Journal of Reproduction and Fertility (1996) **106**, 299–306

Oxygen uptake and carbohydrate metabolism by *in vitro* derived bovine embryos

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The consumption of oxygen, uptake of pyruvate and glucose and production of lactate were determined for groups of bovine embryos produced *in vitro* from the one-cell to the blastocyst stage (day 0–6 of culture). Measurements were made in Hepes-buffered synthetic oviduct fluid medium supplemented with 1.0 mmol pyruvate l^{-1}, 10 mmol D,L-lactate l^{-1} and 1.5 mmol glucose l^{-1} and also 3 mg BSA ml^{-1} and, from day 5 of development, 10% (v/v) fetal calf serum. The amount of ATP production was determined from oxygen consumption and the proportion of glucose taken up that could be accounted for by lactate production. The data revealed that oxygen consumption was relatively constant from days 0–4 of culture (0.24–0.27 nl per embryo h^{-1}), but increased with the initiation of compaction (0.39 nl per embryo h^{-1}) and continued to increase with the formation and expansion of the blastocoel (0.9 nl per embryo h^{-1}). Both pyruvate and glucose uptake followed similar patterns. Furthermore, when plotted against oxygen consumption, both pyruvate and glucose uptake increased significantly ($P < 0.001$) in a linear relationship ($R^2 = 0.61$ and 0.49, respectively). Lactate production also increased with development and accounted for 40% of glucose uptake at day 0 of culture (putative zygotes), increasing to 70% by day 2 (eight-cell stage) and 100% of glucose uptake from day 4 of culture onwards. ATP production followed a similar pattern to that of oxygen consumption (60–85 pmol per embryo h^{-1} from day 0 to day 4) increasing with compaction (124 pmol per embryo h^{-1}) and blastulation (221 pmol per embryo h^{-1}). For precompaction stages, 93–96% of ATP production was derived from oxidative phosphorylation, decreasing to 82% with compaction. ATP produced by oxidative phosphorylation could be accounted for by the uptake of pyruvate, suggesting that bovine embryos produced *in vitro* utilize little endogenous substrates when appropriate exogenous substrates are present in the culture medium. The data revealed that bovine embryos were dependent on oxidative phosphorylation for energy (ATP) production at all stages of pre-elongation development, with perhaps a shift in dependence towards glycolysis in conjunction with compaction. It follows that oxidizable substrates, such as pyruvate and certain amino acids, are preferred in embryo culture medium during development *in vitro*.

Introduction

Despite the plethora of information on the effect of energy substrates on embryo development, energy production or, more accurately, the rate of ATP production by mammalian embryos during development has received little attention. This has been due mainly to an inability to determine the contribution of each potential energy substrate to ATP production. Such sources include not only those added to the culture medium, but also endogenous protein and stores of glycogen and lipid. In a relatively complex medium, which may contain glucose, pyruvate, lactate and 20 amino acids plus protein, possibly in the form of serum, determination of the contribution of each component to ATP production within a single embryo is not yet feasible. However, ATP is produced by only two mechanisms within the eutherian cell: oxidative phosphorylation and glycolysis. By measuring the oxygen consumption, the uptake of glucose and the proportion metabolized to lactate, total ATP production can be determined (Newsholme and Leech, 1983).

Our knowledge of the requirements for bovine embryo development *in vitro* has increased considerably in recent years. This has been due largely to the development of cell coculture-free and serum-free systems to support embryo development *in vitro*. Both Rosenkrans *et al.* (1993) and Takahashi and First (1993) demonstrated that bovine embryos produced *in vitro*

Received 31 August 1995.

