1	58.6	48.2	141.5 ± 11.2	223.5 ± 8.3 [c]

[A]Percentage development from cleaved ova from four replicates; n = 120 cleaved ova per treatment. *Media for hours of culture are defined in section 7.2.2. [B]Data from blastocysts that had reached the blastocyst stage by 144 h culture and were cultured for a further 24 h (values are mean ± SEM). [abc]Like pairs of letters are significantly different, $P < 0.05$.

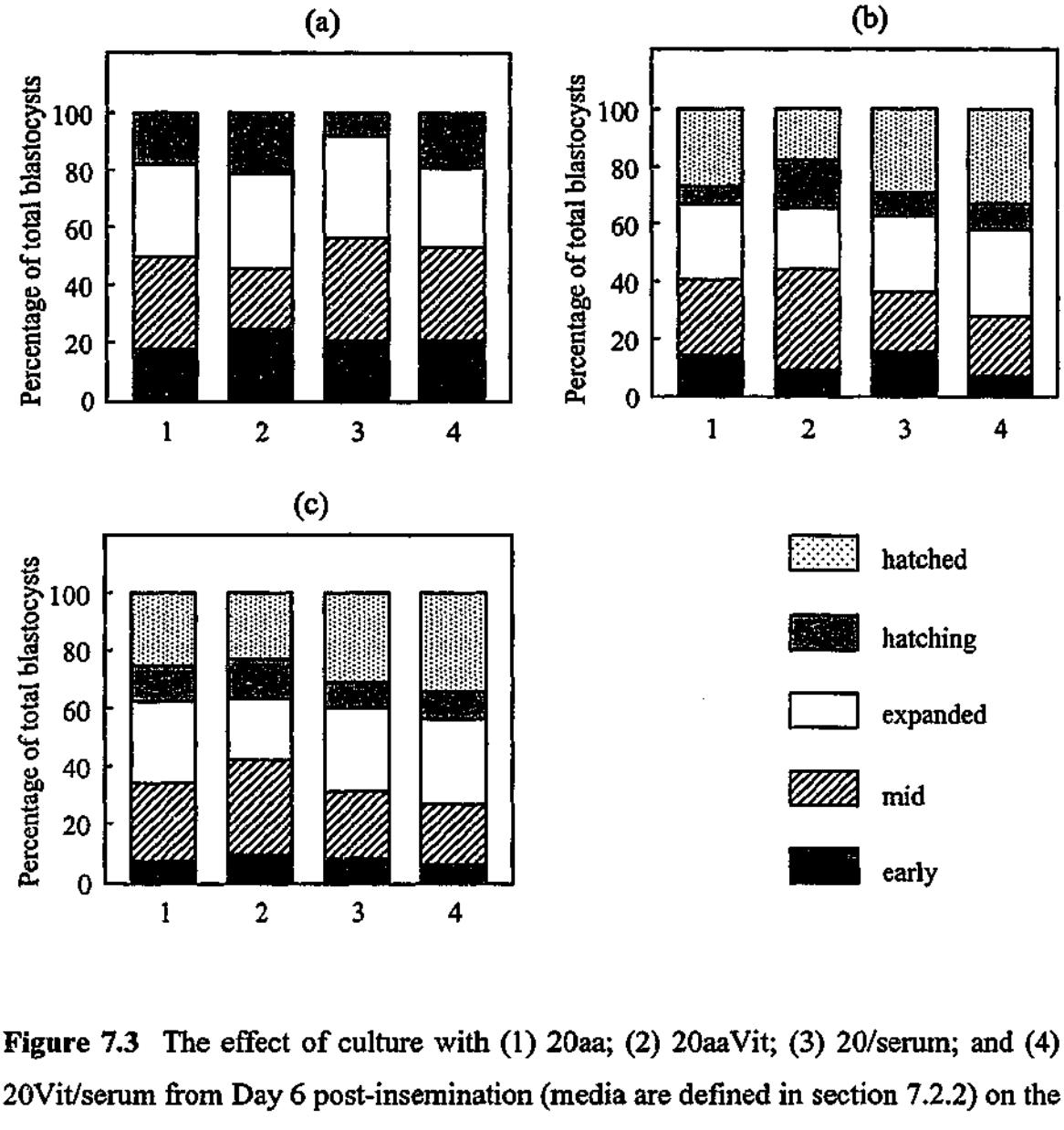


Figure 7.3 The effect of culture with (1) 20aa; (2) 20aaVit; (3) 20/serum; and (4) 20Vit/serum from Day 6 post-insemination (media are defined in section 7.2.2) on the percentage of early, mid, expanded, hatching and hatched blastocysts (of total blastocysts) on (a) Day 7 post-insemination; (b) Day 8 post-insemination; and (c) Day 8 post-insemination for embryos that reached the blastocyst stage on Day 7 post-insemination. All embryos cultured for the first 72 h in NeGln and then in either 20aa or 20aaVit for 48 h to Day 6 post-insemination. Embryos cultured in 20aa were then transferred to either 20aa or 20aa/serum and embryos cultured in 20aaVit were transferred to 20aaVit or 20Vit/serum for culture to Day 8 post-insemination. Data are from four replicates; n = 120 cleaved ova per treatment. No significant differences in blastocyst expansion among treatment groups, $P > 0.05$.

7.3.3 The Effect of Culture with MEM Vitamins on the Metabolism of the Cow Blastocyst.

7.3.3.1 The metabolism of [5-^{3}H] glucose, [2-^{14}C] pyruvate and [G-^{3}H] glutamine by individual blastocysts.

The mean metabolism of [5-^{3}H] glucose by blastocysts was equivalent following culture in 20aa and 20aaVit from Day 4 pi (13.3 ± 1.5 versus 13.3 ± 1.3 pmoles/embryo/h, respectively, $P > 0.05$; Figure 7.4a). For metabolic determinations in the presence of [5-^{3}H] glucose, the mean metabolism of [2-^{14}C] pyruvate was significantly lower for blastocysts cultured in 20aaVit from Day 4 pi, than for blastocysts cultured in 20aa (1.5 ± 0.1 versus 2.0 ± 0.1 pmoles/embryo/h, respectively, $P < 0.05$; Figure 7.4a).

The mean metabolism of [G-^{3}H] glutamine by individual blastocysts was significantly higher following culture in 20aaVit from Day 4 pi, than following culture in 20aa (0.61 ± 0.04 versus 0.50 ± 0.03 pmoles/embryo/h, respectively, $P < 0.05$; Figure 7.4b). For metabolic determinations in the presence of [G-^{3}H] glutamine, the mean metabolism of [2-^{14}C] pyruvate was significantly lower for blastocysts cultured in 20aaVit from Day 4 pi, than for blastocysts cultured in 20aa (1.3 ± 0.01 versus 1.6 ± 0.1 pmoles/embryo/h, respectively, $P < 0.05$; Figure 7.4b).

Blastocyst development was divided into blastocyst stages to further determine the effect of MEM vitamins on blastocyst metabolism. Metabolic determinations for blastocysts at the early and hatching stages were from less than three blastocysts per

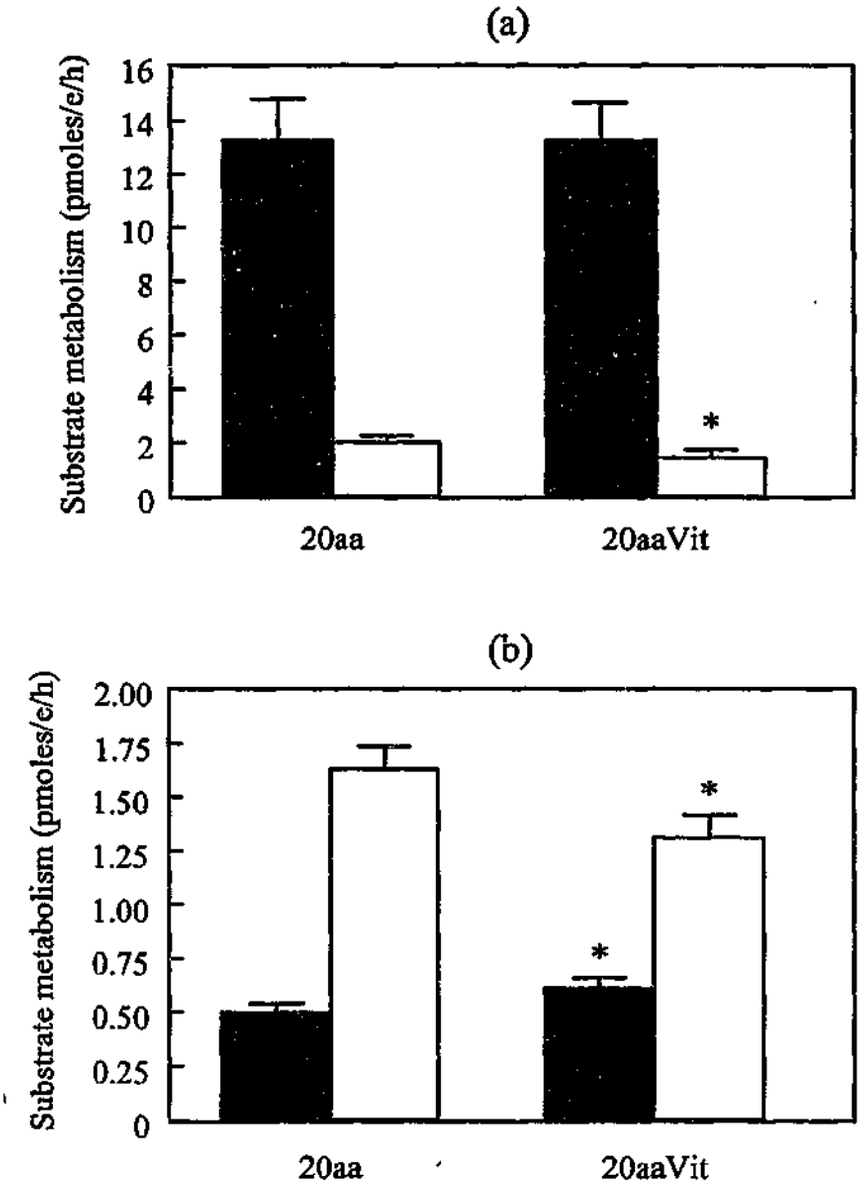


Figure 7.4 Metabolism of (a) [5-^{3}H] glucose (solid bars) and [2-^{14}C] pyruvate (open bars); and (b) [G-^{3}H] glutamine (solid bars) and [2-^{14}C] pyruvate (open bars), by the cow blastocyst (Day 7 post-insemination) following culture in 20aa and 20aaVit for 72 h from Day 4 post-insemination (media are defined in section 7.2.1). Embryos cultured in NeGln from the zygote to Day 4 post-insemination. Metabolic rates are mean ± SEM pmoles/embryo/h (pmoles/e/h). Data are from three replicates for (a) and (b); n ≥ 20 embryos per treatment for (a) and n ≥ 28 embryos per treatment for (b). *Significantly different to 20aa for the same substrate ($P < 0.05$).

treatment per stage. Thus, only the metabolism of blastocysts at the mid and expanded stages was compared for embryos cultured in 20aa and 20aaVit. With respect to culture in 20aa, culture in 20aaVit did not affect the metabolism of [5-^{3}H] glucose by blastocysts at either the mid (10.8 ± 1.2 and 7.7 ± 0.8 pmoles/embryo/h, respectively, $P > 0.05$; Figure 7.5a) or expanded (13.4 ± 1.9 and 15.0 ± 1.8 pmoles/embryo/h, respectively, $P > 0.05$; Figure 7.5a) stages. Culture in 20aaVit did not affect the metabolism of [2-^{14}C] pyruvate by mid blastocysts compared with culture in 20aa (1.1 ± 0.06 and 1.2 ± 0.13 pmoles/embryo/h, respectively, $P > 0.05$; Figure 7.5b) but significantly decreased the metabolism of [2-^{14}C] pyruvate by blastocysts at the expanded stage (1.5 ± 0.1 and 1.9 ± 0.1 pmoles/embryo/h, respectively, $P < 0.01$; Figure 7.5b). Metabolic determinations of [2-^{14}C] pyruvate metabolism were pooled from six replicates for the determination of [2-^{14}C] pyruvate metabolism by different stages of blastocyst development. The metabolism of [G-^{3}H] glutamine by mid blastocysts was not significantly different following culture in 20aa and 20aaVit (0.37 ± 0.07 and 0.44 ± 0.05 pmoles/embryo/h, respectively, $P > 0.05$; Figure 7.5c). The metabolism of [G-^{3}H] glutamine by expanded blastocysts, however, was significantly higher following culture in 20aaVit than in 20aa (0.61 ± 0.04 and 0.51 ± 0.03 pmoles/embryo/h, respectively, $P < 0.05$; Figure 7.5c).

7.3.3.2 The relationship between total blastocyst cell number and the metabolism of [5-^{3}H] glucose, [2-^{14}C] pyruvate and [G-^{3}H] glutamine.

There was a significant positive correlation between blastocyst total cell number and the metabolism of [5-^{3}H] glucose by embryos cultured in both 20aa (r =

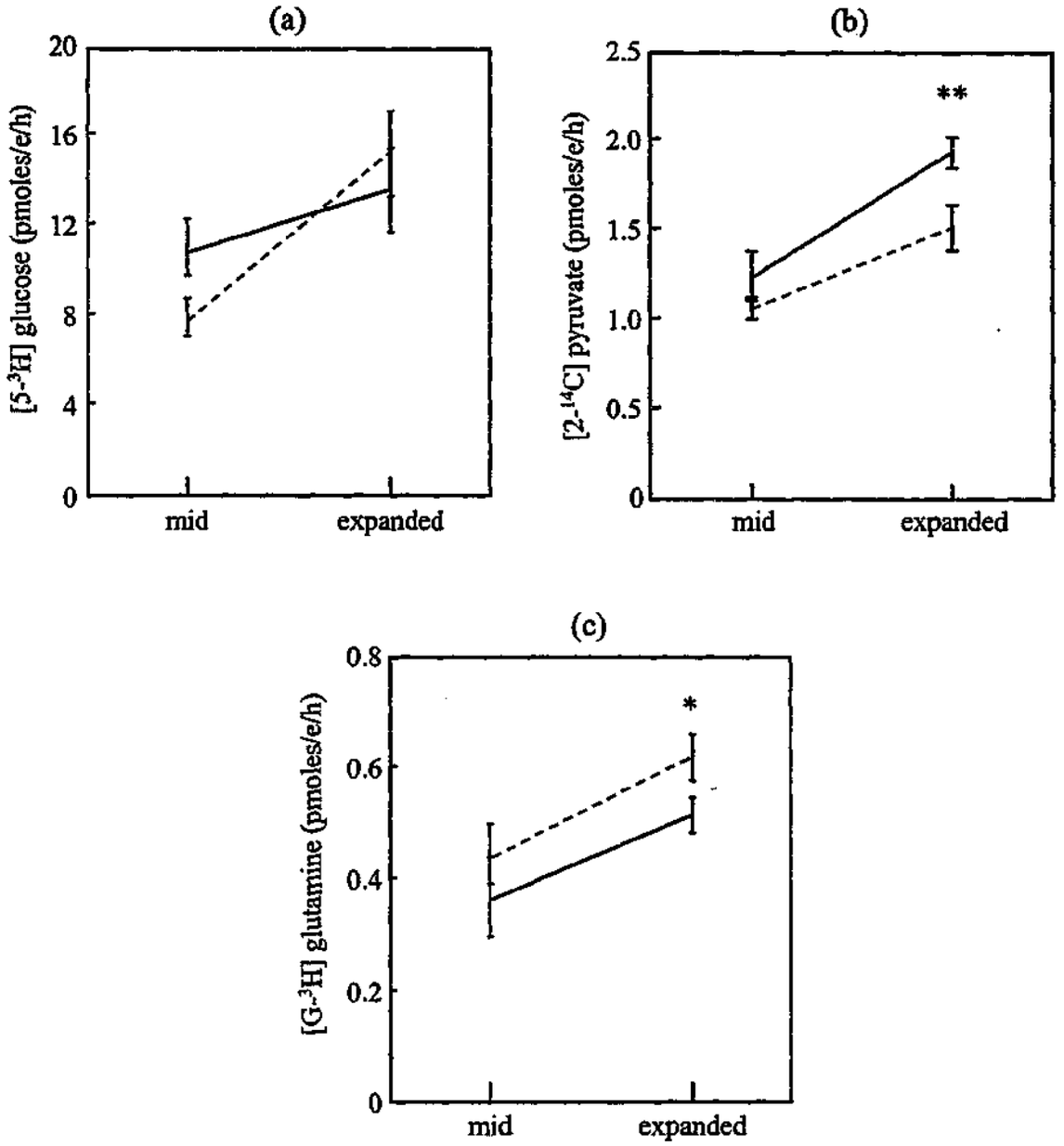


Figure 7.5 Metabolism of (a) [5-^{3}H] glucose; (b) [2-^{14}C] pyruvate; and (c) [G-^{3}H] glutamine by mid and expanded cow blastocysts (Day 7 post-insemination), following culture in 20aa (solid line) and 20aaVit (broken line), for 72 h from Day 4 post-insemination. Embryos cultured in NeGln from the zygote to Day 4 post-insemination. Media are defined in section 7.2.1. Metabolic rates are mean ± SEM pmoles/embryo/h (pmoles/e/h). Data are from three replicates for (a) and (b) and from six replicates for (c). Metabolism significantly different between treatments for same blastocyst stage (*$P < 0.05$, **$P < 0.01$).

