EFFECTS OF NUTRITIONALLY-MEDIATED INCREASES IN THE PLASMA AMMONIA CONCENTRATIONS OF DONOR EWES ON THE *IN VIVO* AND SUBSEQUENT *IN VITRO* DEVELOPMENT AND METABOLISM OF THEIR EMBRYOS.

BY

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" This is a year in the life of a bloke who 'll never drive a Porsche, seldom wear a tie, and doesn't commute to work, because he's there already.
He's been there since the first ewe lambed, the first cow milked, the first field of wheat harvested..... and he still hasn't made any money (or so he says).
But he's still there". (The peasant farmer).

Henry Brewis (1988)

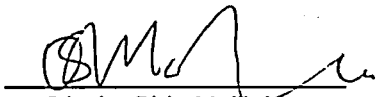
DEDICATION

This is to my brother Goodwill; at last the secret is out; you have always been my inspiration, I love you.

To my GrandDads, Segotso Ramoratwe and Selaelo Segobai; if you had been here today.....

DECLARATION

This thesis is based on work carried out by myself and has not been accepted in any previous application for a degree, neither has it been previously published in any journal. All sources of information have been acknowledged by means of references



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SUMMARY

A review of the literature indicated that high levels of protein in ruminant diets can lower fertility. Ammonia from proteins that are rapidly degraded by the rumen microbes has been suggested as the factor that may affect fertility. The mechanism(s) by which the detrimental effects of ammonia are manifested are speculative. However it seems that the concentration of ammonia in the reproductive tract may be responsible for embryo mortality in sheep as was found in the present study. This study was planned to investigate some of the possible mechanism(s) by which ammonia affects embryo survival in ewes.

Due to the fact that "urea-molasses" blocks are used as a strategy for better utilization of low quality roughages in both hill environments in Britain and in tropical conditions, urea was used as a source of highly degradable nitrogen in the present study.

Twelve ewes were used, balanced for weight and randomly allocated to two treatments. The two treatments were designed to supply the maintenance energy needs of the ewes. The control diet contained 2 g urea/day to meet but not exceed the needs of the rumen microbes for degraded protein, and the "urea-diet" contained 48g urea/day. Therefore rumen degradable protein was in excess of rumen microbial needs to the extent of about 136 g/day in the "urea-diet".

Ewes were given a single injection of $PGF_{2\alpha}$ on the day preceding the introduction of the experimental diets. An exogenous source of progesterone was given via a controlled internal drug release (CIDR) device containing 0.3 g progesterone and this was left in place for 12 days. Superovulation was induced by a series of injections of porcine follicle stimulating hormone (FSH). Daily collection of blood samples started just before insertion of the CIDR device and the plasma was later analysed for progesterone. Starting at 10 hours after the removal of the CIDR device plasma samples were collected at 2-hour interval for the next 30 hours and analysed for LH, glucose, insulin, urea and ammonia.

Laparoscopy was used to deposit semen directly into the uterine horns 52 hours after the removal of the CIDR device. At Day 3

post insemination, embryos were recovered by mid-ventral laparotomy.

Visual assessment of embryos was carried out at the time of recovery using a stereomicroscope (x50 magnification) and thereafter using an inverted microscope (x200 magnification). The embryos were then incubated for 3 hours in synthetic oviductal fluid medium (SOFM) labelled with [5-^{3}H]glucose and [U-^{14}C]glucose immediately following recovery, and after 72 hours in in vitro co-culture. After 96 hours in co-culture the embryos were incubated in ovine culture medium (OCM) labelled with ^{35}S-Methionine for 2 hours.

Progesterone concentrations were 1.91±0.15 in the urea-fed as opposed to 1.55±0.12 ng/ml in the control ewes. However the times of the LH peak and oestrus were not different for the control and the urea-fed animals (20.4±2.48 versus 22.9±1.68 and 18.4±2.93 versus 18.3±1.82 and hrs respectively), neither were ovulation rates (6.5±1.41 and 7.0±1.09) or the numbers of embryos recovered (4.3±1.20 and 4.4±1.19).

Plasma insulin and glucose concentrations were significantly higher ($p<0.001$) in urea- as opposed to control-fed ewes, 48.5±1.97 versus 34.9±1.27 μU/ml, and 3.95±0.053 versus 3.64±0.045 mmol/l, for the urea- and control-fed ewes respectively. Significantly higher concentrations of urea ($p<0.001$), 5.47±0.152 for the urea as opposed to 2.41±0.099 mmol/l for the control ewes, and ammonia ($p<0.01$) 90.4±9.22 and 50.9±6.92 μmo/l were measured in plasma.