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will develop in the presence of relatively high concentrations of lactate alone, but that optimal development occurs when pyruvate and lactate are together. Under their culture conditions, glucose was not required at any stage of development. However, others have found an improvement in embryo development on the addition of glucose before compaction begins (Kim *et al.*, 1993a, b; Matsuyama *et al.*, 1993). These observations follow closely the pattern of carbohydrate uptake and utilization by bovine embryos during development. Substrate uptake and utilization by bovine embryos has been examined by Reiger and Guay (1988), Dorland *et al.* (1991), Javed and Wright (1991), Reiger *et al.* (1992) and Waugh and Wales (1993). Data from the study of Reiger *et al.* (1992) demonstrate that glucose uptake and utilization is low until compaction begins, after the fourth cell cycle. Surprisingly little is known of pyruvate utilization in bovine embryos throughout development. Reiger and Guay (1988) demonstrated that, unlike in the mouse embryo, pyruvate was readily oxidized at the blastocyst stage. Glucose from stored glycogen is another possible source, but little glycogen is formed by ruminant embryos (Pike and Wales, 1979; Waugh and Wales, 1993; Thompson *et al.*, 1995).

Knowledge of the requirement for specific amino acids is limited compared with that of carbohydrates. The addition of amino acids improves embryo development (Kim *et al.*, 1993a; Moore and Bondioli, 1993; Rosenkrans and First, 1994) and metabolism of glutamine through the tricarboxylic acid cycle has been reported by Reiger *et al.* (1992). There are virtually no data on the metabolism of fatty acids by bovine embryos, although they are known to be present in oviductal fluid of cows (Killian *et al.*, 1989; Henault and Killian, 1993a, b). Acetate metabolism has been investigated by Waugh and Wales (1993), who report that, when added to culture medium, little radioisotopic-labelled acetate is metabolized. The role of endogenous and exogenous protein sources as possible substrates has not been investigated, although it has been postulated that these, along with lipids, may be important (Leese, 1991, 1993; Trounson, 1992). The relative importance of carbohydrates, amino acids, proteins or lipids in providing energy for the developing embryo has yet to be evaluated. Such information, along with the contribution of glycolysis or oxidative phosphorylation to ATP production, will be of particular value when designing or optimizing embryo culture systems. We have developed a noninvasive assay for measuring the oxygen consumption by small groups of embryos. This has been used to investigate oxygen uptake of cattle embryos produced in vitro during development, and to determine the contribution of carbohydrate metabolism to ATP production.

Materials and Methods

Production of embryos in vitro

Ovaries were collected from an abattoir and transported to the laboratory in saline at approximately 35°C. The maximum time from slaughter to oocyte collection was approximately 3 h. Cumulus–oocyte complexes (COC) were recovered by aspiration of 1–5 mm follicles using a 19 gauge needle and 10 ml syringe. The COC were collected into Hepes-buffered TCM 199 medium supplemented with 10 μg heparin ml^{-1} (from pig intestinal mucosa; Sigma, St Louis, MO) and 0.4% (w/v) BSA (affinity column purified, 'ABRD'; Immuno-Chemical Products, Auckland). Before *in vitro* maturation, COC were assessed morphologically and only those with a compact, nonatretic cumulus oophorus–corona radiata and an oocyte with homogeneous cytoplasm were selected. All selected COC were washed thoroughly in Hepes-buffered TCM 199 medium (with Earle's salts; Sigma) supplemented with 10% (v/v) fetal calf serum (FCS; Sigma), washed once in maturation medium and placed in 50 μl drops (10 per drop) of the same medium under oil and incubated for 24 h at 39°C under humidified 5% CO_2 in air. The medium used for maturation was TCM 199 supplemented with 10% FCS, 10 μg ovine follicle stimulating hormone ml^{-1} (Ovagen: Immuno-Chemical Products), 0.1 iu hCG ml^{-1} (Chorulon: Intervet, Roskilde) and 1 μg oestradiol ml^{-1} (Sigma).