0.59, P < 0.01; Figure 7.6a) and 20aaVit (r = 0.48, P < 0.05; Figure 7.6b). Culture in 20aaVit versus culture in 20aa decreased the degree of association between total cell number and [5-^{3}H] glucose metabolism, as evidenced by the level of significance of the correlation coefficients (Figure7.6a and b). Blastocyst cell number was also positively correlated with [2-^{14}C] pyruvate metabolism following culture in 20aa (r = 0.57, P < 0.01; Figure 7.6c) and 20aaVit (r = 0.60, P < 0.01; Figure 7.6d). Furthermore, blastocyst cell number was positively correlated with the metabolism of [G-^{3}H] glutamine, following culture in 20aa (r = 0.50, P < 0.01; Figure 7.6e) and 20aaVit (r = 0.53, P < 0.01; Figure 7.6f).

Culture with MEM vitamins affected the relationships between total cell number and substrate metabolism, as evidenced by the slopes of the lines of best fit, for embryos cultured in 20aa and 20aaVit. Culture in 20aaVit versus culture in 20aa, increased the slope of the line of best fit for the relationship between cell number and the metabolism of both [5-^{3}H] glucose (3.8 and 3.2, respectively; Figure 7.6b and a) and [2-^{14}C] pyruvate (43.8 and 34.7, respectively; Figure 7.6d and c) by approximately 20%. Alternatively, culture in 20aaVit versus culture in 20aa decreased the slope of the line of best fit for the relationship between cell number and the metabolism [G-^{3}H] glutamine (90.2 and 108.8, respectively; Figure 7.6f and e) by approximately 20%.

7.3.3.3 The relationships between the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate and the metabolism of [G-^{3}H] glutamine and [2-^{14}C] pyruvate.

There was a significant positive correlation between the metabolism of [5-^{3}H] glucose and [2-^{14}C] pyruvate following culture in both 20aa (r = 0.45, P = 0.05; Figure 7.7a) and 20aaVit (r = 0.47, P < 0.05; Figure 7.7b). Culture with MEM vitamins

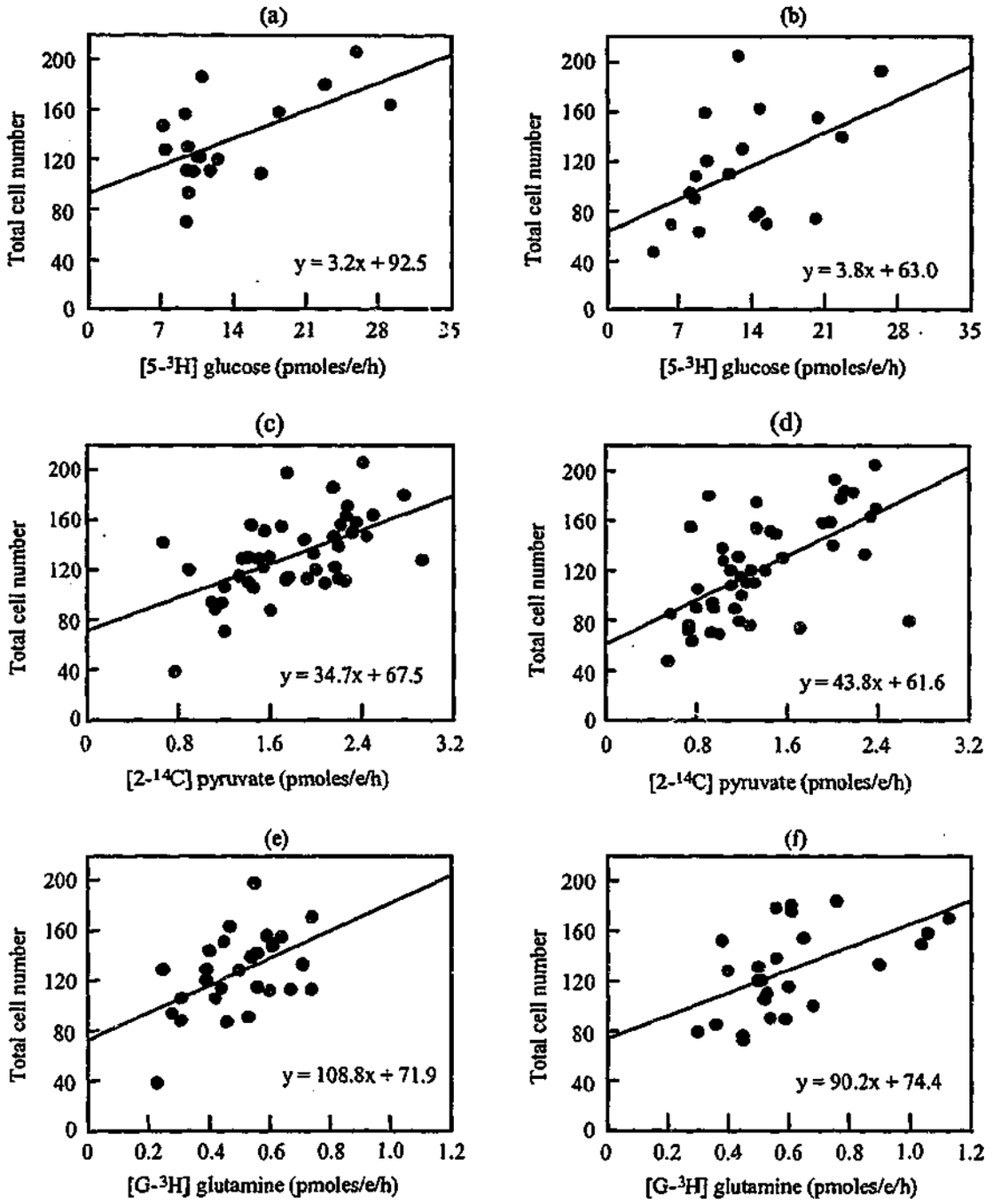


Figure 7.6 Correlations of total blastocyst cell number and (a,b) [5-^{3}H] glucose metabolism, (c,d) [2-^{14}C] pyruvate metabolism and (e,f) [G-^{3}H] glutamine metabolism, by blastocysts cultured from Day 4 pi in (a,c,e) 20aa and (b,d,f) 20aaVit (a: $r = 0.59$, $P < 0.01$; b: $r = 0.48$, $P < 0.05$; c: $r = 0.57$, $P < 0.01$; d: $r = 0.60$, $P < 0.01$; e: $r = 0.50$, $P < 0.01$; f: $r = 0.53$, $P < 0.01$). Metabolic rates are pmoles/embryo/h (pmoles/e/h). Data are from three replicates for a,b,e and f. Data are from six replicates for c and d. Each point represents one embryo.

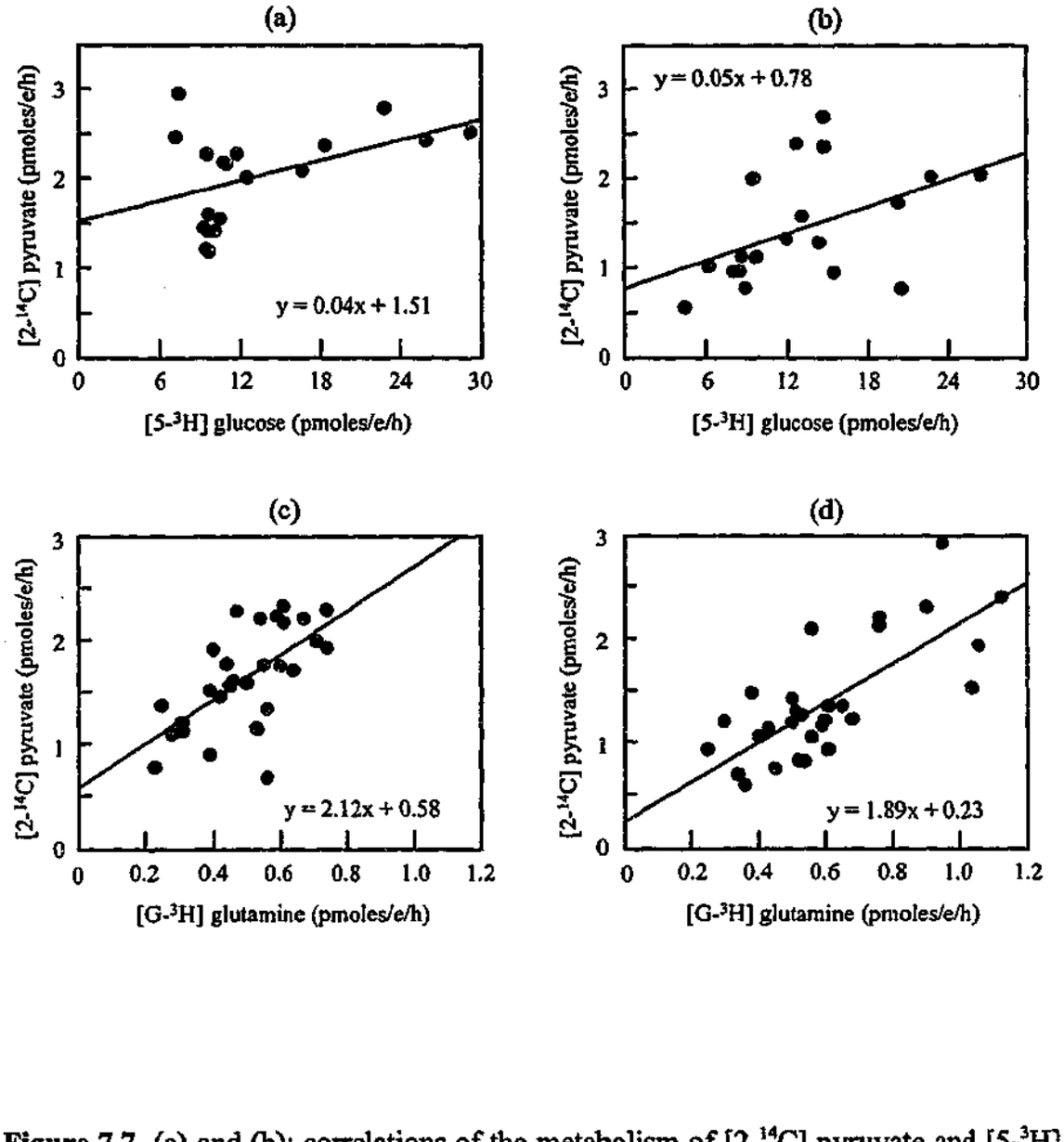


Figure 7.7 (a) and (b): correlations of the metabolism of [2-^{14}C] pyruvate and [5-^{3}H] glucose by cow blastocysts (Day 7 pi), following culture from Day 4 pi in (a) 20aa (r = 0.45, P = 0.05) and (b) 20aaVit (r = 0.47, P < 0.05). (c) and (d): correlations of the metabolism of [2-^{14}C] pyruvate and [G-^{3}H] glutamine by cow blastocysts (Day 7 pi), following culture from Day 4 pi in (c) 20aa (r = 0.63, P < 0.01) and (d) 20aaVit (r = 0.75, P < 0.01). Metabolic rates are pmoles/embryo/h (pmoles/e/h). All embryos were cultured from the zygote to Day 4 pi in NeGln. Media are defined in section 7.2.1. Data are from three replicates for each correlation. Each point represents one embryo.

increased the ratio of [2-^{14}C] pyruvate:[5-^{3}H] glucose metabolism by approximately 25%, as evidenced by the slopes of the lines of best fit for the correlations of [2-^{14}C] pyruvate and [5-^{3}H] glucose metabolism for 20aa (0.04, Figure 7.7a:) and 20aaVit (0.05, Figure 7.7b).

There was a significant positive correlation between the metabolism of [G-^{3}H] glutamine and [2-^{14}C] pyruvate following culture in both 20aa (r = 0.63, P < 0.01; Figure 7.7c) and 20aaVit (r = 0.75, P < 0.01; Figure 7.7d). Culture with MEM vitamins decreased the ratio of [2-^{14}C] pyruvate:[G-^{3}H] glutamine metabolism by approximately 12%, as evidenced by the slopes of the lines of best fit for the correlations of [2-^{14}C] pyruvate and [G-^{3}H] glutamine metabolism for 20aa (2.12, Figure 7.7c) and 20aaVit (1.89, Figure 7.7d).

7.3.4 The Effect of Culture with Specific Vitamins on the Development and Metabolism of the Cow Blastocyst.

7.3.4.1 Development

Culture in Nicotin, Biotin, Folic, Ribo, Myo or Folic/Nicotin/Ribo from Day 4 pi did not affect the development of embryos to the morula and/or blastocyst stages, compared with culture in 20aa (P > 0.05, Table 7.4). Culture with Panto, however, from Day 4 pi significantly increased development of embryos to the blastocyst stage, compared with culture in 20aa (P < 0.05; Table 7.4). Blastocyst development to the early, mid, expanded and hatching stages was not affected by culture in Nicotin, Biotin, Folic, Ribo, Myo, Panto or Folic/Nicotin/Ribo, compared with culture in 20aa

Table 7.4 Effect of culture of the cow embryo with individual vitamins for 72 h from Day 4 post-insemination, on development and total blastocyst cell number.

		Development[A] to various stages after 144 h culture		
Media* 0-72 h	Media** 72-120 h	Morula and blastocyst	Blastocyst	Total cell number[B]
NeGln[1]	20aa	55.5	47.1	95.8 ± 4.8
NeGln[1]	Nicotin	56.7	46.3	95.1 ± 4.6
NeGln[1]	Biotin	52.9	42.8	95.9 ± 5.7
NeGln[1]	Folic	56.7	49.7	92.1 ± 5.2
NeGln[1]	Ribo	53.7	45.1	101.3 ± 5.3
NeGln[2]	20aa	55.4	41.3	96.1 ± 3.8
NeGln[2]	Myo	47.3	39.9	95.5 ± 3.8
NeGln[2]	Panto	51.7	48.9[†]	96.5 ± 3.3
NeGln[2]	Folic/Nicotin/Ribo	52.2	39.7	94.1 ± 4.2

[A]Percentage development from cleaved ova.

