

Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period

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Summary. Large White gilts, 9 to 18 months old, that had exhibited at least two natural oestrous cycles were divided into three groups (phases): unmated pre-ovulatory, unmated post-ovulatory and mated post-ovulatory ($n = 16, 20$ and 18). Oviductal luminal fluid samples were collected under anaesthesia by micropipette from the ampulla and ampullary–isthmic junction and analysed by an ultramicrofluorometric technique. Glucose concentrations (mmol l^{-1} , means combining regions; mean \pm SEM) were significantly higher in blood plasma than in oviductal fluid (4.56 ± 0.20 versus 0.59 ± 0.16 ; $P < 0.0001$; $n = 27$), whereas lactate was higher in the oviduct (5.71 ± 0.53 versus 2.48 ± 0.24 ; $P < 0.0001$; $n = 27$). No significant differences were found between the ampulla and the ampullary–isthmic junction. However, the concentration of glucose was significantly higher ($P < 0.05$) in the ampulla of the pre-ovulatory group (0.97 ± 0.20 ; $n = 13$) compared with the mated group (0.25 ± 0.05 ; $n = 14$) and its concentration in the ampullary–isthmic junction in the pre-ovulatory group (1.65 ± 0.63 ; $n = 13$) was significantly greater ($P < 0.05$) than in the post-ovulatory (0.43 ± 0.11 ; $n = 11$) or mated groups (0.17 ± 0.02 ; $n = 14$). Lactate in the ampulla of mated animals was higher than in the pre-ovulatory group (6.83 ± 0.70 versus 3.86 ± 0.38 ; $P < 0.05$; $n = 15$ and 13), but neither was significantly different from the post-ovulatory group. Furthermore, no change was seen at the ampullary–isthmic junction in lactate concentration with phase. Pyruvate concentrations showed no differences with phase, region or with plasma (oviduct 0.21 ± 0.02 ; plasma 0.14 ± 0.01 ; $n = 27$ and 26). Glucose concentration within the oviduct decreased at a time when gametes or embryos, with their liberated cumulus cells, were present. Consequently it is evident that the microenvironment of the gametes and embryos is changing and, in the light of these measurements, the composition of the media used for *in vitro* culture experiments may require modification to reflect the physiological levels more closely.

Keywords: oviductal fluid; pig; energy substrates; early development

Introduction

The mammalian oviduct permits and regulates the transport, maturation and nutrition of the male and female gametes, allows fertilization of the ovum to occur and maintains the embryo in its initial stages of development.

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Anatomically, the oviduct can be divided into four merging regions: the pre-ampulla, including the fimbriae and the infundibulum, the ampulla, the isthmus and the junctura. Each of these have morphological and histological features that suggest specific functions (Nilsson & Reinius, 1969; Reinius, 1970). Two further subdivisions can be made to include two transitional regions, the ampullary-isthmic and the utero-tubal junctions: the latter is the point of connection of the oviduct with the outer musculature of the uterus. These regions exhibit greater variation in structure between species which may belie a greater complexity of function (Hafez & Blandau, 1969; Hafez, 1973; Hunter, 1988a). The junction between the ampulla and the isthmus is considered to be the site of fertilization, given the rapidity (approximately 30–45 min in the pig) with which eggs are transported to this point following ovulation. Thereafter, the eggs or embryos are maintained in this region for most of their sojourn within the oviduct (Hunter, 1974).

Oviductal fluid is a complex mixture, the ionic composition, pH, osmolarity and macromolecular content of which differ in many important respects from that of plasma (Gandolfi *et al.*, 1989). These differences demonstrate that oviductal fluid is not simply a filtrate of blood plasma (Leese, 1988).