Spermatozoa were prepared from frozen–thawed semen samples from a single bull of proven fertility. The contents of two 0.25 ml straws (each containing approximately 1×10^8 spermatozoa ml^{-1}) were layered on a Percoll gradient (45%/90%) and motile spermatozoa were collected after centrifugation at approximately 1200 ***g*** for 20 min at room temperature. The motile fraction was washed once in Hepes-buffered Tyrode's albumin lactate pyruvate medium (TALP) and then resuspended to a final concentration at insemination of 2×10^6 spermatozoa ml^{-1} in fertilization medium, a modified TALP medium (Lu *et al.*, 1987), supplemented with 0.2 μmol penicillamine l^{-1} (Sigma) and 0.1 μmol hypotaurine l^{-1} (Sigma). Insemination was performed in 50 μl of fertilization medium in microdrops under oil (five oocytes per drop) for 24 h under the same conditions as described for oocyte maturation. After insemination, putative zygotes were removed and washed twice in a Hepes-buffered version of synthetic oviduct fluid medium (H-SOF; Tervit *et al.*, 1972), comprising 20 mmol Hepes l^{-1}, 5 mmol $NaHCO_3$ l^{-1}, 1 mmol pyruvate l^{-1}, 10 mmol D,L-lactate l^{-1}, 1.5 mmol D-glucose ml^{-1} and 3 mg BSA ml^{-1} (fatty acid-free, Sigma). Cultured embryos were placed in 20 μl microdrops of a modified SOFaaBSA medium (Gardner *et al.*, 1994), containing 1 mmol pyruvate l^{-1} and 10 mmol D,L-lactate l^{-1} and incubated under humidified 5% CO_2, 5% O_2 and 90% N_2 at 39°C. Medium was replaced every second day of culture and, from day 4 of culture, was further supplemented with 10% (v/v) FCS. Oxygen consumption determination was performed on putative zygotes (day 0) or cleaved embryos at the two- to four-cell, eight-cell, 16-cell/early morula, compact morula or blastocyst stages (day 1, 2, 4, 5 or 6 of culture, respectively).

Determination of oxygen consumption, pyruvate and glucose uptake and lactate production

Oxygen consumption was determined using the technique described by Houghton *et al.* (in press), using the property of pyrene, an oil-soluble compound which is fluorescent on excitation at 340 nm but the fluorescence of which is quenched proportionately to the oxygen concentration. A 5 μl graduated pipette (PCR micro-pipets: Drummond, Broomall, Pennsylvania) and stainless steel shaft, which acts as a plunger,

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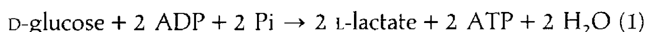
was loaded with 1 µl of 1 mg pyrene ml^{-1} (Sigma) in paraffin oil (BDH, Poole), followed by 2 µl H-SOF (days 0–4 embryos) or H-SOF supplemented with 10% (v/v) FCS (day 5 and day 6 embryos) containing embryos. The number of embryos per assay varied considerably with the stage of development and number available on any given day. The maximum number used in a single assay was 32 putative zygotes on day 0, and the minimum was two blastocysts on day 6 of culture. The pipette was immediately sealed and the plunger fixed in position using sealing wax. A 0% oxygen control was prepared by substituting H-SOF medium with 1 mg baker's yeast ml^{-1} in 60 mmol D-glucose l^{-1}. This was allowed to equilibrate overnight before use. The air-saturated control was prepared by omitting embryos from the medium. Fluorescence was measured using a photometer and photomultiplier attached to a Leica Diavert or Fluorovert fluorescence inverted microscope, as described by Leese and Barton (1984) and Gardner and Leese (1986). At least five measurements were taken during incubation for 4–6 h with the embryos held at 39°C between measurements. A computer-assisted mathematical model, which takes into account the solubilities and diffusion kinetics of oxygen in both aqueous solution and paraffin oil, was used to model the transfer of oxygen from the pyrene–oil phase into the medium in response to oxygen depletion from the medium.

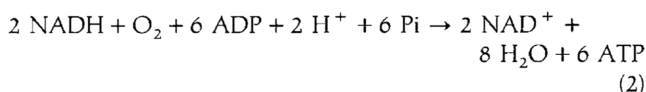
After incubation, embryos and medium were recovered from the pipettes. Samples of embryos from days 4, 5 and 6 were allocated randomly to be fixed and stained with Giemsa to determine the number of cells, as described by Fukui and Ono (1989). A randomly selected sample of embryos from day 0 to day 5 was also immediately returned to culture, where development to the blastocyst stage was assessed after a total of 6 days in culture. The spent H-SOF medium was stored at − 70°C before being analysed for pyruvate, D-glucose and L-lactate concentration using microfluorometric assays as described by Leese and Barton (1984) and Gardner and Leese (1986). Oxygen uptake was expressed as nl per embryo h^{-1}, while pyruvate and glucose uptake and lactate production were expressed as pmol per embryo h^{-1}.