*Media for culture 0-72 h (0 = 18-20 h pi). **Media for culture 72-144 h. Media are defined in section 7.2.4. [B]Blastocyst total cell number (mean ± SEM).

[1,2]Separate experiments; data are from seven replicates; n = 186 (1) and 235 (2) cleaved ova per treatment.

[†]Significantly different to control group (20aa) within experiment, within a column ($P < 0.05$).

($P > 0.05$; Figure 7.8a and b). Culture in Nicotin, Biotin, Folic, Ribo, Myo, Panto or Folic/Nicotin/Ribo from Day 4 pi, did not affect blastocyst total cell number, compared with culture in 20aa ($P > 0.05$, Table 7.4).

7.3.4.2 The metabolism of [G-^{3}H] glutamine and [2-^{14}C] pyruvate.

Culture in Nicotin, Biotin, Folic, Ribo, Myo, Panto or Folic/Nicotin/Ribo did not significantly affect the metabolism of either [G-^{3}H] glutamine or [2-^{14}C] pyruvate by blastocysts, with respect to culture in 20aa ($P > 0.05$, Figure 7.9a and b).

The effect of culture with specific vitamins on the metabolism of [G-^{3}H] glutamine and [2-^{14}C] pyruvate was evident, however, when blastocysts were divided into developmental stages. Compared with 20aa, culture in Ribo did not affect the metabolism of [G-^{3}H] glutamine by blastocysts at the early or mid stages, but significantly increased the metabolism of [G-^{3}H] glutamine by expanded blastocysts ($P < 0.05$, Table 7.5 and Figure 7.10a). Likewise, culture in Myo, Panto or Folic/Nicotin/Ribo, significantly increased the metabolism of [G-^{3}H] glutamine by expanded blastocysts, when compared to culture in the absence of vitamins ($P < 0.05$ for Myo and Panto, $P < 0.01$ for Folic/Nicotin/Ribo; Table 7.5 and Figure 7.11a). Alternately, culture with the combination of riboflavin, nicotinamide and riboflavin significantly decreased the metabolism of [2-^{14}C] pyruvate by early blastocysts with respect to culture in 20aa ($P < 0.05$, Table 7.5, Figure 7.11b), but had no affect on the metabolism of the mid and expanded stages ($P > 0.05$, Table 7.5, Figure 7.11b). Culture with biotin decreased the metabolism of [2-^{14}C] pyruvate by blastocysts at the early, mid and expanded stages compared with culture in 20aa, but metabolism was significantly lower at only the mid blastocyst stage ($P < 0.05$, Table 7.5 and Figure 7.10b).

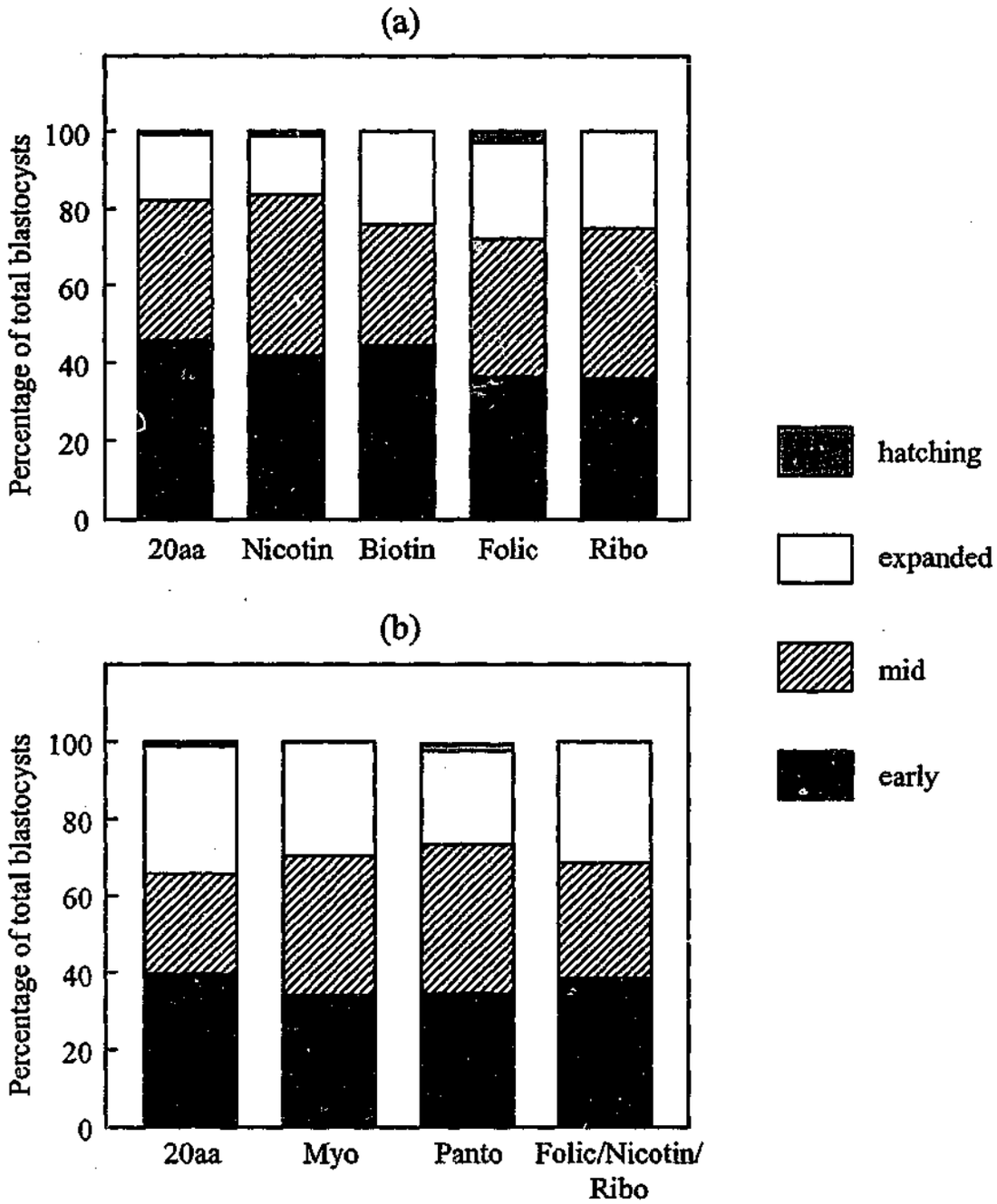


Figure 7.8 The effect of culture with individual vitamins (media are defined in section 7.2.4) for 72 h from Day 4 post-insemination, on the percentage of early, mid, expanded and hatching blastocysts (of total blastocysts). Embryos cultured in NeGln for the first 72 h to Day 4 post-insemination. Data are from seven replicates for (a) and (b). n = 186 (a) and 235 (b) cleaved ova per treatment. No significant differences in blastocyst expansion between treatment groups and control (20aa), $P > 0.05$.

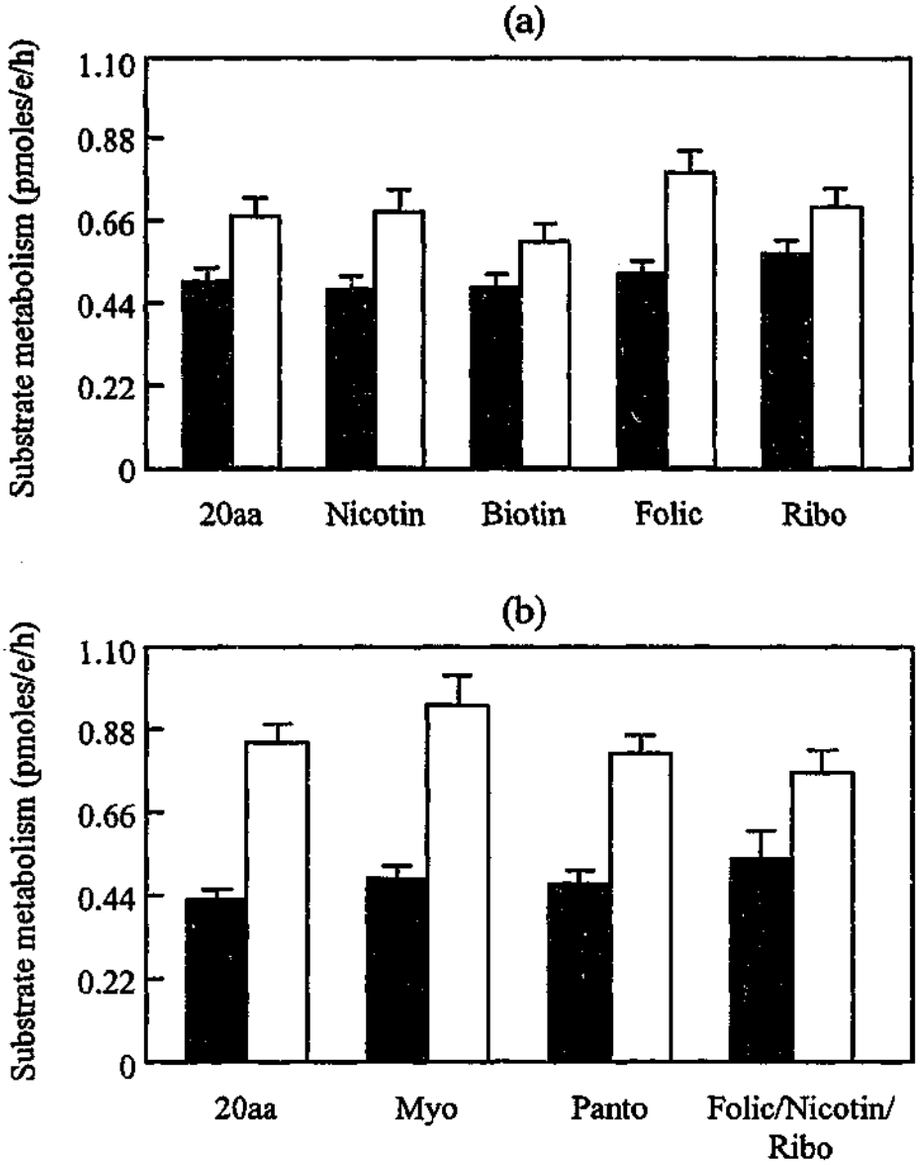


Figure 7.9 (a) and (b): metabolism of [G-^{3}H] glutamine (solid bars) and [2-^{14}C] pyruvate (open bars) by the cow blastocyst (Day 7 post-insemination) following culture with individual vitamins for 72 h from Day 4 post-insemination (media are defined in section 7.2.4). Embryos cultured in NeGln from the zygote to Day 4 post-insemination. Metabolic rates are mean ± SEM pmoles/embryo/h (pmoles/e/h). Data are from six replicates for (a) and (b); $n \geq 50$ embryos per treatment for (a) and $n \geq 58$ embryos per treatment for (b). No significant differences between treatments and 20aa for the same substrate ($P > 0.05$).

Table 7.5 Metabolism of [G-^{3}H] glutamine and [2-^{14}C] pyruvate by various stages of cow blastocysts (Day 7 post-insemination), cultured with individual vitamins for 72 h from Day 4 post-insemination.

	[G-^{3}H] glutamine (pmoles/embryo/h)			[2-^{14}C] pyruvate (pmoles/embryo/h)		
	Early	Mid	Expanded	Early	Mid	Expanded
20aa[1]	0.36 ± 0.04^{a} (18)	0.48 ± 0.03 (21)	0.62 ± 0.05^{a} (17)	0.46 ± 0.05^{a} (18)	0.64 ± 0.04^{a} (21)	0.92 ± 0.06^{a} (17)
Nicotin[1]	0.31 ± 0.03^{ab} (15)	0.45 ± 0.04^{a} (21)	0.63 ± 0.06^{b} (17)	0.39 ± 0.03^{a} (15)	0.67 ± 0.05^{a} (21)	0.92 ± 0.08^{a} (17)
Biotin[1]	0.38 ± 0.05^{a} (15)	0.45 ± 0.05 (16)	0.62 ± 0.05^{a} (19)	0.41 ± 0.05^{a} (15)	0.48 ± 0.05^{b}*(16)	0.83 ± 0.07^{ab} (19)
Folic[1]	0.41 ± 0.05^{a} (14)	0.52 ± 0.04 (16)	0.60 ± 0.04^{a} (22)	0.51 ± 0.06^{a} (14)	0.74 ± 0.08 (16)	0.98 ± 0.07^{a} (22)
Ribo[1]	0.39 ± 0.05^{a} (15)	0.51 ± 0.05^{b} (19)	0.75 ± 0.04^{ab}* (22)	0.42 + 0.04^{a} (15)	0.64 ± 0.05^{a} (19)	0.96 ± 0.06^{a} (22)
20aa[2]	0.28 ± 0.04^{ab} (18)	0.48 ± 0.03^{a} (20)	0.50 ± 0.03^{b} (26)	0.54 ± 0.05^{a} (18)	0.83 ± 0.06^{a} (26)	1.05 ± 0.06^{a} (26)
Myo[2]	0.26 ± 0.02^{ab} (14)	0.50 ± 0.03^{a} (24)	0.61 ± 0.03^{b}* (21)	0.61 ± 0.05^{a} (14)	0.83 ± 0.05^{a} (24)	1.17 ± 0.09^{a} (21)
Panto[2]	0.28 ± 0.02^{a} (16)	0.45 ± 0.03^{a} (21)	0.62 ± 0.04^{a}* (27)	0.46 ± 0.04^{a} (16)	0.77 ± 0.05^{a} (21)	1.06 ± 0.05^{a} (27)
Folic/Nicotin/Ribo[2]	0.21 ± 0.02^{a} (17)	0.46 ± 0.03^{a} (23)	0.67 ± 0.04^{a}**(18)	0.36 ± 0.04^{a}*(17)	0.76 ± 0.04^{a} (23)	1.15 ± 0.06^{a} (18)

[1,2]Separate experiments; data for each experiment are from six replicates; values are mean ± SEM. Media are defined in section 7.2.4. [ab]Like pairs are significantly different within a row for the same substrate. *,**Significantly different to control (20aa) in same experiment, within same column: * $P < 0.05$; ** $P < 0.01$.