The secretory activity of the oviduct depends upon the prevailing balance of oestrogen and progesterone in the blood and hence the stage of the oestrous cycle. The greatest volume of secretion coincides with the period of oestrus and is highest at a time close to or just after ovulation and minimal during the mid-luteal phase or during pregnancy (Restall, 1966; Iritani *et al.*, 1974; Lippes *et al.*, 1981; Hunter, 1988b). The effect of oestrogen in promoting secretion has been demonstrated in ovariectomized rabbits in which there is a reduction in both the volume and pressure of oviductal secretion, which can be restored by systemic injections of oestradiol (Bishop, 1956).

The most commonly investigated substrates in oviductal fluid are glucose, lactate and pyruvate because of their importance to gamete and embryo metabolism (Brinster, 1973; Biggers & Borland, 1976; Leese, 1988). These metabolites have been found in the fluid of all species so far examined, and their concentrations in rabbit fluid increase after ovulation, suggesting a role in the development of the embryos (Holmdahl & Mastroianni, 1965). Lactate has been demonstrated to be an important energy source used by developing embryos *in vitro*. In addition, during progesterone therapy to mimic the luteal phase, the ampulla was found to produce significantly more lactate than the isthmus (Hamner, 1973). Furthermore Carlson *et al.* (1970) using indwelling cannulae in the cow, found a cyclic variation in glucose concentration, the highest values being observed during the first day after oestrus.

Glucose, lactate and pyruvate can be transported from the blood into the oviductal lumen unchanged. Moreover, lactate can also be synthesized within the wall of the oviduct. Similarly, pyruvate can be readily synthesized from the lactate or glucose found in the lumen of the oviduct by the action of the cumulus cells surrounding the oocyte (Leese & Barton, 1985). There is also evidence that glucose can be taken up directly by the early embryo, at least *in vitro*, and possibly *in vivo* (Wales, 1975; Leese & Barton, 1984).

Using the technique of vascular perfusion, Leese & Gray (1985) demonstrated that the glucose and pyruvate that appeared in the lumen originated from blood whereas most of the lactate was derived from glucose within the oviductal wall.

Previous work on the composition of oviductal fluid involved its collection by cannulation (Bishop, 1956) or ligation (David *et al.*, 1969). However, there is the assumption that the fluid collected was homogeneous along the length of the oviduct and that its composition does not change with time. Additionally, because of the chronic manner of collection, the composition of the fluid may have been modified as a result of tissue trauma, and it is difficult to recognize the results as physiological (Biggers & Bellvé, 1974). Despite these reservations, the work by David *et al.* (1969), involving ligation of oviducts to create four segments, demonstrated differences in the composition of the fluid within each. Subsequent work by Biggers & Borland (1976) demonstrated that microenvironments may exist in the vicinity of eggs and embryos. The sampling technique used in the present experiments has enabled the collection of discrete samples of oviductal fluid from different

regions and at different times, thus providing more accurate information on the physiology of the oviduct. This technique was intended to be the best possible compromise between a non-invasive technique and the preservation of the normal physiological state. The underlying objective of this work was to gain a better knowledge of the environment, particularly the microenvironment within the oviductal lumen, to which the gametes and early embryos are exposed (Hunter, 1990).

Materials and Methods

Animals

Large White gilts, 9 to 18 months old, were penned indoors under a natural lighting regimen in groups of five or six. They were fed twice a day on a normal commercial diet with free access to water. They had exhibited at least two spontaneous oestrous cycles, verified by daily heat checks, and had not been used in any previous experimental work.

The gilts were divided into three groups: (i) unmated pre-ovulatory, (ii) unmated post-ovulatory and (iii) mated post-ovulatory ($n = 16, 20$ and 18 , respectively). The mated animals were served twice by a boar of proven fertility, initially when they were first discovered to be in oestrus and again approximately 24 h later. Surgery was scheduled for pre- and post-ovulatory groups in relation to the commencement of standing heat, in the knowledge that ovulation would occur 40–44 h later (Hunter, 1974). Ovulation was confirmed at the time of surgery in groups (i) and (ii) by inspection of the ovaries. This also confirmed the timing of surgery relative to ovulation.