There were higher levels of ammonia and urea in the flushings from the reproductive tracts of the urea-fed than control ewes (63.6±7.50 μmol/l and 0.414±0.084 mmol/l versus 35.7±4.30 μmol/l and 0.185±0.044 mmol/l respectively)

The percentage of well-developed embryos from the urea-fed ewes was greater than for the controls(90% versus 79%) at the time of recovery. There was also a greater number of viable embryos from the urea-supplemented ewes than from the controls. After 72 hours in co-culture the percentages of viable embryos for the control ewes were 59% compared with 46% for the "urea-fed" ewes.

Glucose metabolism by the embryos as measured by the production of $^{3}H_2O$ and $^{14}CO_2$ was positively correlated to stage of development. On the day of embryo collection viable embryos from "urea-fed" ewes were metabolically more active than those from the control animals. There was a close association between the amounts of radio-active H_2O and CO_2 generated by viable embryos from both treatment groups ($r=0.470$, $p=0.012$). On the third day of <u>in vitro</u> culture there was however no significant difference in the glucose metabolism between viable embryos from urea and control animals ($p=0.61$); 13.21 ± 2.50 versus 11.53 ± 1.91 pmol/embryo of $^{3}H_2O$, and 0.344 ± 0.059 versus 0.262 ± 0.043 pmol/embryo of $^{14}CO_2$.

Protein synthesis by viable embryos was not different between the treatment groups. There was a positive correlation between the rate of protein synthesis by the embryo and its glucose metabolism.

The study indicates that there is scope for the application of measurements of embryonic metabolic activity in the assessment of embryo viability.

It is concluded that ammonia from "urea-supplemented" diets affects embryo development. The initial response of the embryo appears to involve an enhanced rate of development which in the present study was associated with an enhanced metabolic activity and an associated increase in protein synthesis. In view of the eventual reduction in embryo survival it is assumed that this initial response is eventually detrimental although it may also explain the enhanced birth weight of lambs produced from the small number of embryos that survive. There was no evidence in the present study that the high-urea diet gave rise to embryos with an altered proportion of their energy metabolism occurring via the pentose-phosphate shunt as opposed to the glycolytic pathway.

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1.0.0 INTRODUCTION

In the face of rapidly expanding biotechnology and more discerning markets, the goal set for the animal producers has been altered from maximizing animal production to maximizing the efficiency of production (McBride, Burton and Burton 1988). This applies to situations where production in-puts may be limiting and also to those which are considered to be adequate. In order to achieve greater efficiency, animal producers must increasingly understand factors which affect animal productivity and try to incorporate improved technologies into their production strategies. Factors which affect animal productivity include reproduction, growth rates (prenatal and postnatal) and feed conversion efficiency.

Although a number of other factors for example, daylength, environmental heat stress and animal social interactions can affect reproductive function, nutrition plays a major role. Obviously undernutrition will compromise the ability of animals to reproduce, but it is becoming a well recognised fact that overfeeding can cause reproductive wastage.

In temperate climates availability of feed may not be the limiting factor to reproduction, except in hill environments (Robinson 1990). Nevertheless nature has evolved a pattern in grazing animals (sheep, goats and deer) such that offspring are born when grazing is plentiful in spring (Martin et. al. 1992). Animals have a clearly defined breeding season which starts after exposure to decreasing photoperiod in autumn. The start of

oestrous cyclicity is associated with an increase in the frequency of pulsatile LH secretion and the removal of the responsiveness of the hypothalamus to the negative feedback of ovarian steroids and peptide hormones (Chemineau et. al. 1988).

In the tropics natural pastures account for a large part of the diet throughout the year. Grass which is the major component of the diet of grazing ruminants has the advantage of being to a large degree, a complete diet for ruminants in most circumstances (Lee 1988). However, in semi-arid and arid conditions, during the dry season, the quality and quantity of feed is often limiting. The quality of grasses, as illustrated by crude protein and crude fibre, often reaches values of less than 3% and greater than 30% for CP and CF respectively (McDonald, et. al. 1988). So this strong seasonal pattern of food availability has resulted in an evolution of reproductive mechanism in which plentiful nutrients are a cue to initiate reproductive function (Martin et. al. 1992).

During extended droughts animals can undergo a long anoestrous period solely due to nutritional deprivation and stress. However due to mixed crop and livestock farming practices in tropical and subtropical latitudes, there is always some crop residues left after harvesting. These include cereal and leguminous straws, hulms and bran. But the nutritive value of crop residues, especially straws and stover, is generally low. According to Mbayta (1975), small grain straw furnishes less than one percent digestible protein. Furthermore Sudana and Lee (1986) included

in the list of deficient nutrients, minerals for both the growth of microbial cells and the amount of dietary protein that escapes rumen degradation.