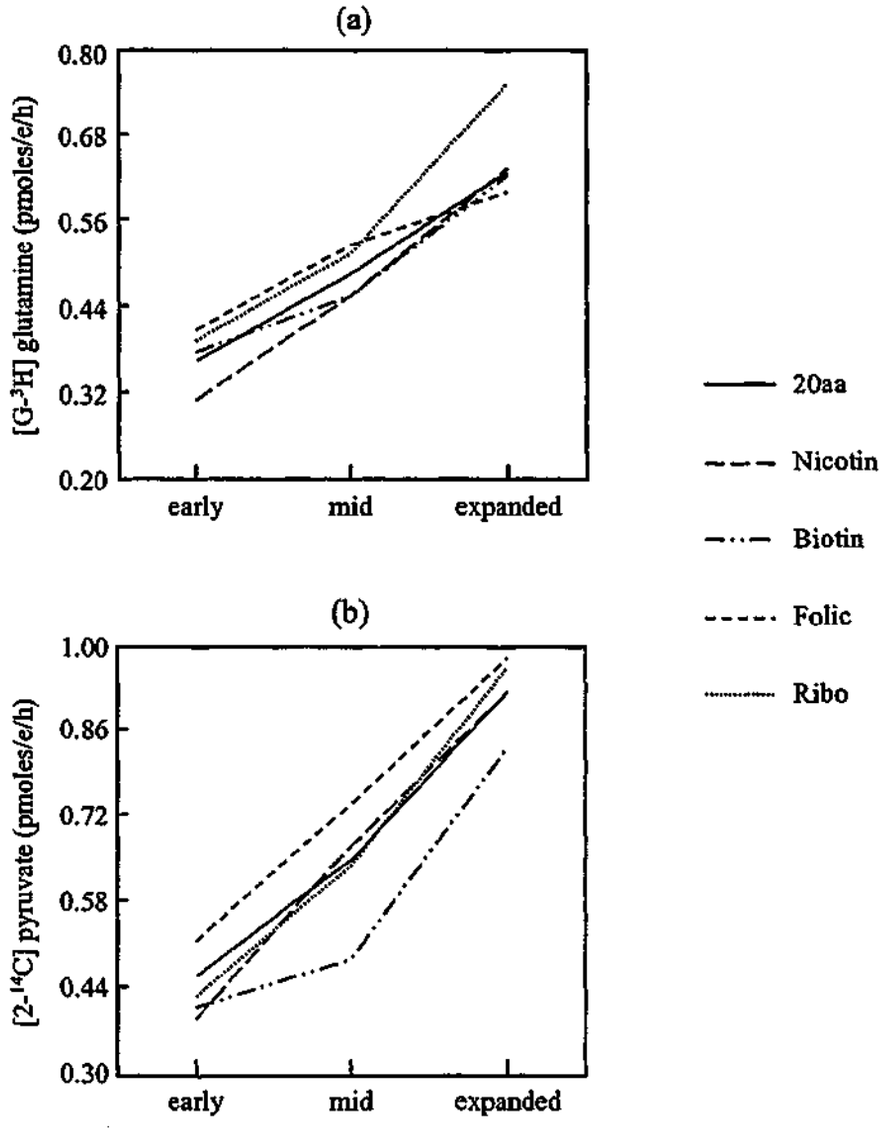


Figure 7.10 Metabolism of (a) [G-^{3}H] glutamine; and (b) [2-^{14}C] pyruvate by early, mid and expanded cow blastocysts (Day 7 post-insemination) following culture in 20aa, Nico, Biotin, Folic and Ribo for 72 h from Day 4 post-insemination. Embryos were cultured in NeGln from the zygote to Day 4 post-insemination. Media are defined in section 7.2.4. Data are from six replicates. Metabolic rates are mean pmoles/embryo/h (pmoles/e/h). Note that figures do not show the SEM of each mean nor the significant differences among means. These are reported in Table 7.5.

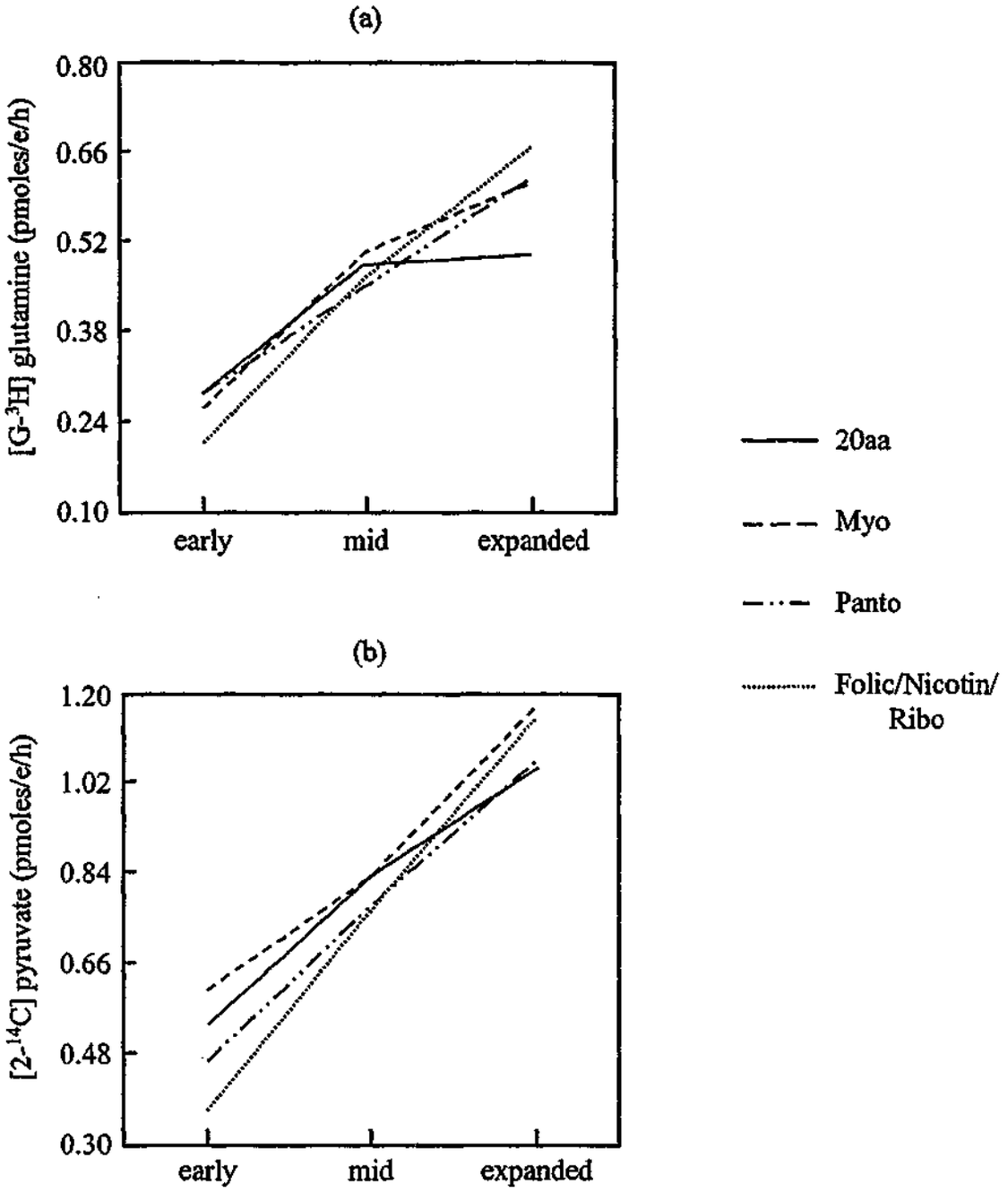


Figure 7.11 Metabolism of (a) [G-^{3}H] glutamine; and (b) [2-^{14}C] pyruvate by early, mid and expanded cow blastocysts (Day 7 post-insemination), following culture in 20aa, Myo, Panto, Folic/Nicotin/Ribo, for 72 h from Day 4 post-insemination. Embryos were cultured in NeGln from the zygote to Day 4 post-insemination. Metabolic rates are mean ± SEM pmoles/embryo/h (pmoles/e/h). Media are defined in section 7.2.4. Data are from six replicates. Note that figures do not show the SEM of each mean or the significant differences among means. These are reported in Table 7.5.

There was a significant increase in the metabolism of [G-^{3}H] glutamine between blastocysts at the early and expanded stages, in all treatment groups ($P < 0.05$, Table 7.5). The metabolism of [G-^{3}H] glutamine was not significantly different between the mid and expanded stages, for embryos cultured in 20aa ($P > 0.05$, Table 7.5). Following culture in Ribo, Panto and Folic/Nicotin/Ribo, however, the metabolism of [G-^{3}H] glutamine significantly increased between the mid and expanded stages ($P < 0.05$, Table 7.5).

The metabolism of [2-^{14}C] pyruvate significantly increased between the early, mid and expanded stages in all treatment groups ($P < 0.05$, Table 7.5) except for Folic. Following culture in Folic, the metabolism of [2-^{14}C] pyruvate by expanded blastocysts was significantly higher than the metabolism of early blastocysts ($P < 0.05$, Table 7.5), however, the metabolism of mid blastocysts was not significantly different to the metabolism of either early or expanded blastocysts ($P > 0.05$).

7.4 DISCUSSION

Despite the fact that the water soluble B-complex vitamins play an important role in cellular metabolism as components of coenzymes, there have been very few attempts to determine their role in the development of the in vitro produced cow embryo. This has largely been due to reports of the null affect of culture with MEM vitamins on cow blastocyst development (Takahashi and First, 1992; Rosenkrans and First, 1994) and cell number (Takahashi and First, 1992). The fact that MEM vitamins do not stimulate development of the cow embryo to the blastocyst stage, however, does not rule out the possibility that such vitamins could have an important role in sustaining embryo viability. Similarly, this was demonstrated for amino acids during culture of the mouse embryo (Lane and Gardner, 1997a). The present study revealed that vitamins affected development of the cleavage stage cow embryo and expansion of the cow blastocyst. Further, the study showed that vitamins had a significant impact on the metabolism of glutamine and pyruvate by the cow blastocyst.

The present study confirmed previous findings that culture with MEM vitamins did not stimulate development of the cow embryo to the morula and blastocyst stages on Day 7 pi (Takahashi and First, 1992; Rosenkrans and First, 1994). Rosenkrans and First (1994) found that culture with MEM vitamins actually slightly suppressed blastocyst development in the cow. Although subsequent blastocyst development and cell number were not affected in the present study, culture of the early cleavage stage cow embryo for the initial 72 h with MEM vitamins, inhibited development beyond the 8-cell stage. The inhibitory effect of MEM vitamins was not evident, however, after either 22 h or 52 h culture. Thus, vitamins were omitted from culture media for the first 72 h, for the remainder of the study.

Culture with MEM vitamins beyond Day 4 pi, did not affect blastocyst cell number or development of the blastocyst to the early, mid, expanded or hatched stages, on Day 7 pi. It has previously been reported that the hamster embryo required vitamins for hatching (Kane and Bavister, 1988b), while vitamins were found to be necessary for expansion of the rabbit blastocyst (Kane, 1988). Due to the fact that few cow blastocysts had commenced hatching on Day 7 pi, together with the observation that approximately 30% of cow blastocysts were at the early stage and had thus not commenced expansion, the effect of MEM vitamins was also studied on Day 8 pi. Culture with MEM vitamins did not affect development to the blastocyst or cell number on Day 8 pi and did not affect the number of blastocysts at the expanded, hatching or hatched stages. MEM vitamins did, however, affect expansion of Day 7 blastocysts on Day 8 pi, as evidenced by an increase in blastocyst diameter following culture with MEM vitamins. Myo-inositol, pyridoxine, riboflavin and nicotinamide could be the vitamins responsible for the observed increase in blastocyst diameter, as these vitamins increased expansion of the rabbit blastocyst (Kane, 1989). Myo-inositol has been shown to be involved in the regulation of cell volume in somatic cells (Isaacks *et al.*, 1994; Burg, 1995). Pyridoxine is involved in the transamination of amino acids, while riboflavin and nicotinamide both have an important role in cellular metabolism, as components of coenzymes for the oxidation of energy substrates.

Culture of Day 7 blastocysts to Day 8 pi in the presence of serum, resulted in an equivalent increase in blastocyst diameter as culture with MEM vitamins. Thompson *et al.* (1998b) reported a similar effect of serum on blastocyst diameter following culture from the zygote to Day 7 pi. Of significance is the fact that the diameter of

blastocysts cultured in the presence of serum was equivalent to that of in vivo derived blastocysts (Thompson *et al.*, 1998b). It is not known whether myo-inositol is a component of cow serum, however, it is not present in human serum (see Table 1.5). The stimulatory effect of serum on blastocyst diameter may be due to the presence of growth factors (Kane *et al.*, 1997). Recently, Giliam and Gardner (unpublished data) found that both serum and vitamins were necessary for culture of the cow embryo, for an extended period beyond Day 7 pi. One of the aims of the present study was to determine whether the synergistic effect between MEM vitamins and serum on blastocyst development and expansion, was evident by Day 8 pi when many blastocysts are transferred to recipient cows. It has been suggested that vitamins, specifically inositol, may be associated with the action of specific growth factors (Fahy and Kane, 1993). In the present study, however, there was no evidence of a synergistic effect between MEM vitamins and serum on blastocyst development, expansion or cell number, on Day 8 pi. The fact that blastocyst development and cell number were equivalent following culture with serum and BSA, contradicts previous reports of the stimulatory effects of serum on cow embryo development (Pinyopummintr and Bavister, 1994; Van Langendonckt *et al.*, 1997; Thompson *et al.*, 1998a). In the present study, however, serum was added to media on Day 6 pi. Previous studies have shown that the stimulatory effects of serum were dependent on the time of the addition of serum, with the stimulatory effects occurring between the 9- to 16-cell and morula stages (Pinyopummintr and Bavister, 1994; Van Langendonckt *et al.*, 1997). In the present study, embryos were cultured with serum from Day 6 pi for two reasons. Firstly, as stated above, MEM vitamins and serum were both found to be required beyond Day 7 pi for growth and expansion of the cow

blastocyst (Gardner and Giliam, unpublished data). Secondly, despite the fact that culture of cow embryos with serum from Day 5 pi improved blastocyst development, embryo survival post-transfer was reduced with respect to embryos cultured with BSA in the absence of serum (Thompson *et al.*, 1998a) Extended culture of ruminant embryos with serum has been found to result in abnormal mitochondrial ultrastructure (Dorland *et al.*, 1994; Thompson *et al.*, 1995), abnormally early blastocoel formation (Walker *et al.*, 1992; Thompson *et al.*, 1995) and perturbations in energy metabolism (Gardner, 1994).

Previous studies in the cow embryo have used only morphological criteria to assess the effect of vitamins. The present study revealed that, as well as increasing blastocyst diameter on Day 8 pi, MEM vitamins had a significant effect on the activity of the TCA cycle in the cow blastocyst, Day 7 pi. Culture with MEM vitamins resulted in a significant increase in glutamine oxidation, with a concomitant decrease in pyruvate oxidation. Interestingly, the effect of MEM vitamins on the metabolism of glutamine and pyruvate was dependent on the stage of blastocyst development, with metabolism affected in the expanded blastocyst, but not in the mid blastocyst. This effect was also evident in the change of the rate of substrate metabolism with an increase in blastocyst cell number, following culture in the presence and absence of vitamins. In the presence of MEM vitamins, pyruvate oxidation was suppressed and glutamine oxidation was elevated with an increase in blastocyst cell number, compared with embryos cultured in the absence of vitamins. Furthermore, the correlation of pyruvate and glutamine metabolism in individual blastocysts revealed that culture with MEM vitamins reduced the ratio of pyruvate:glutamine metabolism. Thus, MEM vitamins increased the activity of one section of the TCA cycle, but

decreased the activity of another section. This indicates that specific vitamins may inhibit the activity of the TCA cycle in the section from oxaloacetate to α-ketoglutarate. The observed increase in glutamine oxidation could be due either to compensation for the reduction in the activity in the first half of the TCA cycle, or to stimulation of the TCA cycle in the section from α-ketoglutarate to oxaloacetate by specific vitamins.

Surprisingly, culture with MEM vitamins did not significantly affect the glycolytic activity of the cow blastocyst. While the relationship between cell number and glycolytic activity appeared to be affected by the presence of MEM vitamins, this was most likely due to sample size, as the correlations between the two parameters were not equivalent in 20aa and 20aaVit. Gardner *et al.* (1994b) found that the addition of MEM vitamins to SOF containing amino acids, significantly increased both the uptake of glucose and the production of lactate by the sheep blastocysts on a per cell basis. Metabolic rates were not corrected for cell number in the present study, as blastocyst cell number was not significantly affected by culture with MEM vitamins. The discrepancy in the effect of MEM vitamins on glycolytic activity is likely due to the more advanced development of embryos in the study by Gardner *et al.* (1994b). In that study, the proportion of hatching blastocysts was three to four times higher than the highest rate of hatching observed in the present study on Day 7 pi, and blastocyst development was approximately twice that in the present study. In the study by Gardner *et al.* (1994b), cultured embryos were from in vivo matured and in vivo fertilized oocytes. Thus, the stimulatory effects of MEM vitamins on the glycolytic activity of ruminant blastocysts may not be evident in the earlier stages of blastocyst development. MEM vitamins did not significantly affect the rates of

glucose uptake and lactate production in the mouse blastocyst when added to media containing amino acids, however, there was a synergistic effect between amino acids and vitamins in reducing the proportion of glucose converted to lactate (Lane and Gardner, 1998). Importantly, culture of in vivo produced mouse blastocysts for 6 h in media containing amino acids and MEM vitamins, sustained embryo viability equivalent to that of in vivo blastocysts, compared with a decrease in viability of embryos cultured in the absence of amino acids and vitamins.