Surgery and fluid sampling

Animals were starved for 24 h before surgery. Anaesthesia was induced by an intravenous injection of sodium pentobarbital (Somnotol: CDMV Inc., Quebec), with a dose sufficient to permit endotracheal intubation and was thereafter maintained on a closed circuit apparatus using halothane (Fluothane: ICI Pharmaceuticals: distributed by CDMV Inc., Quebec), nitrous oxide and oxygen. Strict aseptic techniques were used and the reproductive tract was exposed by a mid-ventral incision; the oviducts were gently exteriorized, one at a time, to obtain the samples. Manipulation of the tissues was kept to a minimum to avoid trauma, and the bulk of the genital tract was left within the abdomen to prevent dehydration.

Possible regional differences in oviductal fluid composition were investigated by collecting samples from the ampulla and the ampullary–isthmic junction.

The samples were collected as non-invasively as possible, using sterile fine bore Pasteur pipettes, which had been hand-drawn and thereafter selected for an internal diameter of approximately 0.5 mm. Their tips were flame-polished to avoid tissue damage and contamination of the samples by blood. All samples were collected by capillary action alone; no form of suction was used. This preserved the physiological and regional integrity of the samples and ensured the steady-state concentration of the substrates which they contained when related to time. Samples containing blood were rejected.

The ampullary sample was obtained by introducing the pipette between the fimbria and into the ampulla to a depth of approximately 3–4 cm. The sample from the ampullary–isthmic junction was obtained by puncturing the wall of the oviduct in the vicinity of the sampling site with a blunt, polished, dissecting needle. Conspicuous blood vessels were avoided. After its withdrawal, and the removal of any potentially contaminating blood with a sterile surgical gauze, the pipette was introduced into the lumen to the correct position to collect the sample. The pipette was then withdrawn using a finger to seal the end thus preventing the loss of the sample into the lumen. Sampling was acute and required only a few seconds.

The volume of samples in individual pipettes ranged from 5 to 50 μl , although it was frequently possible to collect duplicate samples. Immediately after sampling, the pipettes were coded and frozen at -25°C .

For comparison, a peripheral blood sample was taken at the time of surgery.

Assays for energy substrates

Samples were transported to the laboratory on dry ice and maintained at -80°C until analysis by ultramicrofluorometry using the enzymes hexokinase and glucose-6-phosphate dehydrogenase to determine the glucose concentration and lactate dehydrogenase for lactate and pyruvate. These were essentially a miniaturization of conventional methods of analysis in which the reduced pyridine nucleotides, NADH and NADPH, are generated or consumed in coupled reactions (see Leese & Barton, 1984; Leese *et al.*, 1984; Gardner & Leese, 1990).

Statistical analysis

After decoding, the results were analysed statistically by SAS (1985) general linear models procedure for analysis of variance followed by the Student–Newman–Keuls test for differences between groups. For statistical purposes they

were separated into two groups, the first comparing blood plasma with oviductal fluid concentrations, and the second comparing the ampulla with the ampullary–isthmic junction. In the latter comparison it was found that the variances between groups were not equal. The statistical analyses were therefore performed on arcsine transformed data and the variances were homogeneous according to O'Brien's (1979) test of equal variances. Regional differences in substrate concentrations along the length of the oviduct, and differences between unmated pre- or post-ovulatory and mated post-ovulatory, were considered significant at $P < 0.05$.