Calculation of ATP production

The relative proportion of D-glucose that was metabolized to L-lactate was used to calculate the rate of ATP production from the following equations:



$$\text{D-glucose} + 2\ \text{ADP} + 2\ \text{Pi} \rightarrow 2\ \text{L-lactate} + 2\ \text{ATP} + 2\ H_2O \quad (1)$$



$$2\ \text{NADH} + O_2 + 6\ \text{ADP} + 2\ H^+ + 6\ \text{Pi} \rightarrow 2\ \text{NAD}^+ + 8\ H_2O + 6\ \text{ATP} \quad (2)$$

Oxygen consumption by day 6 embryos cultured under serum-free conditions

Embryos were cultured for 6 days under serum-free conditions (i.e. in SOFaaBSA throughout the incubation period, changing to fresh medium every second day) but otherwise in the same conditions as described above to determine whether the addition of serum from day 4 of culture affected the rate of oxygen uptake. Five oxygen uptake assays were carried out, using 4–7 blastocysts per assay. During the assay, embryos were incubated in H-SOF medium without FCS.

Statistical analysis

The pattern of oxygen, pyruvate and glucose uptake, and lactate and ATP production during development from day 0 to day 6 was analysed by one-way analysis of variance. Differences between means were examined using Fisher's test. A simple regression was conducted for both pyruvate and glucose uptake values against corresponding oxygen consumption values. Comparison between serum-treated and serum-free embryos was made using an unpaired *t* test. Analyses were conducted using the MINITAB statistical package (Ver. 10.1, Minitab, State College).

Results

Oxygen and carbohydrate uptake

Analysis of variance revealed that, overall, the uptake of oxygen by bovine embryos produced *in vitro* increased with development ($P < 0.001$). Little change was observed from day 0 (one-cell stage) to day 4 (16-cell/early morula stage) embryos (Table 1 and Fig. 1a). However, as embryo compaction was initiated, oxygen uptake rose, so that by day 6 (blastocyst stage) a significant increase was observed ($P < 0.05$). As with oxygen consumption, an overall increase in pyruvate uptake with development was observed ($P < 0.001$; Table 1 and Fig. 1a). Initially, pyruvate uptake was low, but after day 0 it was relatively constant up to day 5. Blastocyst formation was accompanied by a large increase in pyruvate consumption ($P < 0.05$). Glucose consumption followed a broadly similar pattern (Table 1 and Fig. 1a), with the exception that compaction was characterized by a significant ($P < 0.05$) rise in glucose uptake. When pyruvate uptake was plotted against oxygen consumption (Fig. 2a), a significant linear increase ($P < 0.001$) was observed ($R^2 = 0.61$). The same was the case for glucose uptake and oxygen consumption ($P < 0.001$, $R^2 = 0.49$; Fig. 2b).

Lactate and ATP production

Lactate production followed a similar pattern to the uptake of other measured carbohydrates, in that concentrations increased with development ($P < 0.001$), especially after blastulation. Assuming that lactate production reflects glycolytic activity rather than reduction of exogenously derived pyruvate by lactate dehydrogenase (E.C. 1.1.1.27), we have calculated the amount of ATP production from the proportion of glucose converted to lactate and the uptake of oxygen (Eqns 1 and 2). As lactate production was not measured for day 5 embryos (late morulae), we assumed approximately 100% of glucose could be accounted for by lactate, on the basis that it was likely to be similar to the proportion observed for both day 4 and day 6 embryos (see Table 1). From these assumptions, the rate of ATP production by bovine embryos was relatively constant for day 0 to day 2 embryos (eight-cell stage; Table 1 and