The determination of the effect of specific MEM vitamins on blastocyst metabolism revealed that riboflavin, myoinositol and pantothenate all significantly increased glutamine metabolism in the expanded blastocyst, without affecting pyruvate oxidation. Riboflavin is a component of the coenzyme FAD and pantothenate is a pre-cursor of coenzyme A (CoA). Both FAD and CoA are cofactors in the oxidative carboxylation of α-ketoglutarate which is the entry point for glutamine into the TCA cycle (Barbehenn *et al.*, 1974; Anbari and Schultz, 1993). FAD and CoA are also cofactors in the oxidative carboxylation of pyruvate, however, pyruvate oxidation was not affected. Rieger *et al.* (1992b) reported a similar increase in glutamine oxidation in the cow blastocyst without a concomitant increase in pyruvate oxidation. As stated by Rieger *et al.* (1992b), this phenomenon may be similar to that observed in proliferating adrenocortical cells in vitro (Hornsby and Gill, 1981), possibly resulting from discrepancies in the sensitivities of NAD^+-substrate-dehydrogenases to inhibition by NADH, in different sections of the TCA cycle (Hornsby, 1982). It is interesting that, in the study by Rieger *et al.* (1992b), pyruvate oxidation did not significantly increase with blastocyst development. In the present study, pyruvate oxidation significantly increased between the early and

expanded blastocyst stages, both in the presence and absence of specific vitamins. The reason for this is unknown, however, vastly different culture systems were used in the two studies. In the study by Rieger *et al.* (1992b), embryos were cultured in complex medium in a co-culture system, containing serum. Interestingly, glutamine oxidation by expanded blastocysts was four to five times higher in the study by Rieger *et al.* (1992b) than in the present study. Thus, if there is in fact differential sensitivity to NADH in different sections of the TCA cycle, the higher rate of glutamine metabolism by blastocysts in the study by Rieger *et al.* (1992b), along with the incongruous metabolism of glutamine and pyruvate, may indicate that a NADH sensitivity threshold had already been reached.

Blastocyst formation and development are energetically demanding. Unlike the mouse, the ruminant blastocyst undergoes an extensive period of proliferation and expansion prior to attaching to the uterine wall. An increase in metabolic activity would be required for the production of biosynthetic intermediates and ATP, necessary for the marked increase in protein synthesis (Frei *et al.*, 1989). Furthermore, energy demands would increase to support the activity of Na^+-K^+ ATPase, for generation, expansion and maintenance of the blastocoel (Overstrom *et al.*, 1989; Donnay and Leese, 1999). It was recently demonstrated that ATP production via oxidative phosphorylation was indeed important for cow embryo development from Day 5 to Day 7 pi (Thompson *et al.*, 2000). An increase in glutamine metabolism through the TCA cycle will ultimately have an effect on oxidative phosphorylation via the generation of NADH and $FADH_2$. Thus, riboflavin, myo-inositol and pantothenate may be important components of culture media for the developing cow blastocyst, through their stimulation of the activity of part of the TCA cycle. Pantothenate was, in

fact, the only vitamin found to stimulate development to the blastocyst stage. Pantothenate was also recently reported to increase development of hamster embryos to the blastocyst stage (McKiernan and Bavister, 2000). Interestingly, culture of hamster embryos with pantothenate also increased embryo viability, as evidenced by an increase in the percentage of live fetuses recovered following transfer of embryos to recipient hamsters (McKiernan and Bavister, 2000). It would be most interesting to determine whether the observed increase in viability of hamster embryos following culture with pantothenate, was associated with an increase in glutamine metabolism. Recently, Holm *et al.* (1999) reported that the addition of myo-inositol to SOFaa containing citrate, overcame the negative effect on cow embryo development and cell number of the removal of protein from the culture medium. Riboflavin significantly increased blastocyst cell number in the mouse (Tsai and Gardner, 1994) but, like all other vitamins tested, had no effect on cell number in the present study. In the study by Tsai and Gardner (1994), however, the stimulatory effect of riboflavin on cell number was determined in the absence of amino acids. Interestingly, riboflavin, myo-inositol and pantothenate were all required for normal development of rat embryos, either explanted on Day 9 of gestation (Cockroft, 1988) or cultured in bottles at the head-fold stage (Cockroft, 1979).

Folic acid and nicotinamide were also required by the post-implantation rat embryo (Cockroft, 1979; Cockroft, 1988). Nicotinamide stimulated expansion of the rabbit blastocyst (Kane, 1988), however, nicotinamide was at the concentration found in Ham's F-10 medium and was thus almost four times the concentration of nicotinamide in MEM vitamins. Culture of the mouse embryo with nicotinamide at the concentration present in Ham's F-10 medium significantly reduced embryo

viability, as evidenced by a reduced blastocyst cell number and a decreased pregnancy rate and fetal weight (Tsai and Gardner, 1994). Folic acid and nicotinamide, however, did not affect blastocyst development, cell number or metabolism of the cow blastocyst at any stage. Furthermore, the addition of both folic acid and nicotinamide to medium containing riboflavin, did not result in a synergistic effect on the metabolism of glutamine or pyruvate by the expanded blastocyst, nor did they affect the stimulatory action of riboflavin on glutamine metabolism. It is not known why culture in Folic/Nicotin/Ribo significantly reduced pyruvate oxidation at the early blastocyst stage, however, pyruvate oxidation was equivalent to blastocysts in 20aa, by the expanded stage.

While several of the individual MEM vitamins tested could account for the observed increase in glutamine metabolism by the expanded blastocyst in the presence of MEM vitamins, none of the vitamins tested could account for the observed decrease in pyruvate metabolism. The fact that MEM vitamins significantly increased the diameter of Day 7 blastocysts on Day 8 pi, indicates that energy production was not compromised in these blastocysts, compared with those cultured in the absence of MEM vitamins. Interestingly, Donnay and Leese (1999) recently reported that while oxidative metabolism had a role in expansion of the cow blastocoel, expansion of the blastocoel was not in fact dependent on oxidative phosphorylation. Donnay and Leese (1999) suggested that the embryo could adapt to an inhibition of oxidative phosphorylation by increasing energy production via glycolysis. In the present study, however, glycolytic activity was not affected by vitamins, indicating that a reduction in pyruvate oxidation was not compensated for by an increase in glycolysis. Pyridoxine, a vitamin present in MEM vitamins, is involved in the transamination of

amino acids. Thus, the observed decrease in pyruvate oxidation may have reflected a decrease in exogenous pyruvate metabolism, resulting from an increase in the utilization of amino acids as alternative energy sources.

The BME vitamin solution is identical to MEM vitamins, with the exception of the presence of biotin in the BME solution. The concentration of biotin in the BME solution is approximately four times greater than the concentration of biotin that was shown to inhibit development of the mouse embryo (Shirley, 1989). Thus, the effect of biotin at the concentration in BME vitamins was tested in the present study. Biotin did not affect blastocyst development, cell number or glutamine metabolism, but significantly reduced the metabolism of pyruvate by blastocysts at the mid stage. Although not significant, pyruvate metabolism was also lower at the expanded stage, following culture in biotin. Thus, at the concentration present in BME vitamin solution, biotin may be detrimental to the cow embryo.

In conclusion, the effect of vitamins in culture of the cow embryo increased with development of the blastocyst. Vitamins significantly increased the activity of part of the TCA cycle in expanded blastocysts on Day 7 pi, specifically the segment from α-ketoglutarate to oxaloacetate. This increase in metabolic activity on Day 7 pi could be associated with the observed increase in the diameter of the Day 7 blastocyst by Day 8 pi. The individual vitamins riboflavin, myo-inositol and pantothenate, which have been shown to stimulate expansion of the rabbit blastocyst and post-implantation development in the rat embryo, all significantly increased the activity of the TCA cycle in the expanded blastocyst, with respect to glutamine metabolism, but did not affect the earlier stages. Due to the fact that vitamins do not appear to be required prior to the blastocyst stage, along with the fact that vitamins inhibited cleavage

during the first 72 h, it is recommended that vitamins are added to culture media from Day 4 pi. This study revealed that vitamins do affect the cow embryo and that the addition of vitamins to culture media for the developing blastocyst may be important for improving embryo viability. Future studies are, thus, required to assess the role of vitamins in cow blastocyst viability following uterine transfer.

CHAPTER 8

CONCLUDING DISCUSSION

8.1 CARBOHYDRATES

This study is the first report of the non invasive determination of the linear rate of carbohydrate uptakes by individual cow embryos, under conditions similar to those in which embryos are routinely cultured. That is, nutrient uptakes were measured in the presence of glucose, lactate, pyruvate, glutamine and the non-essential and essential amino acids, at 5% CO_2, 7% O_2 and 88% N_2. In addition, this study reports the effects of culture conditions on the relationship between carbohydrate metabolism and embryo development in cow embryos.

8.1.1 Glucose

In the present study, glucose uptake by cow embryos was found to be low and relatively constant during development from the zygote to the 8- to 16-cell stage (2.4 to 3.0 pmoles/embryo/h). Glucose uptake then increased exponentially from the 16-cell stage to the blastocyst. The main increase in glucose uptake was from the morula stage to the blastocyst stage, 6.7 pmoles/embryo/h to 17.6 pmoles/embryo/h, respectively. Interestingly, the observed pattern and level of glucose uptakes were similar to those reported by Thompson *et al.* (1996) despite the fact that, in the study by Thompson *et al.* (1996), uptakes were determined in the absence of amino acids in a HEPES-buffered culture system, at 20% O_2, with serum present from Day 4 of culture. Furthermore, nutrient uptakes were end-point determinations taken as the average uptake from a group of embryos (Thompson *et al.*, 1996).

The metabolism of glucose through the EMP was also found to increase markedly from the 8- to 16-cell stage to the blastocyst, from 1 pmole/embryo/h to

13.9 pmoles/embryo/h, respectively. Interestingly, the metabolism of glucose via the EMP by the cow oocyte after 24 h maturation, was approximately equivalent to that determined for the 8- to 16-cell embryo (0.77 pmoles/embryo/h and 1.0 pmole/embryo/h, respectively). This is reflective of the fact that the oocyte and early cleavage stage embryo are largely under regulation of the maternal genome. The major onset of activation of the embryonic genome in cow embryos occurs at the 8-cell stage (Telford *et al.*, 1990). Thus, the physiology of the early cleavage stage embryo is likely to be similar to that of the oocyte. This was further evident in the oxidation of pyruvate by the mature oocyte (0.65 pmoles/oocyte/h) and the 8- 16-cell embryo (0.43 pmoles/embryo/h) when compared to the oxidation of pyruvate by the blastocyst (1.6 pmoles/embryo/h).

In the present study, glucose uptake by cow embryos at the 8- to 16-cell stage in 20aa was found to be 2.7 pmoles/embryo/h, while the metabolism of glucose via the EMP was found to be 1.0 pmole/embryo/h. Direct statistical comparisons could not be made of glucose uptake and the metabolism of glucose through the EMP, as measurements were not taken simultaneously, from the same cohort of embryos. The observed values, however, are likely not significantly different. The determination of the metabolism of glucose through the EMP is in fact reflective of total glucose metabolism by the embryo. The metabolism of [5-^{3}H] glucose is in fact a measure of the activity of both the EMP and the PPP (Tiffen *et al.*, 1991). In addition, very little glucose is oxidized through the TCA cycle in cow embryos (Rieger and Guay, 1988; Rieger *et al.*, 1992a, 1992b). At the blastocyst stage, the metabolism of glucose via the EMP in 20aa (17.6 pmoles/embryo/h) was similar to the observed uptake of glucose by cow blastocysts in 20aa (13.9 pmoles/embryo/h).

Lactate production from glucose at the 8- to 16-cell stage was 2.9 pmoles/embryo/h. In the absence of amino acids, glucose metabolism via the EMP was found to be 1.5 pmoles/embryo/h. Since one molecule of glucose forms two molecules of lactate, lactate production could account for the observed metabolism of glucose through the EMP. At the blastocyst stage, lactate production from glucose was found to be 42.8 pmoles/embryo/h. This can more than account for the observed uptake of glucose and the metabolism of glucose through the EMP. This is in keeping with previous studies indicating that nearly 100% of the glucose taken up by cow (Thompson *et al.*, 1996) and sheep (Gardner *et al.*, 1993) blastocysts could be accounted for by lactate production. Thus, the present results support the fact that there is not a block to glycolysis at pyruvate kinase in the cow embryo as was suggested by Rieger and Guay (1988). Consideration must be given to the fact that lactate production was determined in medium with glucose as the sole energy substrate and glycolytic activity was determined in medium containing pyruvate and lactate. Interestingly, Thompson *et al.* (1991) found that the presence of pyruvate and lactate had no effect on the glycolytic activity of cow embryos at the blastocyst stage. The effect, however, of pyruvate and lactate on the glycolytic activity of the cleavage stage cow embryo has not been determined. Furthermore, glucose was used at a concentration of 0.5 mM for the determination of glucose uptake and lactate production, while 1.5 mM glucose was used for the determination of the metabolism of glucose through the EMP. The concentration of glucose has been found to drive glycolysis in the cleavage stage mouse embryo (Vella *et al.*, 1997) thus, glucose metabolism by the 8- to 16-cell cow embryo may have in fact been higher in the presence of a higher concentration of glucose. This did not appear to be the case in the

present study as approximately 1.5 pmoles/embryo/h of glucose would have been taken up by the cow embryo to produce 2.9 pmoles/embryo/h of lactate.

Interestingly, glucose metabolism by 8- to 16-cell embryos was negatively correlated with development to the 8- to 16-cell stage. Thus, the glycolytic activity of the 8- to 16-cell embryo was most likely reflective of the glycolytic activity of embryos during the first 72 h culture. Further studies are needed to determine the effect of the glycolytic activity of earlier cleavage stage cow embryos on subsequent developmental competence.

The present findings indicate that the cow embryo is similar to the mouse embryo in that a high glycolytic activity was found to inhibit development of the early cleavage stages (Gardner and Lane, 1993b). Unlike the 2-cell mouse embryo (Gardner and Lane, 1993b), however, a reduction in glycolytic activity was not associated with an increase in the oxidation of pyruvate in the 8- to 16-cell cow embryo. Thus, a Crabtree-like effect did not appear to be responsible for the observed impaired developmental capacity of the cow embryo under certain culture conditions. The presence of a Crabtree-effect in the cleavage stage cow embryo, however, cannot be ruled out exclusively on the basis of exogenous pyruvate oxidation. Factors responsible for the observed reduction in glycolytic activity, namely amino acids, may have merely decreased the utilization of exogenous pyruvate but increased overall oxidative capacity (competition of substrates for entry into the TCA cycle is discussed in more detail in section 8.2).

The fact that both glucose uptake and glycolytic activity increased with development from the 8- to 16-cell stage to the blastocyst indicates that a high glycolytic activity is not detrimental to development of the embryo beyond the 8- to

16-cell stage. Further, there was no correlation between glycolytic activity, on a per cell basis, and blastocyst development. Thus, the switch from the maternal to the embryonic genome is accompanied by changes in the regulatory mechanisms of embryo development. Such changes may include an alleviation of the inhibition of PFK as a result of a decrease in the ratio of ATP:ADP, with development from the 8- to 16-cell stage to the blastocyst (Leese *et al.*, 1984; Rozell *et al.*, 1992). Furthermore, an increase in hexokinase mRNA's at the morula stage may allow for an increased glycolytic capacity. Interestingly, the isoform of hexokinase present in the mouse embryo was found to change with development from the zygote to the blastocyst stage (Edwards and Gardner, 1995).