Results

Glucose concentrations (mmol l^{-1} , mean \pm SEM) were significantly higher in plasma than in the oviductal fluid ($P < 0.0001$), whereas lactate was higher in the oviduct than in the plasma ($P < 0.0001$) (Fig. 1). No significant regional differences were found between the ampulla and the ampullary–isthmic junction. However, the concentration of glucose at the ampullary–isthmic junction of the pre-ovulatory group (1.65 ± 0.63 ; $n = 13$) was significantly greater than in the post-ovulatory (0.43 ± 0.11 ; $n = 11$) or mated groups (0.17 ± 0.02 ; $n = 14$). The concentration of glucose was also significantly higher ($P < 0.05$) in the ampulla of the pre-ovulatory group (0.97 ± 0.20 ; $n = 13$) than in the ampulla of the mated group (0.25 ± 0.05 ; $n = 14$). Lactate concentration was significantly higher in the ampulla of mated animals than in the pre-ovulatory group (6.83 versus 3.86 ; $P < 0.05$; $n = 15$ and 13), but neither was significantly different from the post-ovulatory group. Furthermore, no change was seen at the ampullary–isthmic junction in lactate concentration with phase. Pyruvate concentrations showed no difference either with phase, region or with plasma (oviduct 0.21 ± 0.02 ; plasma 0.14 ± 0.01).

Attempts were made to obtain samples from the isthmic portion of the uterotubal junction, but owing to the extremely restricted internal diameter of the oviduct at this point and also because the secretions in this region were predominantly mucoid in nature, no samples were collected.

Discussion

The glucose concentration within the ampulla of the oviduct was significantly lower in the mated animals than the pre-ovulatory value and there was a corresponding rise in the concentration of lactate. Similarly, in the ampullary–isthmic junction, the glucose concentration also decreased, but the concomitant rise in the amount of lactate was not statistically significant. Glucose metabolism did not lead to an accumulation of pyruvate as the amount of pyruvate did not increase and, as its relative concentration was one tenth that of glucose, any increases would have been clearly evident.

Critical changes occur in an embryo during the time that it is maintained in the oviduct. These changes are important to its immediate and long-term survival. They are primarily related to the development of its metabolic competence and thus ability to metabolize the various energy substrates found in oviductal fluid; these substrates are used in ascending order of complexity (Biggers *et al.*, 1967; Brinster, 1973; Biggers & Borland, 1976). A knowledge of the natural concentrations of substrates upon which an embryo depends *in vivo* could therefore be of considerable benefit and can be applied directly to the formulation of media for *in vitro* studies. The development of an embryo *in vitro* to the blastocyst stage has often been used as an index of the beneficial effects of the composition of one medium rather than another. This index indicates only that the embryo has achieved that relatively early stage and frequently does not consider its future developmental potential which should encompass both fetal development and the birth of normal live young.

Studies in other species, although based on collection by cannulation, identified oviductal substrate concentrations as, 1.57 – 1.76 $\text{mmol glucose l}^{-1}$, 1.67 – 2.51 $\text{mmol lactate l}^{-1}$ and 0.15 $\text{mmol pyruvate l}^{-1}$, in sheep (Hamner, 1973). In cattle, Carlson *et al.*, (1970) identified mean glucose concentrations during the period of oestrus in two cannulated animals as 0.04 and 0.02 mmol l^{-1} , respectively. With a more discrete method of collection, the concentrations in the mouse were found to be 3.4 $\text{mmol glucose l}^{-1}$, 4.79 $\text{mmol lactate l}^{-1}$ and 0.37 $\text{mmol pyruvate l}^{-1}$ (Gardner &