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Table 1. The uptake of oxygen, pyruvate and glucose and the production of lactate and ATP* by bovine embryos produced *in vitro*

Day of culture	Stage	Mean number of embryos per replicate (range)	Oxygen uptake (nl per embryo h^{-1}) (replicates)	Pyruvate uptake (pmol per embryo h^{-1}) (replicates)	Glucose uptake (pmol per embryo h^{-1}) (replicates)	Lactate production (pmol per embryo h^{-1}) (replicates)	ATP* production (pmol per embryo h^{-1}) (replicates)	% ATP from oxidation
0	1-cell	19 (10–32)	0.24 ± 0.02^{a} (8)	2.8 ± 0.7^{a} (7)	1.5 ± 0.4^{a} (4)	1.1 ± 0.5^{a} (4)	60 ± 10^{a} (4)	96
1	2- to 4-cell	12 (10–15)	0.21 ± 0.02^{a} (6)	$6.0 \pm 0.9^{a,b}$ (6)	2.6 ± 0.9^{a} (5)	3.0 ± 0.9^{a} (4)	58 ± 6^{a} (5)	92
2	8-cell	13 (9–20)	0.19 ± 0.02^{a} (6)	$5.7 \pm 0.7^{a,b}$ (6)	3.1 ± 0.5^{a} (5)	4.6 ± 1.4^{a} (6)	54 ± 6^{a} (5)	92
4	16-cell–morula	9 (7–11)	0.27 ± 0.06^{a} (4)	10.5 ± 0.7^{b} (4)	3.0 ± 0.4^{a} (3)	6.2 ± 0.2^{a} (3)	$85 \pm 4^{a,b}$ (3)	93
5	Compact morula	8 (6–11)	0.39 ± 0.07^{a} (6)	9.9 ± 2.6^{b} (6)	10.6 ± 3.3^{b} (6)	—	124 ± 23^{b} (6)	82
6	Blastocyst	5 (2–8)	0.90 ± 0.13^{b} (9)	20.5 ± 2.4^{c} (9)	14.6 ± 0.9^{b} (6)	31.9 ± 4.6^{b} (4)	221 ± 32^{c} (6)	86

*ATP production was calculated using an estimate of the proportion of glucose metabolized to lactate.
Values in the same column with different superscripts are significantly different ($P < 0.05$).

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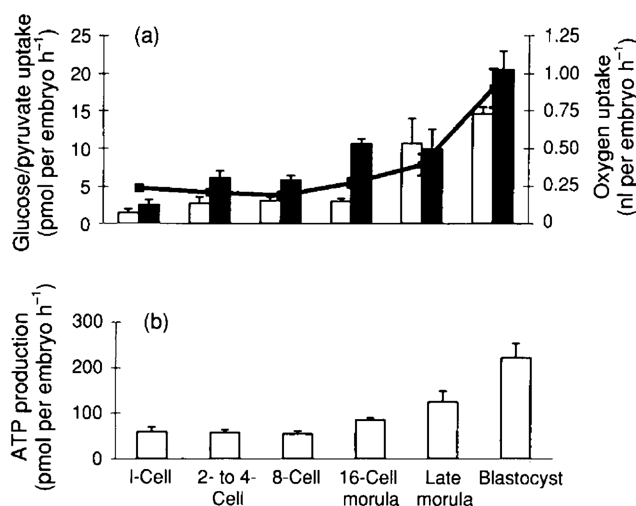

Fig. 1. Uptake of (a) oxygen (nl per embryo h^{-1}, ▬), glucose (pmol per embryo h^{-1}, □) and pyruvate (pmol per embryo h^{-1}, ■) and (b) production of ATP (pmol per embryo h^{-1}) by bovine putative zygotes and embryos produced *in vitro*.

Fig. 1b). From day 4, rate of ATP production increased with each developmental stage. However, as embryo development proceeded, the rate of production of ATP per cell decreased with each developmental stage, until day 4 (late morula stage; Table 2). The proportion of ATP produced via oxidative phosphorylation remained relatively constant at approximately 92–95% until the compact morula stage, at which time it dropped to approximately 82% of all ATP produced.