8.1.2 Pyruvate

Pyruvate uptake by the cow embryo was slightly higher than that of glucose up to the 8- to 16-cell stage, ranging between 3.5 to 5.1 pmoles/embryo/h. Pyruvate uptake, however, remained relatively constant from the 1-cell stage (4.6 pmoles/embryo/h) to the morula stage (4.1 pmoles/embryo/h), increasing only with blastocyst development (12.6 pmoles/embryo/h). The only other study to non-invasively determine the uptake of pyruvate by cow embryos from the zygote to the blastocyst stage found equivalent levels of pyruvate uptake from the 1-cell to the 8-cell stages, but found that pyruvate uptake was approximately twice as high at the 16-cell to morula stages as in the present study (Thompson *et al.*, 1996). In addition, Thompson *et al.* (1996) found that pyruvate uptake at the blastocyst stage (20.5 pmoles/embryo/h) was higher than that determined in the present study. The observed differences are likely to be due to differences in the two culture systems and methods

of determination. In the study by Thompson *et al.* (1996), pyruvate uptakes were determined in the absence of amino acids. In the present study, culture with amino acids significantly reduced the oxidation of exogenous pyruvate by embryos at the 8- to 16-cell stage and the blastocyst. In addition, Thompson *et al.* (1996) have shown that the presence of other TCA cycle intermediates, such as amino acids, reduced the oxidation of pyruvate by sheep embryos at the 8-cell and early blastocyst stages. Thus, the higher levels of pyruvate uptake beyond the 8- to 16-cell stages as determined by Thompson *et al.* (1996), were possibly a function of the absence of amino acids. Other factors could also have contributed to the observed differences in pyruvate uptakes. In the study by Thompson *et al.* (1996), pyruvate uptakes were averages of groups of embryos and were end-point determinations. All uptakes were measured in a HEPES-buffered medium, at 20% O_2 (Thompson *et al.*, 1996). Furthermore, uptakes by compacted morulae and blastocysts were measured in the presence of serum (Thompson *et al.*, 1996). Menke and McLaren (1970) in fact reported that culture of mouse embryos with serum increased oxidative metabolism. Determinations of pyruvate uptake in the present study were linear rates of uptakes by individual embryos under conditions similar to those within which embryos were cultured. Thus, the observed levels and pattern of pyruvate uptake in the present study most likely reflect the uptake of pyruvate by cow embryos during culture in 20aa.

Pyruvate oxidation by embryos cultured in 20aa to the 8- to 16-cell stage was 0.43 pmoles/embryo/h. Thus, only about 8% of the pyruvate taken up by the embryo at the 8- to 16-cell stage appeared to be oxidized. Likewise, only 9.5% of the pyruvate taken up at the blastocyst stage appeared to be oxidized. Thus, it would seem that the majority of pyruvate is used for means other than the production of energy through the

TCA cycle. In support of the present findings, Rieger *et al.* (1992b), using radiolabelled substrates, found that only 14% of pyruvate taken up by cow blastocysts was oxidized through the TCA cycle. Gardner *et al.* (1993) reported that around 65% of pyruvate taken up by the sheep blastocyst was converted to lactate. Considerable amounts of lactate were in fact produced from pyruvate at both the 8- to 16-cell (6.7 pmoles/embryo/h) and blastocyst (12.1 pmoles/embryo/h) stages in the present study. The present study indicates that approximately 100% of the pyruvate taken up by cow embryos was converted to lactate. Lactate production, however, was determined in the absence of glucose, lactate and amino acids. Pyruvate uptakes were likely to be higher in the absence of other substrates (Dorland *et al.*, 1991; Lane and Gardner, 2000a), therefore, lactate production would have been overestimated. Despite this, the fact that less than 10% of the pyruvate taken up by the embryo was oxidized indicates that significant amounts of pyruvate are converted to lactate by cow embryos. Thus, pyruvate may have a significant role in the production of NAD^+ for glycolysis. While the ratio of pyruvate:lactate has been determined to be a critical factor for the development of mouse (Cross and Brinster, 1973) and sheep embryos (Thompson *et al.*, 1993), the sensitivity of cow embryos to an appropriate redox state has been questioned (Rosenkrans *et al.*, 1993; Edwards *et al.*, 1997). Apparent differences between species may in fact be due to different culture media components, with pyruvate being required for the maintenance of an appropriate redox state under certain culture conditions. This remains to be determined.

Pyruvate may also have a role as an antioxidant through its direct reaction with hydrogen peroxide (Andrae *et al.*, 1985). Pyruvate has in fact been shown to reduce levels of hydrogen peroxide in cow embryos (Morales *et al.*, 1999). Morales *et al.*

(1999) found that, in the absence of pyruvate (0.33 mM), cow embryos were sensitive to increased levels of hydrogen peroxide, particularly up to the 8-cell stage and at the blastocyst stage.

Interestingly, the oxidation of pyruvate was negatively correlated with development at both the 8- to 16-cell stage and the blastocyst stage on a per cell basis. This would indicate that culturing embryos under conditions that reduce the level of pyruvate oxidation is an important factor in supporting cow embryo development. This appears to contradict the fact that pyruvate uptake and metabolism increase with development to the blastocyst stage. Furthermore, Thompson *et al.* (1996) found that oxygen consumption increased with embryo development, indicating that blastocyst development is accompanied by an increase in oxidative metabolism and thus activity of the TCA cycle. As discussed in section 8.2, the observed decrease in pyruvate oxidation with an increase in embryo development was not likely to be reflective of an overall decrease in pyruvate oxidation or a reduction in the activity of the TCA cycle. In the presence of factors affecting pyruvate oxidation, namely amino acids, the utilization of exogenous pyruvate could have been reduced in response to either an increase in the endogenous pool of amino acids and/or the successful competition of amino acids for entry into the TCA cycle.

There was no correlation observed between the oxidation of pyruvate and the metabolism of glucose through the EMP at the 8- to 16-cell stage, when carbohydrate uptakes and metabolism were relatively low. Conversely, glucose metabolism was positively correlated with pyruvate oxidation at the blastocyst stage, when the uptake and metabolism of carbohydrates were found to be highest. Thus, the dynamics of the

relationship between the activities of the EMP and TCA cycle change from the 8- to 16-cell stage to the blastocyst.

8.2 AMINO ACIDS

Amino acids were found to have a significant effect on embryo development, metabolism, pHi and cell differentiation. Furthermore, many of the effects of amino acids during culture of cow embryos from the zygote to the blastocyst stage were found to be temporal.

While previous studies have shown that culture of cow embryos from the zygote to the blastocyst stage is improved following the addition of glutamine and Eagle's non-essential and essential amino acids to culture media, the present study revealed that the cow embryo appears to require the essential amino acids only during development from the 8- to 16-cell stage to the blastocyst. Development during the first 72 h culture and subsequent blastocyst development, cell number and allocation of cells to the TE and ICM were equivalent in NeGln and 20aa. Beyond the 8- to 16-cell stage, however, the cow embryo showed a requirement for glutamine and the non-essential and essential amino acids. Blastocyst development, cell number, the allocation of cells to the TE and ICM and the proportion of cells in the ICM were all higher following culture in 20aa than in SOF, Gln, NeGln or EssGln.

The temporal and differential effects of amino acids during culture of the cow embryo were in fact found to be different to those reported for the mouse. The presence of the essential amino acids during culture of the early cleavage stage cow embryo did not alter the stimulatory effects of the non-essential amino acids and glutamine as was found during culture of the cleavage stage mouse embryo (Lane and

Gardner, 1994). Furthermore, cow blastocyst development was not stimulated by the non-essential amino acids and allocation of cells to the ICM was not stimulated by the essential amino acids, as was found in the mouse (Lane and Gardner, 1997a). Rather, blastocyst development was stimulated only by the presence of all amino acid groups (20aa) and allocation of cells to the ICM appeared to be stimulated by the non-essential amino acids and glutamine.

The apparent differences between the effects of amino acids on the development and differentiation of mouse and cow blastocysts is possibly due to differences in active amino acid transport systems. A comparison of the uptake and output of individual amino acids by mouse (Lamb and Leese, 1994) and cow (Partridge and Leese, 1996) blastocysts reveals that there are in fact differences between the two species with respect to both non-essential and essential amino acids. Interestingly, significantly more essential amino acids were found to be taken up by the mouse blastocyst compared with cow blastocysts. With respect to the non-essential amino acids, significant amounts of alanine and asparagine were put out and taken up, respectively, by the cow blastocyst compared with the mouse. Such findings could well reflect differences in amino acid transport systems between the mouse and the cow and could likely account for the observed differences in the effects of the non-essential and essential amino acids on blastocyst development and cell differentiation.

The present study indicated that the concentration of amino acids in Eagle's essential group was too high for the cow embryo during both development of the early cleavage stages and development beyond the 8- to 16-cell stage. Although there were no significant effects observed on cell number or allocation of cells to the TE and ICM when the essential amino acids were used at either half or one quarter the given

concentrations, there was a significant negative correlation between embryo development and the concentration of essential amino acids during both the first and second 72 h culture. The concentrations of individual essential amino acids in uterine fluid of the cow have in fact been shown to be significantly lower than the concentrations of essential amino acids found in Eagle's essential amino acids (Table 1.3). A reduction in the concentration of Eagle's essential amino acids by 50% and 75% results in concentrations of amino acids more similar to that found in uterine fluid. Interestingly, a reduction in the concentration of specific essential amino acids alleviated their inhibitory effects during culture of hamster embryos (McKiernan *et al.*, 1995). Further research is needed to determine whether decreasing the concentration of individual essential amino acids would be conducive to an improved development of cow embryos during culture.

While glutamine is undoubtedly used as an energy source during development from the zygote to the blastocyst stage, with metabolism through the TCA cycle, the pattern of glutamine metabolism is in fact different to that of glucose and pyruvate metabolism. Unlike glycolytic activity and pyruvate oxidation, glutamine oxidation did not increase between the 8- to 16-cell stage (0.6 pmoles/embryo/h) and the blastocyst stage (0.5 pmoles/embryo/h). Similarly, previous studies have shown that the metabolism of glutamine through the TCA cycle was relatively low during development of the cow embryo, peaking at the 2- to 4-cell stages but not increasing with development to the blastocyst stage (Rieger *et al.*, 1992a, 1992b). Glutamine metabolism in fact increased with blastocyst expansion (Tiffin *et al.*, 1991; Rieger *et al.*, 1992a, 1992b), rising approximately two fold between the expanded and hatching stages (Rieger *et al.*, 1992b). In the present study, glutamine oxidation did increase

with blastocyst expansion from 0.36 pmoles/embryo/h in the early blastocyst to 0.62 pmoles/embryo/h in the expanded blastocyst, following culture in 20aa. Interestingly, Tiffen *et al.* (1991) reported a five to six fold increase in glutamine oxidation between the early (0.48 pmoles/embryo/h) and expanded (2.66 pmoles/embryo/h) stages in in vivo produced cow blastocysts. This indicates that glutamine oxidation may in fact be reduced in in vitro produced cow embryos, which could have serious implications for energy production and thus embryo viability.

Rieger *et al.* (1992a) found that glutamine oxidation by in vitro produced cow blastocysts at the expanded stage was approximately 4.11 pmoles/embryo/3h, which is equivalent to twice that found in the present study, following culture in 20aa. In the study by Rieger *et al.* (1992a) embryos were cultured in a co-culture system in complex medium with serum. Thus, growth factors present in the serum or secreted by the somatic support cells or components present in the complex medium (Menezo B2) may have led to an increased glutamine metabolism. Interestingly, in the present study, glutamine oxidation by blastocysts at the expanded stage was increased in the presence of MEM vitamins, although it was not as high as that reported by Rieger *et al.* (1992a). Thus, the metabolism of glutamine by the expanding cow blastocyst may be impaired in in vitro produced embryos. Further studies are needed to determine the effects of media components such as vitamins and growth factors on the metabolism of glutamine by the expanded cow blastocyst, particularly with respect to embryo viability.

The fact that the pattern of glutamine oxidation during development from the zygote to the blastocyst stage differs to that of the metabolism of pyruvate via the TCA cycle and glucose through the EMP, indicates that the cow embryo has a

preference for a particular substrate at a given time and that the requirement for that substrate may extend beyond the production of ATP. This was particularly evident during oocyte maturation when, relative to the metabolism of glucose and pyruvate, glutamine metabolism increased linearly from 0.45 pmoles/oocyte/h in the immature oocyte to 1.2 pmoles/oocyte/h after 24 h maturation. The present study revealed that significant amounts of both glucose and pyruvate were converted to lactate by the cow embryo, possibly for the production of NAD^+ to drive glycolysis, and the maintenance of an appropriate redox state in the embryo (although as previously discussed, it is not certain whether this is critical for embryo development in the cow). Relatively little or no lactate, however, was produced from glutamine indicating that glutamine does not function in the production of cytoplasmic NAD^+ and is not involved in the maintenance of the cytoplasmic redox potential.

There was some indication in the present study that glutamine functions as an osmolyte during development of the early cleavage stage embryo. The removal of glutamine from NeGln, during the first 72 h culture had a negative effect on subsequent blastocyst development. The fact that replacing glutamine with the osmolyte betaine, during the first 72 h, helped maintain subsequent blastocyst development, indicates that glutamine may provide some osmotic support during development of the early cleavage stages. Although culture of cow embryos in medium with an osmolarity of around 270 mOsm can support comparatively high levels of embryo development, it cannot be assumed that the embryo is not under some degree of osmotic stress. Recent evidence indicates that the osmolality of the reproductive tract is 290 to 300 mOs/kg, however, in vivo, embryos are in the presence of potential organic osmolytes such as amino acids. While glutamine has

been found to function as an osmolyte for the mouse embryo with detrimental increases in osmolarity above 300 mOsm (Biggers *et al.*, 1993; Dawson and Baltz, 1997), this is the first study to show that glutamine appears to have a function as an osmolyte for the early cleavage stage embryo under conventional culture conditions.

Glutamine may also be required during development of the early cleavage stage embryo for its role in the regulation of pHi. The addition of glutamine to SOF during the first 72 h culture significantly decreased the pHi of 8- to 16-cell embryos. The addition of the non-essential acids to glutamine further decreased the pHi of 8- to 16-cell embryos, however, culture with the essential amino acids negated the effects of glutamine and the non-essential amino acids on pHi. Thus, glutamine and the non-essential amino acids appear to have an important role in the regulation of pHi in the early cleavage stage cow embryo. Interestingly, Lane and Bavister (1999) found that cleavage stage cow embryos were unable to recover from an alkaline load, despite the presence of the HCO_3^-/Cl^- exchanger. Similarly, irrespective of the presence of the HCO_3^-/Cl^- exchanger in early cleavage stage mouse embryos, amino acids were found to act as buffers in response to an increasing alkaline load (Edwards *et al.*, 1998b). Lane and Bavister (1999) suggested that prolonged culture of embryos may reduce the ability of the HCO_3^-/Cl^- exchanger to function normally. Interestingly, the pHi of 8- to 16-cell embryos in the present study was negatively correlated with development to the 8- to 16-cell stage. Thus, as suggested by Bavister (1995), culture conditions have a significant effect on the pHi of the embryo, which in turn has an impact on developmental competence. The present study indicated that culture of cow embryos in the absence of the non-essential amino acids and glutamine resulted in an elevated pHi which was associated with a reduced developmental capacity.