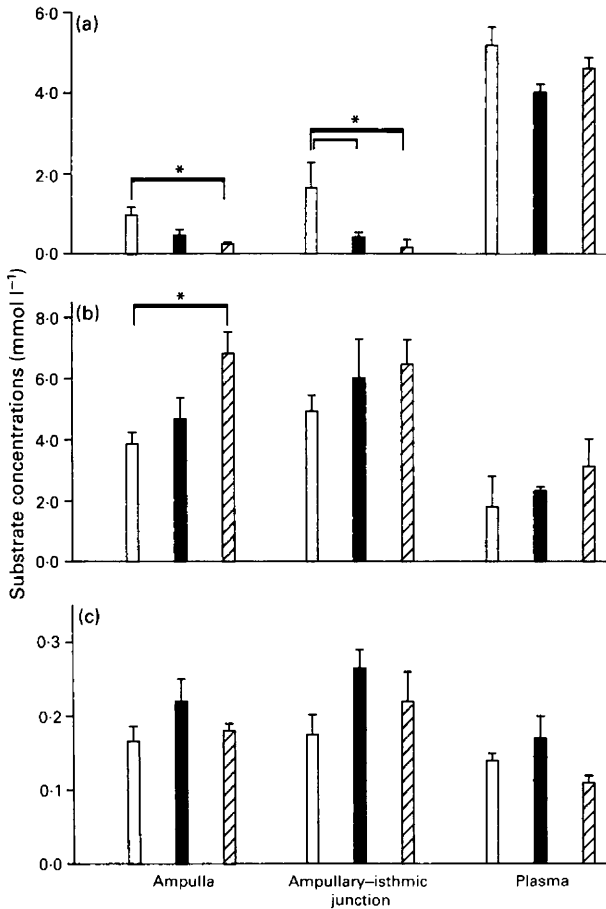


Fig. 1. Substrate concentrations ($\text{mmol l}^{-1} \pm \text{SEM}$) (a) glucose (b) lactate and (c) pyruvate in oviductal fluid sampled from regions (ampulla and ampullary-isthmic junction) or plasma during different phases (\square) unmated pre-ovulatory; (\blacksquare) unmated post-ovulatory; (▨) mated post-ovulatory. *Values at each end of the bar are significantly different ($P < 0.05$).

Table 1. Concentrations (mean \pm SEM) of glucose, lactate and pyruvate in blood plasma and fluid taken from two regions (ampulla and ampullary-isthmic junction) of the pig oviduct during the peri-ovulatory period

| Samples | Phase | Glucose (mmol l^{-1}) (n) | Lactate (mmol l^{-1}) | Pyruvate (mmol l^{-1}) |
|----------------------------|----------------|---|-------------------------------------|--------------------------------------|
| Ampulla | Pre-ovulatory | 0.97 ± 0.20 (13) | 3.86 ± 0.38 (13) | 0.17 ± 0.02 (15) |
| | Post-ovulatory | 0.47 ± 0.14 (11) | 4.67 ± 0.70 (12) | 0.22 ± 0.03 (14) |
| | Mated | 0.25 ± 0.05 (14)* | 6.83 ± 0.70 (15)* | 0.18 ± 0.01 (15) |
| Ampullary-isthmic junction | Pre-ovulatory | 1.65 ± 0.63 (13) | 4.93 ± 0.53 (13) | 0.18 ± 0.03 (15) |
| | Post-ovulatory | 0.43 ± 0.11 (11)* | 6.02 ± 1.28 (12) | 0.27 ± 0.03 (14) |
| | Mated | 0.17 ± 0.02 (14)* | 6.48 ± 0.80 (15) | 0.22 ± 0.04 (15) |
| Blood plasma | Pre-ovulatory | 5.19 ± 0.45 (8) | 2.05 ± 0.43 (8) | 0.14 ± 0.01 (8) |
| | Post-ovulatory | 4.02 ± 0.20 (10) | 1.93 ± 0.19 (10) | 0.17 ± 0.02 (10) |
| | Mated | 4.62 ± 0.26 (8) | 3.47 ± 0.46 (9) | 0.11 ± 0.01 (9) |

*Significantly different from pre-ovulatory group $P < 0.05$.

n: number of observations.

Leese, 1990). Although these differences may be due in part to the different methods of collection, they clearly show that species differences exist in the concentrations of substrates found in oviductal fluid.

Results reported by Hamner (1973) showed that lactate increased in the ampulla of the rabbit after ovulation and the present data show a trend in this direction, and also in the ampullary–isthmic junction, but in neither case was the increase statistically significant. However, the concentration of lactate in the pig oviduct was greater than that found in plasma in contrast to the findings of Holmdahl & Mastroianni (1965) for the rabbit oviduct.