Development after the assay

Development to the blastocyst stage from cultured embryos after the assay period was as follows: day 0 = 5 of 15 (33%); day 1 = 3 of 12 (25%); day 2 = 42 of 64 (66%); day 4 = 9 of 16 (56%); day 5 = 5 of 7 (71%).

Effect of serum

The mean ($\pm$ SEM) oxygen uptake by blastocysts cultured under serum-free conditions was 0.76 ± 0.19 nl per embryo h^{-1}. This was not significantly different from that of embryos incubated in the presence of serum (0.90 ± 0.13 nl per embryo h^{-1}).

Discussion

This study demonstrates that the demand for energy production during development of bovine embryos produced *in vitro* remains constant throughout precompaction development, but then increases as compaction is initiated and the blastocoel is formed. Moreover, the profile of oxygen consumption, detailed here for bovine embryos for the first time, follows this developmental pattern and is similar to that reported for mice (Mills and Brinster, 1967; Houghton *et al.*, in press). The only other data that we are aware of that describes oxygen consumption for bovine embryos are that of Overstrom *et al.*

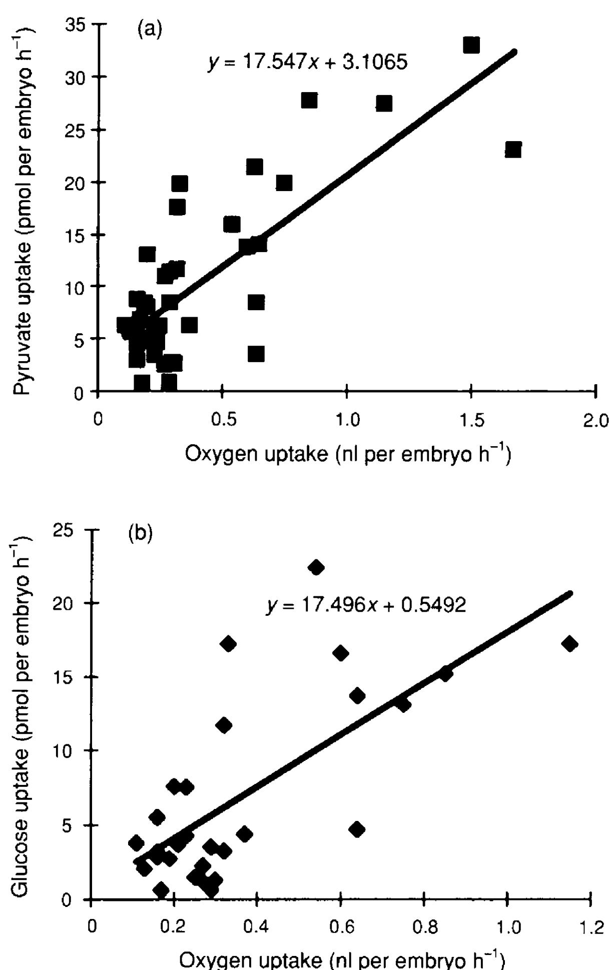

Fig. 2. Regression plots of (a) oxygen uptake (nl per embryo h^{-1}) against pyruvate uptake (pmol per embryo h^{-1}); or (b) glucose uptake (pmol per embryo h^{-1}) for bovine embryos (all stages) produced *in vitro*.

(1992). Using a phosphate-buffered medium and a sensitive polarographic electrode, they reported that day 7 bovine blastocysts derived *in vivo* consumed 1.47 ± 0.44 nl O_2 per embryo h^{-1}. We have measured oxygen consumption for the equivalent stage and the source of blastocysts using the same technique and medium described here, at 0.66 ± 0.08 nl per embryo h^{-1} (Thompson *et al.*, in press). The corresponding estimated rate of ATP production was 205 ± 23 pmol per embryo h^{-1}, of which approximately 85% derived from oxidative phosphorylation (Thompson *et al.*, in press). These values are similar to those reported here, indicating that at least in terms of ATP production, there is little difference between blastocyst stage embryos derived *in vivo* and *in vitro*. Differences between our data and that of Overstrom *et al.* (1992) may be attributed to differences in incubation conditions and methodology for determining oxygen uptake.