Amino acids were also found to have a significant impact on embryo metabolism. While the glycolytic activity of 8- to 16-cell embryos decreased following culture for the first 72 h with each amino acid combination, culture in NeGln significantly reduced glycolytic activity with respect to culture in the absence of amino acids. As with pHi, the glycolytic activity of 8- to 16-cell embryos was negatively correlated with development to the 8- to 16-cell stage. While glycolytic activity can affect pHi (Eisner *et al.*, 1989), pHi can also affect glycolytic activity (Spriet, 1991; Edwards *et al.*, 1998a; Miccoli *et al.*, 1998). Irrespective of the relationship between glycolytic activity and pHi, amino acids, specifically the non-essential amino acids and glutamine, had a profound effect on glycolytic activity and pHi, either directly or indirectly, which in turn was associated with developmental capacity.

Culture for the first 72 h with amino acids significantly reduced pyruvate oxidation by 8- to 16-cell embryos. This was also evident at the blastocyst stage following culture for the second 72 h with amino acids. In addition, culture with the non-essential and/or the essential amino acids during the second 72 h altered the observed relationship between pyruvate and glucose metabolism. Culture in NegIn, EssGln or 20aa decreased the ratio of pyruvate:glucose metabolism by approximately three fold, compared with culture in the absence of amino acids or with glutamine as the sole amino acid. On a per cell basis, pyruvate oxidation was in fact significantly reduced at the blastocyst stage in the presence of amino acids, particularly following culture in NeGln.

The observed reduction in pyruvate oxidation by cow embryos in the presence of amino acids was not necessarily indicative of an overall reduction in the activity of

the TCA cycle. It is possible that amino acids successfully competed with pyruvate for entry into the TCA cycle (Thompson *et al.*, 1993). Furthermore, the embryo's requirements for exogenous pyruvate may decrease following the transamination of specific amino acids to pyruvate, resulting in an increase in the endogenous pool of pyruvate. The metabolism of both glucose and pyruvate were found to increase with blastocyst cell number, most likely in response to an increase in energy demands. Compared with culture in the absence of amino acids, however, the increase in pyruvate oxidation with cell number was not as great following culture in 20aa. This likely indicates that blastocysts were using exogenous amino acids in preference to exogenous pyruvate.

Hence, the present study revealed that amino acids not only improve embryo development but that they have a temporal role during culture. Furthermore, amino acids have a significant effect on the allocation of cells to the ICM and TE in the cow blastocyst and effect the proportion of cells allocated to the ICM. This is the first study to indicate that a key role of amino acids during development of the early cleavages stage cow embryo is in the regulation of both glycolytic activity and pHi. Importantly, factors affecting metabolism and pHi of the cow embryo can have a profound effect on embryo development.

8.3 VITAMINS

Apart from the studies by Takahashi and First (1992) and Rosenkrans and First (1994) there has been little attempt to ascertain the effects of culture with vitamins on the cow embryo. The present study revealed that vitamins have an effect on both the development and metabolism of the cow embryo.

Following culture from the zygote with MEM vitamins, fewer embryos had developed beyond the 8-cell stage compared with embryos cultured in the absence of vitamins. Equivalent proportions of embryos, however, had at least reached the 8-cell stage after 72 h culture in media with and without MEM vitamins. There was thus no evidence of the inhibitory effect of vitamins during the first 72 h culture on subsequent blastocyst development, blastocyst expansion or blastocyst cell number.

There was no evidence of an effect of vitamins on blastocyst development when embryos were cultured for 72 h from Day 4 pi in the presence of MEM vitamins. Development to the blastocyst stage, blastocyst expansion and total blastocyst cell number were equivalent in the presence and absence of MEM vitamins. The fact that culture from Day 4 pi with pantothenate present as the only vitamin significantly increased blastocyst development on Day 7 pi, indicates that one or more of the vitamins present in the MEM group counteracted the stimulatory effect of pantothenate. Similarly, Tsai and Gardner (1994) found that the beneficial effect of riboflavin on the development of mouse blastocysts was negated in the presence of other vitamins. Of the individual MEM vitamins, folic acid, myoinositol, riboflavin and nicotinamide, looked at in the present study, there was no evidence of an inhibitory effect on blastocyst development.

Interestingly, continued culture of Day 7 blastocysts for an additional 24 h in the presence of MEM vitamins resulted in a significant increase in blastocyst diameter, compared with embryos cultured from Day 4 pi in the absence of vitamins. The stimulatory effect of MEM vitamins on blastocyst diameter was equivalent to the observed effect of culture with serum from Day 6 pi. There was no synergistic effect

between MEM vitamins and serum on blastocyst diameter, indicating that the stimulatory effect of serum was plausibly due to vitamins present in the serum.

The increase in blastocyst diameter in the presence of MEM vitamins represents an increase in blastocoel expansion. An increase in ATP production is undoubtedly required to support the activity of Na^+-K^+ ATPase, for the movement of fluid into the blastocoel cavity (Overstrom *et al.*, 1989). Donnay and Leese (1999) recently documented that oxidative metabolism does indeed have a role in blastocyst expansion. In light of this, it is interesting that culture with MEM vitamins increased the metabolism of glutamine through the TCA cycle by blastocysts on Day 7 pi. Thus an increase in blastocyst diameter on Day 8 pi may well be reflective of an increased ATP production on Day 7 pi in the presence of MEM vitamins.

Interestingly, there was a discrepancy between the metabolism of glutamine and pyruvate through the TCA cycle, at the blastocyst stage, following culture with MEM vitamins. MEM vitamins increased the metabolism of glutamine but decreased the metabolism of pyruvate through the TCA cycle. This discrepancy was also observed during oocyte maturation when glutamine metabolism increased significantly between 12 h and 24 h maturation, while pyruvate oxidation decreased. Furthermore, culture with different amino acids during the first 72 h changed the observed relationship between the metabolism of pyruvate and glutamine by 8- to 16-cell embryos. Thus the activity of the TCA cycle does not appear to be uniform in all segments of the cycle. This is interesting in light of the fact that pyruvate metabolism through the TCA cycle is often used as an indicator of oxidative metabolism. The present data would also suggest that glutamine is the preferred energy source over pyruvate for the maturing cow oocyte, 8- to 16-cell embryo and the blastocyst. Rieger *et al.* (1992b) in fact

suggested that the discrepancy observed between glutamine and pyruvate oxidation with blastocyst development could be due to differences in the sensitivities of NAD^+-substrate dehydrogenases to NADH in different sections of the TCA cycle.

Alternatively, as discussed in section 8.2, the observed rates of exogenous pyruvate oxidation in the presence of MEM vitamins may not be indicative of total pyruvate oxidation. As the embryo's requirements for amino acids such as alanine, glycine, cysteine, serine, threonine and tryptophan increase, the endogenous pool of pyruvate would also increase. This would result in a decrease in the embryo's requirements for exogenous pyruvate and thus an underestimation of the amount of pyruvate oxidized by the embryo. Thus, the observed decrease in pyruvate oxidation at the expanded blastocyst stage in the presence of MEM vitamins could have been due to an increase in the utilization of amino acids in response to the presence of pyridoxine which functions as a cofactor in amino acid transamination. This theory is supported by the fact that culture with riboflavin, myoinositol, pantothenate or the combination of folic acid, nicotinamide and riboflavin increased the metabolism of glutamine at the expanded blastocyst stage but had no effect on the oxidation of exogenous pyruvate.

Unlike culture with all MEM vitamins, the stimulatory effect of riboflavin, myoinositol, pantothenate and the combination of folic acid, nicotinamide and riboflavin on glutamine metabolism, was apparent only at the expanded blastocyst stage and not across all blastocyst stages. While the observed stimulatory effect of MEM vitamins on glutamine metabolism at the blastocyst stage could have been due to the synergistic effects of two or more vitamins, it could alternatively be reflective of the proportions of blastocysts measured at each stage of development. That is, the

metabolism of very few early blastocysts was measured following culture in the presence and absence of MEM vitamins, whereas approximately one third of the blastocysts included in the determination of the effects of individual vitamins of energy metabolism were at the early blastocyst stage.

The fact that, with the exception of pantothenate, the stimulatory effects of vitamins on the cow embryo were not evident until the expanded blastocyst stage, indicates that the embryo's main requirement for vitamins is at the commencement of the period of extended growth of the blastocyst. Vitamins were in fact found to be a critical component of culture media for extended culture of the cow embryo beyond Day 7 pi (Giliam and Gardner, unpublished data).

The effects of other vitamins such as pyridoxine, choline and thiamine on embryo development and metabolism need to be determined, along with the combined effects of specific vitamins. As discussed in section 2.8, the metabolism of glutamine by in vitro produced cow blastocysts at the expanded blastocyst stage appears to be lower than that reported for in vivo produced blastocysts. Thus, the stimulatory effect of vitamins on glutamine metabolism, may well indicate that the presence of vitamins in culture media is a significant factor in improving the viability of in vitro produced embryos. In light of the recent report that pantothenate improved blastocyst development and viability of hamster embryos following transfer of embryos to recipients (McKiernan and Bavister, 2000), the observed effects of pantothenate on embryo development and glutamine metabolism in the present study are most interesting. Further studies are needed to determine whether there is in fact a relationship between glutamine metabolism and embryo viability following transfer of cow and hamster embryos cultured with pantothenate.

8.4 PRE-PUBERTAL COWS

In the present study, the development of embryos from oocytes retrieved from pre-pubertal cows at two to three months of age (unstimulated) and five to seven months of age (stimulated) was significantly reduced compared with that observed for embryos produced from the ovaries of slaughterhouse adult cows.

With respect to embryos from adult cows, blastocyst development was significantly reduced in embryos derived from the oocytes of five to seven month old calves. The observed differences in both the level and pattern of pyruvate uptakes by pre-pubertal and adult cows during development of the early cleavage stages were likely reflective of the differences in developmental capacity. In support of this, Hardy *et al.* (1989b) found that pyruvate uptake by cleavage stage human embryos was indicative of subsequent developmental competence. Perturbations in the uptake of pyruvate by early cleavage stage pre-pubertal embryos could indicate a reduced oxidative capacity, an inability to maintain an appropriate redox potential and/or a reduced capacity to counteract oxidative stress.

Interestingly, pyruvate oxidation was also found to be compromised during the maturation of oocytes from pre-pubertal cows (two to three months old). Peak pyruvate oxidation at 12 h maturation was significantly lower in oocytes from pre-pubertal cows compared with oocytes from adult cows. Furthermore, peak glutamine oxidation and glycolytic activity at 24 h maturation were also significantly lower in pre-pubertal animals. Perturbations in energy substrate metabolism by pre-pubertal oocytes have previously been reported in cows (Gandolfi *et al.*, 1998) and sheep (O'Brien *et al.*, 1996), suggesting that energy metabolism is compromised in pre-pubertal oocytes. The present study, however, revealed that the observed metabolic

perturbations in pre-pubertal cow oocytes were in fact a function of oocyte size. The oocytes retrieved from pre-pubertal cows were significantly smaller at all stages of maturation than those retrieved from the ovaries of adult cows.

Oocyte size is a critical factor for subsequent developmental competence (Arlotto *et al.*, 1996; Otoi *et al.*, 1997). Blastocyst development was indeed substantially lower from oocytes derived from pre-pubertal cows (two to three months old). Previous studies have shown that protein content (Schultz and Wassarman, 1977) and RNA synthesis (Fair *et al.*, 1995) are both indicative of oocyte size. Interestingly, Levesque and Sirard (1994) found that pre-pubertal cow oocytes had a different protein profile to oocytes from adult cows. Furthermore, the protein profiles of pre-pubertal and adult cow oocytes were reflective of subsequent developmental competence (Levesque and Sirard, 1994). Thus, the observed reduced developmental capacity of oocytes from pre-pubertal cows in the present study may well have been the result of insufficient proteins in embryos produced from oocytes that had not completed the growth phase.

Oocyte size can also affect the maturation of oocytes to MII (Fair *et al.*, 1995). This was not observed in the present study, however, the proportion of oocytes reaching MII was determined only after 24 h maturation. Oocyte size has in fact been positively correlated with the speed of oocyte maturation (Tsafriri and Channing, 1975; Fukui and Sakuma, 1980; Arlotto *et al.*, 1996). In addition, the speed at which oocytes reach MII has been recently linked to subsequent developmental capacity (Dominko and First, 1997). The fact that GVBD was delayed in oocytes from pre-pubertal cows in the present study, indicates that the progression of meiosis was in fact slower in pre-pubertal oocytes. Interestingly, the delay in GVBD was concomitant

with a delay in the increase of glycolytic activity in the pre-pubertal oocyte. Further studies are required to determine the role of glucose in the nuclear maturation of the pre-pubertal and adult oocyte.

Despite perturbations in embryo development and the uptake of pyruvate by early cleavage stage pre-pubertal embryos, those embryos that reached the blastocyst stage were not compromised with respect to the uptake of glucose and pyruvate or total cell number. This is interesting in light of the fact that both the uptake of glucose by cow blastocysts (Renard *et al.*, 1980) and blastocyst cell number in the mouse (Lane and Gardner, 1997a) have been positively correlated with viability. Thus, pre-pubertal embryos do not appear to be compromised following activation of the embryonic genome. The poor developmental competence of embryos derived from the oocytes of pre-pubertal cows is therefore likely to be a function of factors inherited from the oocyte. Further studies are required to investigate the dynamics of oocyte growth and maturation in pre-pubertal cows in order to improve subsequent development to the blastocyst stage.

8.5 CONCLUSION

The present study has contributed to a greater understanding of the ways in which specific media components affect the in vitro produced cow embryo. Amino acids were found to have both a temporal and differential effect on development and blastocyst cell number during culture from the zygote to the blastocyst stage. Several mechanisms for the action of amino acids during culture of the cleavage stage cow embryo were elucidated. Amino acids were shown to regulate glycolytic activity and buffer pHi in the 8- to 16-cell embryo, with both mechanisms having a significant

correlation with developmental competence during the first 72 h culture. In addition, glutamine appeared to function as an osmolyte during development of the cleavage stage cow embryo. There was also strong evidence that amino acids successfully competed with pyruvate for entry into the TCA cycle at both the 8- to 16-cell and blastocyst stages. Thus, the apparent non-existence of a Crabtree-like effect at the 8- to 16-cell stage could not be conclusive when glucose and pyruvate metabolism were determined in the presence of amino acids. MEM vitamins did not affect development or cleavage to the blastocyst but did stimulate blastocyst expansion. Furthermore, MEM vitamins had a significant effect on oxidative metabolism in the cow blastocyst but did not affect glycolytic activity. Pantothenate significantly improved development of embryos to the blastocyst stage and increased glutamine oxidation.

This study was the first to determine linear rates of pyruvate and glucose uptake by cow embryos at successive stages of development, from the zygote to the blastocyst, under conditions similar to those in which embryos were cultured. The uptake of both glucose and pyruvate was low during the early cleavage stages, increasing significantly with development to the blastocyst stage. Determinations of the metabolism of oocytes revealed that both glycolytic activity and glutamine oxidation increased during maturation of the cow oocyte. The present study highlighted several differences between oocytes and embryos from pre-pubertal cows and adult cows. A smaller oocyte size, a delay in the increase in glycolytic activity during maturation, a delay in reaching the GVBD stage and a different pattern of pyruvate uptake by early cleavage stage pre-pubertal embryos, when compared to oocytes and embryos from adult cows, may be factors accounting for the observed differences in developmental competence. Importantly, those embryos that reached

the blastocyst stage from pre-pubertal oocytes were not compromised with respect to cell number or nutrient uptakes.

While the present study highlights the marked effects that amino acids and vitamins have with respect to development, cleavage, differentiation to the ICM and TE, metabolic activity and pHi during culture of the cow embryo, the relationship of these parameters to embryo viability can only be hypothesised. Future studies are required to determine the effects of culturing cow embryos with amino acids and vitamins on fetal development following transfer of embryos to recipient cows.

APPENDICES

Appendix 1 Composition of stocks for the preparation of maturation medium and maturation handling medium (H-199).