Leese & Gray (1985) investigated the source of these substrates in rabbit oviductal fluid, using a combined vascular and luminal perfusion system. They concluded that these substrates, except for lactate, are derived mainly from the blood. When glucose was added to the medium, lactate appeared in the lumen, signifying that it was being formed metabolically from glucose within the oviductal wall. They estimated that 75% of the lactate appearing in the lumen *in vivo* is derived from vascular glucose and the remaining 25% from vascular lactate. The concentration of pyruvate found in oviductal fluid is not necessarily derived solely from the blood, since Biggers *et al.* (1967) showed that, in the mouse, cumulus cells surrounding the ova or embryos can synthesize pyruvate from glucose. This is linked to their observation that one-cell mouse embryos without cumulus cells *in vitro* could divide only in the presence of pyruvate, and cannot metabolize glucose at this stage. Subsequently, Leese & Barton (1985) and Gardner & Leese (1990) also demonstrated the importance of the cumulus cells and suggested that they provide energy substrates for the eggs and embryos. In pigs, the cumulus cells disperse and are seldom found attached to the zona pellucida more than 6 h after ovulation (Szöllözi & Hunter, 1973). However they may still provide energy sources, since they remain in suspension in the immediate vicinity of the eggs. In rabbits and probably in ruminants, pyruvate may also be formed metabolically from short chain fatty acids such as propionate in the oviductal wall (Leese, 1980).

Possible explanations for the post-ovulatory decrease in the amount of glucose in the oviductal fluid include (i) a higher demand being placed on the substrate by the musculature of the oviduct, (ii) its use by eggs, embryos or cumulus cells and (iii) changes in the dynamics of glucose transport through the oviductal wall.

If the decrease in the concentration of glucose did stem from an increased demand by the musculature, it may reflect specialized changes occurring in the contractile activity of the musculature in response to the presence of eggs or embryos. Whereas glucose may be used by eggs or embryos, Flood & Wiebold (1988) showed that pig eggs and embryos up to the four-cell stage consume only about 0.1–0.2 pmol glucose per embryo per hour. It is unlikely that the decrease in the amount of glucose found in our studies (1 mmol l^{-1}) would be solely attributable to the metabolic activity of eggs and embryos. Moreover, if the requirement of embryos for glucose is so low, then a normal physiological response of the oviduct to the presence of eggs or embryos may be to decrease the rate of transport of glucose into the lumen. It has been shown that supra-physiological concentrations of glucose may interfere with normal embryonic development *in vitro* (Lawitts & Biggers, 1991). The relationship of these substrates in oviductal fluid to the presence of embryos will be investigated in our laboratories by the use of a unilaterally ovariectomized model.

When metabolic competence is compromised, as frequently occurs *in vitro*, it manifests itself as a 'block' to embryonic development, occurring at the four-cell stage in the pig (Polge & Frederick, 1968; Rundell & Vincent, 1968, 1969; Davis & Day, 1978). Such 'blocks' should be overcome by using a medium based upon the physiological composition of oviductal fluid. Recent work by Lawitts & Biggers (1991) established that the two-cell 'block' in mouse embryos is due to unsuitable concentrations of components being used in a medium (M16). Their work demonstrated that the concentrations of glucose, lactate and pyruvate were too high and this corresponds to our data and supports our hypothesis that modification to a more physiological level should be beneficial.