The first constraint to better utilisation of fibrous crop residues and standing hay is the imbalance in the nutrients made available from rumen fermentation (Preston and Lee 1986). In realisation of this fact, an adopted method in recent years has been to upgrade (Tuah, Lufadeju and ϕrskov 1986, ϕrskov 1993) and supplement (van Niekerk 1982, Ghosh, Alam and Akbar 1993) fibrous forages. This can be done by chemically treating straw with sodium hydroxide or ammonia and/or by supplementing with urea, bran or leguminous forages. Supplying urea to maintain high levels of ammonia in the rumen (ie. >200 mg N/l) should optimise intake, increase the rate and extend of digestion and increase microbial protein yield relative to volatile fatty acid production (Preston and Leng 1987). Multi-nutrient blocks (urea-molasses block) are being used as a strategy for correcting the imbalance of nutrients made available to the host animal from the rumen in hill environments in Britain and the practice is being extended to tropical regions to enhance roughage intake.

There is always a word of caution when using concentrates containing urea because of its toxicity if it becomes wet (Preston and Leng 1987, Macala 1992) or intake exceeds 30g/kg. Recently it has been found that urea, even at non-toxic levels to the animal might still have detrimental effects on embryo survival in sheep (Bishonga 1993).

In pursuit of optimising milk production during early lactation, farmers often increase nutrient density of diets in order to offset low feed intake (Elrod and Butler 1993, Elrod et. al. 1993). However high levels of protein for dairy animals have been observed in some studies (Jordon and Swanson 1979, Jordon et. al. 1983, Kaim et. al. 1983) to have negative effects on fertility. These effects have been attributed to excess ammonia which may either interfere with the energy balance of the animals (Kaim et. al. 1983, Carroll et. al. 1988, Canfield et. al. 1990, Elrod and Bulter 1993) or change the ionic composition of the uterus (Jordon et. al. 1983, Canfield et. al. 1990) and hence alter the pH of the uterine secretions (Elrod et. al. 1993, Elrod and Bulter 1993). Alternatively it may alter reproductive hormones (Saitoh and Takahashi 1977, Jordon et. al. 1983, Carroll et. al. 1988) and intermediary metabolism. The mechanism by which ammonia could be compromising reproduction is still speculative. The present study will attempt to assess the effects of readily degradable nitrogen in the form of added urea on embryo survival in ewes and investigate the mechanisms involved.

2.0.0 LITERATURE REVIEW

2.1.0 REPRODUCTION AND FACTORS AFFECTING EMBRYO SURVIVAL

The significant contribution of sheep to regional production in the arid and semi-arid zone of Africa is lessened by reproductive wastage (Mukasa-Mugerwa, Mutiga and Girma 1992). The same limitation to productivity also occurs in temperate conditions. Reproduction in farm animals is a cumulative result of a number of interacting factors which may differ from one species to another (cattle verses pigs) and from one environment to another (temperate verses tropical). Though maximal capacity of animals to reproduce is set by genetics, factors like environment, nutrition and social interaction may modulate and compromise this potential. Of particular interest in hot climatic conditions is the effects of environmental stress. It has been reported by Gwazdauskas et al (1973), Ingraham et al (1974), Thatcher (1974), Bandinga et al (1985), that often heat stress causes transient infertility in cattle. Social interaction between animals has been found to influence reproductive activity. This is evident when a male animal is introduced to females to advance their breeding season, (Martin et. al. 1988). However the most important factor that affects reproduction is nutrition. The influences of nutrition include, among other things, effects on attainment of puberty, duration of postpartum anoestrus, gametogenesis, ovulation rate, embryonic mortality (Robinson 1990), litter size and lactational performance (Williams 1992).

2.1.1 Genetics

The maximal capacity for reproduction is set by genotype. According to Murray et al (1985) early embryonic loss attributable to abnormal karyotypes ranges between 0.6 and 10.5 percent. Of this polyploidy is the most common contributor, especially in cattle.

2.1.2 Environmental stress

Reduced fertility in animals in hot environments is associated with elevated body temperature (Gwazdauskas et. al. 1973). Though the effects of heat stress is manifested in both the male and the female animal, seasonal depression of fertility due to heat stress has being mainly attributed to the female. According to Putney et al (1986) the main reason for low fertility in the bovine female exposed to heat stress is the fact that the early bovine embryo (day 1-7 of development) is extremely sensitive to harmful effects of maternal hyperthermia. This results in an increased frequency of non-viable, abnormal and retarded embryos. Biggers et al (1987) reported that maternal heat stress between day 8-16 after insemination reduces conceptus weight. An in vitro study whereby Day 17 conceptuses were subjected to heat showed that protein synthesis and secretion into the medium was markedly decreased (Putney et. al. 1987). From these observations it could be argued that in conjunction with retarded growth of the conceptuses, there may be an inability on the part of the conceptus to signal its presence to the mother. Altered amounts and frequency of circulating hormones in the pregnant animal may

also explain embryonic mortality due to heat stress. According to Lewis et al (1984) pre-partum heat stress increases the concentration of 13,14-dihydro-15 $PGF_{2\alpha}$ (PGFM) during the postpartum period in dairy cattle. Increased concentration of systemic PGFM may reflect greater uterine secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Putney, Gross and Thatcher 1988).