The patterns of energy substrate utilization described here follow qualitatively those described by Javed and Wright (1991) and Reiger *et al.* (1992). Javed and Wright (1991) characterized the utilization of radio-isotope-labelled glucose within embryos derived *in vivo*. Glycolytic metabolism (as measured by the release of tritiated water from 5-[^{3}H]glucose)

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Table 2. Mean (± SEM) number of cells and estimated ATP production per cell for randomly selected embryos assayed on day 4, 5 or 6 of culture

Day	Stage	n	Mean (± SEM) number of cells	Range	ATP production (pmol per cell h^{-1})
4	16-Cell–morula	20	20.5 ± 1.9	11–41	4.1
5	Compact morula	32	57.8 ± 6.4	9–141	2.1
6	Blastocyst	33	94.6 ± 6.7	30–210	2.3

rose markedly from the 16-cell stage through to blastulation. However, their values for glucose consumption are substantially below those reported here; up to 50 times in some cases. In contrast, our data are quantitatively similar (within a threefold range) to those of Reiger *et al.* (1992). As with our study, Reiger *et al.* (1992) used embryos produced *in vitro*, but used a coculture system for embryo development and radioisotope-labelled glucose and glutamine to measure metabolic pathway activity. For the first time, we have measured lactate production from bovine embryos derived *in vitro*. Our data demonstrate that lactate production for the one-cell stage is approximately 40% of glucose metabolized, increasing to 70% by the eight-cell stage and 100% by the 16-cell–early morula and subsequent stages. This pattern fits in well with the data of Reiger *et al.* (1992), who showed that glucose metabolism is low from the two-cell to the eight-cell stage; glycolytic metabolism then increased continuously to blastulation. In contrast, little increase was observed in glucose oxidation through the tricarboxylic acid cycle (as indicated by the release of $^{14}CO_2$ from [6-^{14}C]glucose). Our data provide further evidence that the major metabolic fate of glucose in post-compaction bovine embryos is lactate production. A similar conclusion was drawn by both Javed and Wright (1991) and Waugh and Wales (1993), using [U-^{14}C]glucose. Furthermore, Gardner *et al.* (1993) have demonstrated that lactate production can account for 100% of the glucose consumed by post-compaction ovine embryos. The overwhelming evidence is that a significant amount of aerobic glycolysis occurs (i.e. conversion of glucose to lactate in the presence of oxygen) when glucose is available in the medium. The physiological significance of these observations has yet to be resolved. However, Thompson *et al.* (1993) suggested that the system for shuttling reducing equivalents from the cytosol across into the mitochondria may be impaired in embryos from these species and that maintenance of the cytoplasmic redox equilibrium of [NADH]:[NAD^+] necessitates the production of L-lactate.

In contrast to glucose metabolism, there are little data pertaining to the fate of pyruvate in bovine embryos, with the exception of those of Reiger and Guay (1988), which show that significant quantities of pyruvate are oxidized by day 7 blastocysts derived *in vivo*. However, studies using ovine embryos have demonstrated that pyruvate uptake and oxidation occur in significant quantities throughout pre-elongation development, and increase during compaction and blastulation (Gardner *et al.*, 1993; Thompson *et al.*, 1993). Here, we report that pyruvate uptake increases with development, particularly as embryos begin compaction. This situation differs from that in mouse embryos, in which pyruvate uptake decreases after the eight-cell stage as substrate preference switches from pyruvate to glucose (Gardner and Leese, 1986; Martin and Leese, 1995). However, in contrast to mouse embryos, human embryos display a similar pattern of pyruvate metabolism to that described here for the bovine embryo (Hardy *et al.*, 1989; Conaghen *et al.*, 1993). On the basis of the following reaction:

$$\text{Pyruvate} + 2.5\ O_2 \rightarrow 3\ CO_2 + 2\ H_2O \qquad (3)$$

the amount of pyruvate taken up can more than account for that consumed in complete oxidation at all stages, with the exception of embryos from day 0 of culture (i.e. putative zygotes). It is, therefore, unlikely that endogenous substrates, such as cytoplasmic lipids, proteins or glycogen, contribute greatly, if at all, to the generation of ATP in bovine embryos.