However, there have been conflicting reports on the effects of pyruvate and lactate on the development of pig embryos in culture. Davis & Day (1978) reported that pyruvate and lactate interfere with development to the blastocyst stage. The medium used was a modified Krebs-Ringer bicarbonate containing 5.56 mmol glucose l^{-1} , 25 mmol lactate l^{-1} and 0.25 mmol pyruvate l^{-1} . Subsequently, Davis (1985) reported no detrimental effects with respect to pyruvate alone (0.025–0.25 mmol l^{-1}), but that a combination of lactate (25 mmol l^{-1}) and pyruvate (0.025 mmol l^{-1}) inhibited embryonic development. Using similar concentrations of glucose, lactate and pyruvate, Stone *et al.* (1984) found that blastocyst formation was inhibited by the combination of all three substrates (although this was not statistically significant) but that hatching was completely prevented. On the other hand, glucose alone supported blastocyst formation, hatching and zona expansion. It was also evident from these studies that the medium used could partially compensate for the inhibitory effects since these were less evident when Minimum Essential Medium was used as opposed to Krebs-Ringer bicarbonate.

Our results have quantified the physiological concentrations of energy substrates in the natural environment for embryos. The data suggest that the concentrations of lactate and glucose used in media for studying pig embryonic development have been too high, whereas pyruvate concentrations have been close to physiological. It would seem, therefore, that a paradox exists between embryonic development in culture media and *in vivo*. The inhibition of embryonic development *in vitro*, using pyruvate and lactate concentrations that are close to physiological, proves that other components in a medium such as the amino acids (Stone *et al.*, 1984) and electrolytes (Borland *et al.*, 1977) are important and that *in vitro* these may be able to compensate for the absence of natural components that are present in oviductal fluid.

The objective of this study was to identify the actual concentrations of substrates within the oviductal lumen of pigs during the peri-ovulatory period. The most non-invasive sampling technique was used to avoid artefacts that a chronic sampling system would have introduced through tissue trauma. We would anticipate that a medium based on the range of concentrations found in the post-ovulatory or mated groups in the ampullary-isthmic region should give the best results for culturing pig embryos (glucose 0.2–0.5 mmol l^{-1} ; lactate 6.3–6.5 mmol l^{-1} ; pyruvate 0.23–0.27 mmol l^{-1}). Consideration should also be given to frequent replacement of medium to reflect the dynamic environment within the oviduct and to avoid their depletion.

Whereas the emphasis has perhaps been placed on sustaining the eggs and embryos, it should not be forgotten that these substrates have also been shown to be important to spermatozoa through their effects on motility, capacitation (Rogers & Perreault, 1990) and the acrosome reaction (Miyamoto & Chang, 1973). Spermatozoa may use pyruvate and lactate (Storey & Kane, 1978), although high concentrations of lactate inhibit capacitation and hyperactivation, and thus depress the fertilization rate (Neill & Olds-Clarke, 1988).

If embryonic development is compromised by the use of media that are not based on physiological measurements, embryonic mortality may be potentiated even some time after blastocyst formation, as a result of damage incurred by the embryo during earlier stages (Renard *et al.*, 1980). This is supported by the observations that when embryos develop successfully *in vitro* from zygotes to blastocysts, they are retarded by 18–24 h compared with the situation *in vivo* (Bowman & McLaren, 1970; Harlow & Quinn, 1982). Because of the delay between cause and effect, embryonic deterioration may be erroneously attributed to other factors. Bavister (1988) has itemized a range of factors that could contribute to either the failure or success of an *in vitro* system. Ultimately, confidence that a medium contains the precise physiological concentrations of substrates should circumvent these problems and permit the design of better culture media than are currently available. Despite the many studies that have been made on the nutrient requirements of the embryo *in vitro*, studies on nutrient availability during this period of development *in vivo* are rare and largely derived from experimentation on small laboratory animals. Similar investigations in large commercial species have been neglected; this can perhaps now be remedied by the adoption of this analytical technique.

We should like to express our special thanks to K. Martin and P. Humpherson for their help with the sample analysis and also to the staff at the CRRRA experimental farm for the care of the animals and assistance at times of surgery. This work was supported by NSERC of Canada and MESST (Quebec).

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Received 21 October 1991