Compound	Base medium for TCM-199 (g)	Maturation medium stock	H-199 stock	FSH stock	LH stock
TCM-199 powder†	1 bottle (for 1litre)	-	-	-	-
$NaHCO_3$	-	1.05 g	0.21 g	-	-
Pyruvate	0.022	-	-	-	-
Penicillin	0.06	-	-	-	-
Streptomycin	0.05	-	-	-	-
HEPES	-	-	2.1 g	-	-
H_20	500 ml	250 ml	250 ml	-	-
Base Medium for TCM-199	-	250 ml	250 ml	-	-
LH	-	-	-	-	5 U
FSH	-	-	-	5 U	-
H-199 Stock	-	-	-	1 ml	1 ml

†TCM-199 powder from Sigma.

Appendix 2 Preparation of maturation medium and maturation handling medium (H-199) from stock solutions.

Component	Maturation medium (ml)	Maturation handling medium (H-199) (ml)
Maturation medium stock†	25.00	-
H-199 stock†	-	50.00
LH stock†	0.05	-
FSH stock†	0.05	-
FCS	2.50	-
BSA	-	0.20 g

†Composition of stock solutions in Appendix 1.

Appendix 3 Preparation of Percoll gradients from stock solutions.

Components	Sperm-TALP	Percoll stock	Percoll gradients (ml)		
			45%	70%	90%
Sperm-TALP stock*	2.00	-	-	-	-
Percoll	-	2.00	-	-	-
Sperm-TALP	-	18.00	-	-	-
Percoll stock	-	-	4.50	7.00	4.50
H_2O	18.00	-	5.50	3.00	0.50
BSA	0.12 g	-	-	-	-

* Composition of stock solution in Appendix 4.

Appendix 4 Preparation of Sperm-TALP stock solution.

Compound	Sperm-TALP stock 10 x concentration (g)
NaCl	5.840
KCl	0.231
$NaHCO_3$	2.100
$NaH_2PO_4.H_2O$	0.040
Na Lactate	4.030
HEPES	2.380
Na Pyruvate	0.110
Phenol Red	0.010
$CaCl_2$*	0.294
$MgCl_2.6H_2O$	0.305
BSA	-
H_2O	100.00 ml

*Dissolved in 10 ml H_2O before added (part of 100 ml H_2O).

Appendix 5 Preparation of Fert-TALP from stock solutions.

Stocks	Compound		Fert-TALP (ml)
Fert-TALP Stock A		(g/100ml)	1.00
(10 x concentration)	NaCl	6.660	
*3 months	KCL	0.238	
	$NaH_2PO_4.H_2O$	0.048	
	Na Lactate	1.860	
	$MgCl_2$	0.102	
Fert-TALP Stock B		(g/50ml)	1.00
(10 x concentration)	$NaHCO_3$	1.050	
*2 weeks at 4°C	Phenol red	0.005	
Fert-TALP Stock C		(g/10ml)	0.100
(100 x concentration)	Na Pyruvate	0.028	
*1 week at 4°C			
Fert-TALP Stock D		(g/10ml)	0.100
(100 x concentration)	$CaCl_2$	0.290	
*1 week at 4°C			
H_2O			7.800 ml
BSA			0.06 g

* Indicates length of time stock was kept before discarding and at what temperature.

Appendix 6 Preparation of cow embryo culture media from stocks for amino acid experiments.

Stock†	SOF (ml)	Gln (ml)	NeGln (ml)	EssGln (ml)	20aa (ml)	19aa (ml)	Bet (ml)	NeBet (ml)	19aaBet (ml)	NeGln plus 0.25x Eagle's essential amino acids* (ml)	NeGln plus 0.5x Eagle's essential amino acids* (ml)
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
D	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
F	0.10	0.10	0.05	0.05	-	-	0.10	0.05	-	0.038	0.025
G	-	0.10	0.10	0.10	0.10	-	-	-	-	0.10	0.10
H	-	-	-	-	-	-	0.10	0.10	0.10	-	-
I	-	-	1.00	-	1.00	1.00	-	1.00	1.00	1.00	1.00
J	-	-	-	0.20	0.20	0.20	-	-	0.20	0.05	0.10
H_2O	7.70	7.60	6.65	7.45	6.5	6.6	7.60	6.55	6.50	6.612	6.575
BSA	0.08g	0.08 g	0.08 g	0.08 g	0.08 g	0.08 g	0.08 g	0.08 g	0.08 g	0.08 g	0.08 g

†Composition of stock solutions in Appendix 9.
*Defined in Appendix 9.

Appendix 7 Preparation of cow embryo culture media from stocks for vitamin experiments.

Stock[†]	NeGlnVit	20aaVit	20aaVit/serum	20aa/serum	Folic	Nicotin	Ribo	Biotin	Panto	Myo	Folic/Nicotin /Ribo
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
D	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
F	0.05	-	-	-	-	-	-	-	-	-	-
G	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
I	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
J	-	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
K	0.10	0.10	0.10	-	-	-	-	-	-	-	-
L	-	-	-	-	1.00	-	-	-	-	-	1.00
M	-	-	-	-	-	0.010	-	-	-	-	0.01
N	-	-	-	-	-	-	0.10	-	-	-	0.10
O	-	-	-	-	-	-	-	0.10	-	-	-
P	-	-	-	-	-	-	-	-	0.10	-	-
Q	-	-	-	-	-	-	-	-	-	0.10	-
FCS	-	-	1.00	1.00	-	-	-	-	-	-	-
H_2O	6.55	6.40	5.40	5.50	-	-	-	-	-	-	-
BSA	0.08g	0.08 g	-	-	0.08g	0.08 g	0.08g	0.08 g	0.08g	0.08 g	0.08 g

[†]Composition of stock solutions in Appendix 9.

Appendix 8 Preparation of HEPES-buffered media from stock solutions.

Stock†	H-SOF (ml)	H-Gln (ml)	H-NeGln (ml)	H-EssGln (ml)	H-20aa (ml)
A	1.00	1.00	1.00	1.00	1.00
B	0.16	0.16	0.16	0.16	0.16
C	0.10	0.10	0.10	0.10	0.10
D	0.10	0.10	0.10	0.10	0.10
E	0.84	0.84	0.84	0.84	0.84
F	0.10	0.10	0.05	0.05	-
G	-	0.10	0.10	0.10	0.10
H	-	-	-	-	-
I	-	-	1.00	-	1.00
J	-	-	-	0.20	0.20
H_2O	7.70	7.60	6.65	7.45	6.5
BSA	0.04g	0.04 g	0.084g	0.04 g	0.08 g
pH*	7.40	7.40	7.40	7.40	7.40

†Composition of stock solutions in Appendix 9.

* pH adjusted with NaOH solution of individual media.

Appendix 9 Composition of stocks for the preparation of culture media for cow embryos.

Stocks	Compound	
Stock A		(g/100ml)
(10 x concentration)	NaCl	5.827
*3 months at 4°C	KCL	0.534
	KH_2PO_4	0.162
	$MgCl_2.6H_2O$	0.099
	Na Lactate (D/L-isomer)	0.616
	Glucose	0.270
	Penicillin	0.060
	Streptomycin	0.050
metabolism Stock A		(g/100ml)
(10 x concentration)	NaCl	5.827
*3 months at 4°C	KCL	0.534
	KH_2PO_4	0.162
	$MgCl_2.6H_2O$	0.099
	Penicillin	0.060
	Streptomycin	0.050
Stock B		(g/50ml)
(10 x concentration)	$NaHCO_3$	1.050
*2 weeks at 4°C	Phenol red	0.005
Stock C		(g/10ml)
(100 x concentration)	Na Pyruvate	0.036
*2 weeks at 4°C		

Appendix 9 continued

Stocks	Compound	
Stock D		(g/10ml)
(100 x concentration)	$CaCl_2.6H_2O$	0.252
*2 weeks at 4°C		
Stock E		(g/100ml)
(10 x concentration)	HEPES	5.958
*3 months at 4°C	Phenol red	0.010
Stock F		(g/10ml)
(100 x concentration)	NaCl	0.468
*1 month at 4°C		
Stock G		(g/10ml)
(100 x concentration)	Glutamine	0.146
*2 weeks at 4°C		
Stock H		(g/10ml)
(100 x concentration)	Betaine	0.117
*2 weeks at 4°C		
Stock I		(mg/100ml)
Eagle's non-essential amino	Alanine	8.910
acids†	Asparagine	14.640
(10 x concentration)	Aspartate	13.310
*6 months at 4°C	Glutamate	14.710
	Glycine	7.510
	Proline	11.510
	Serine	10.510

Appendix 9 continued

Stocks	Compound	
Stock J'		(g/l)
Eagle's essential amino acids†	L-Arginine.HCl	6.320
(50 x concentration)	L-Cystine $Na_2.H_2O$	1.511
*12 months at 4°C	L-Histidine.HCl.H_2O	2.095
	L-Isoleucine	2.625
	L-Leucine	2.625
	L-Lysine.HCl	3.653
	L-Methionine	0.745
	L-Phenylalanine	1.651
	L-Threonine	2.382
	L-Tryptophan	0.510
	L-Tyrosine $Na_2.2H_2O$	2.595
	L-Valine	2.345
Stock K'		(g/l)
MEM vitamins	Pantothenate	0.100
(100 x concentration)	Choline	0.100
*12 months, at -20°C	Folic Acid	0.100
	I-Inositol	0.200
	Nicotinamide	0.100
	Pyridoxine	0.100
	Riboflavin	0.010
	Thiamine	0.100
Stock L		(g/l)
(10 x concentration)	Folic Acid	0.01
*12 months, at -20°C		

Appendix 9 continued

Stocks	Compound	
Stock M	Nicotinamide	(g/10 ml)
(1000 x concentration)		0.01
*12 months, at -20°C		
Stock N		(g/100 ml)
(100 x concentration)	Riboflavin	0.001
*12 months, at -20°C		
Stock O		(g/100 ml)
(100 x concentration)	Biotin	0.01
*12 months, at -20°C		
Stock P		(g/100 ml)
(100 x concentration)	Pantothenate	0.01
*12 months, at -20°C		
Stock Q		(g/100 ml)
(100 x concentration)	Myo-Inositol	0.02
*12 months, at -20°C		
Stock R		(g/10ml)
(100 x concentration)	Na Pyruvate	0.055
*2 weeks at 4°C		
Stock S		(g/10ml)
(100 x concentration)	Glucose	0.090
*2 weeks at 4°C		

Appendix 9 continued

Stocks	Compound	
Stock T	Glutamine	(g/10ml)
(100 x concentration)		0.073
*2 weeks at 4°C		

*Indicates length of time stock was kept before discarding and at what temperature.

'Purchased stock.

†Amino acids as classified by Eagle (1959): Eagle's MEM non-essential amino acids and MEM essential amino acids. Note the Eagle's essential amino acids do not contain glutamine.

Appendix 10 Preparation of media from stocks for the measurement of cow embryo substrate metabolism in the presence of different amino acids and for the measurement of cow oocyte substrate metabolism.

Stock†	metSOF (ml)	metGln (ml)	metNeGln (ml)	metEssGln (ml)	met20aa (ml)	mmet20aa (ml)
met A	-	-	-	-	-	1.00
A	1.00	1.00	1.00	1.00	1.00	-
B	1.00	1.00	1.00	1.00	1.00	1.00
C	-	-	-	-	-	-
D	0.10	0.10	0.10	0.10	0.10	0.10
F	0.10	0.10	0.05	0.05	-	-
G	-	0.10	0.10	0.10	0.10	0.10
I	-	-	1.00	-	1.00	1.00
J	-	-	-	0.20	0.20	0.20
S	-	-	-	-	-	0.10
H_2O	7.80	7.70	6.75	7.55	6.60	6.50
BSA	0.08g	0.08g	0.08g	0.08 g	0.08 g	0.04 g

†Composition of stock solutions in Appendix 9

Appendix 11 Preparation of culture media from stocks for the measurement of cow embryo metabolism in the presence of different vitamins.

Stock †	met20aa (ml)	met20aaVit (ml)	metFolic (ml)	metNicotin (ml)	metRibo (ml)	metBiotin (ml)	metPanto (ml)	metMyo (ml)	metFolic/Nicotin/Ribo (ml)
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
G	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
I	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
J	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
K	-	0.10	-	-	-	-	-	-	-
L	-	-	1.00	-	-	-	-	-	1.00
M	-	-	-	0.010	-	-	-	-	0.01
N	-	-	-	-	0.10	-	-	-	0.10
O	-	-	-	-	-	0.10	-	-	-
P	-	-	-	-	-	-	0.10	-	-
Q	-	-	-	-	-	-	-	0.10	-
H_2O	6.60	6.50	5.60	6.59	6.50	6.50	6.50	6.50	5.49
BSA	0.08g	0.08 g	0.08g	0.08 g	0.08g	0.08 g	0.08g	0.08g	0.08g

†Composition of stock solutions in Appendix 9

Appendix 12 Composition of EPPS buffer and Glycine Hydrazine buffer for microfluorescence biochemical assays.

Compound	EPPS buffer	Glycine hydrazine buffer
EPPS†	2.52 g	-
Streptomycin	0.01 g	-
Penicillin	0.01 g	-
Glycine	-	7.50 g
Hydrazine	-	5.20 g
EDTA	-	0.20 g
H_2O	200 ml	100 ml
pH*	8.0	9.4

†(N-[2-Hydroxyethyl]piperazine-N'-[3-propanesulfonic acid]: HEPPS).

*pH of buffers adjusted with NaOH.

Appendix 13. The preparation of metabolic media from stocks for the measurement of substrate uptake and metabolite production by microfluorimetry.

Stock†	cmet20aa (ml)	metnil (ml)	metpyr (ml)	metglc (ml)	metgln (ml)	metglc/gln (ml)
met A	1.00	1.00	1.00	1.00	1.00	1.00
B	1.00	1.00	1.00	1.00	1.00	1.00
C	-	-	-	-	-	-
D	0.10	0.10	0.10	0.10	0.10	0.10
F	-	0.10	0.10	0.10	0.10	0.10
G	0.10	-	-	-	-	-
I	1.00	-	-	-	-	-
J	0.20	-	-	-	-	-
R	0.10	-	0.10	-	-	-
S	0.10	-	-	0.10	-	0.10
T	-	-	-	-	0.10	0.10
H_2O	6.40	7.80	7.70	7.70	7.70	7.60
BSA	0.04 g	0.04 g	0.04 g	0.04 g	0.04 g	0.04 g

†Composition of stock solutions in Appendix 9.

Appendix 14. Composition of stock solutions of SNARF-1 and nigericin used in the determination of intracellular pH.

Compound	SNARF-1 stock[A]	Nigericin stock[B]
SNARF-1	0.050 mg	-
DMSO[†] (dehydrated)	0.080 ml	-
Nigericin[‡]	-	10.00 mg
Methanol (100%)[‡]	-	0.100 ml
H_2O (MQ)	-	10.00 ml

[A] Stored in 20 µl aliquots at -20°C for approximately 1 week.

[B] Stored in 250 ml aliquots at -20°C for approximately 1 week.

[†]Dimethylsulphoxide.

[‡] Nigericin dissolved in methanol before the addition of H_2O.

Appendix 15. Composition of calibration buffer[†] for the calibration of SNARF-1 with intracellular pH.

Compound	mM	g/l
KCl	140.0	10.438
KH_2PO_4	1.0	0.136
$MgSO_4$	1.0	0.247
Glucose	10	0.179
HEPES	20.0	4.766
BSA	-	4.000

[†]Buffer without BSA was stored at 4°C for up to 1 month. Just prior to use, BSA was added and buffer filtered (0.2 μm filter). The pH of the buffer was then adjusted to 6.9, 7.0, 7.1, 7.2, 7.3 and 7.4 with KOH dissolved in calibration buffer. The buffers of various pH were then stored at 4°C for a maximum or 1 week.

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