This report, for the first time, describes the rate of ATP production for bovine embryos. We have made several assumptions concerning the amount of glycolysis and the proportion of oxygen taken up which acts as the terminal electron acceptor in oxidative phosphorylation (100% in this case). However, we believe that these assumptions are justified on the evidence of our data. Production of ATP is constant from day 0 to day 2 of culture (one-cell to eight-cell stage), but increases as embryos approach compaction, and continues to increase with blastulation. This pattern fits in well with the observation that both protein synthesis and activity of the Na^+–K^+ ATPase increase during compaction and blastocoel formation in mammalian embryos, and therefore the demand for ATP will increase (Leese, 1991). The ATP production per cell within embryos, however, steadily declined until compaction. This may merely reflect the decreasing volume in cell size as embryos develop, therefore, decreasing the number of mitochondria per cell. Perhaps of more interest was the decrease in proportional contribution to ATP production from oxidative phosphorylation as the amount of glycolysis increased. Such a strategy may be required by embryos to produce ATP as they pass from the oviduct to the uterus, especially if the concentration of oxygen decreases, as has been reported in some mammals (Fischer and Bavister, 1993). This relationship between the amount of glycolysis and ATP production fits well with the observation that there is no absolute requirement for glucose for bovine pre-elongation development (Kim *et al.*, 1993b; Matsuyama *et al.*, 1993; Rosenkrans and First, 1994), although a beneficial effect has been observed if added near to the time of compaction (Ellington *et al.*, 1990; Kim *et al.*, 1993b). Whether or not glycolysis can support sufficient amounts of ATP production to support blastulation, as has been found in rats (Brison and Leese, 1994), has yet to be determined.

The data presented here demonstrate that oxidation of substrates provides the majority of ATP throughout the development. This supports well the observations that addition of either pyruvate or lactate greatly facilitates development of early bovine embryos (Kim *et al.*, 1993a, b; Matsuyama *et al.*, 1993; Takahashi and First, 1993). However, there is evidence that ovine embryos will develop to the blastocyst stage, albeit at low rates, in the absence of carbohydrates and presence of amino acids (Thompson *et al.*, 1993). This is further supported

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by the evidence that glutamine was oxidized by bovine embryos in much the same developmental pattern as pyruvate (Reiger *et al.*, 1992). Coupled with the data presented here, this suggests that some form of oxidizable substrate present in the culture medium is required for development, but that pyruvate is much the preferred substrate. A similar conclusion was made by Thompson *et al.* (1993) for development of ovine embryos *in vitro*.

The measurement of oxygen consumption for short periods had little detrimental effect on embryo viability, as assessed by further development *in vitro*. It is generally accepted that between 30% and 40% of zygotes will reach the blastocyst stage if *in vitro* production procedures are followed (Bavister *et al.*, 1992). The rates of development after culture reported here are similar to those reported elsewhere using these culture techniques (Thompson *et al.*, 1992). We believe that the technique we have described for measuring oxygen consumption may be used for the biochemical determination of embryo viability. Consumption of oxygen has already been identified as a possible viability indicator for bovine embryos (Overstrom *et al.*, 1992). The significant linear relationships observed when both pyruvate and glucose uptake values were plotted against oxygen consumption demonstrate that, as the demand for ATP increases with development, it is likely that consumption of all substrates increases. We speculate that if a metabolic test of embryo viability is to be devised, it is likely to involve the measurement of more than one substrate, with oxygen as one major candidate. As the sensitivity of our technique could almost certainly be improved with further engineering, we propose that it may find application in determining the viability of preimplantation stage embryos.

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