Heat stress in the male is likely to damage spermatogenesis. Reduced fertility in females bred to heat stressed males may result in increased embryonic loss. This was confirmed in a study by Quintana Casares et al (1992) who found a significant increase in embryonic loss as early as 4 days of gestation in ewes inseminated with semen from heated rams.

2.1.3 Nutritional Effects

There is still uncertainty about the earliest stage of development at which nutrition influences an animal's subsequent ability to achieve its genetic potential for reproduction (Robinson 1990). However it has been demonstrated that extreme nutritional regimes are detrimental to embryonic development. Regardless, there are certain instances whereby a mild degree of undernutrition of females in good condition actually enhances placental growth and improves birth weights (Faichney and White 1987).

a) Effects of undernutrution on the embryo

It is well recognised that undernutrition compromises embryo survival in all animals. For example it has been noted that

severe undernutrition causes embryo loss in the ewe (Robinson 1986) and goat (Mani, McKelvey and Watson 1992) during the first weeks of pregnancy. Undernutrition also reduces fetal growth in ewes (Nordby et. al. 1987) and goats (Mani, Watson and McKelvey 1993). Underfeeding probably causes embryonic loss due to the inadequacy of the reproductive tract to supply nutrients to the embryo or an altered uterine environment arising from a nutritionally-induced change in the endocrine status.

Embryos use glucose as a source of energy (Liggins 1982) and it is likely that a reduced supply will result in retarded growth and eventual death. However, during early pregnancy the nutrient demand of the embryo is insignificant as compared to total nutrient demand of the mother or those of foetuses in mid- and late pregnancy (Parr et. al. 1982). It is however very specific. This means that there has to be a severe nutritional deprivation to cause embryonic loss. Hostile uterine conditions may be created by underfeeding possibly through altered uterine pH, change in the protein profiles and later on, according to Mani et al (1993) reduced fetal fluids and placental tissues.

Williams and Cumming (1982) found that undernutrition was associated with higher concentrations of circulating progesterone while Parr, Cumming and Clarke (1982) observed an increased plasma progesterone in ovariectomised ewes given exogenous progesterone and undernourished immediately after mating. Although it is possible that the endocrine status of nutritionally stressed pregnant animals may be altered, it is not

clear if alterations in secretion rates or metabolic clearance rates caused increased plasma progesterone. Associated with high levels of progesterone, Parr et al (1982) observed smaller and less developed embryos and Williams and Cumming (1982) recorded low embryo survival. This is contrary to the observations found by Ashworth, Sales and Wilmut (1989) in which a periovulatory rise in plasma progesterone was associated with improved embryo survival. This scenario probably points to different mechanism(s) by which raised progesterone concentrations influence embryo survival in undernourished ewes and those on sub-maintenance feeding. For example a feeding level below maintenance may mask the benefits to embryo survival of a rise in progesterone.

b) High plane of nutrition and embryo survival

Although undernutrition can decrease embryo survival, too much food can also be detrimental. There are several reports which point out that overfeeding during early stages of pregnancy leads to embryonic loss in both pigs (Kirkwood and Thatcher 1988, Dyck and Strain 1983, Williams 1992) and sheep (McKelvey and Robinson 1986, Robinson 1990, Parr et. al. 1993). Recently it has been found that overfeeding can also reduce the proportion of viable embryos of superovulated ewes (McEvoy et. al. 1993). In contrast to these reports, studies by Toplis and Ginesi (1983) and Prime, Varley and Symonds (1988) showed no significant effect on litter size in multiparous sows given different patterns of feed intake pre-conception. This lack of effect of food intake (Prime et. al. 1988) on litter size may be due to differences in endocrine status in multiparous sows as opposed to gilts.

Kirkwood and Thatcher (1988) reported that although embryo survival in gilts is depressed by high energy intakes, the total numbers of surviving embryos are not, thus indicating that high feeding regimes enhance ovulation rate.

Low embryo survival has been attributed to the reduced circulating progesterone levels that accompany high levels of feeding in the sow (Kirkwood and Thatcher 1988, Prime and Symonds 1992), ewe (McKelvey and Robinson 1986, Parr et al. 1987, Parr et al. 1993) and superovulated ewes given artificial progesterone (McEvoy et al. 1993). Altered steroid metabolism in the liver due to dietary induction of the hepatic mixed function oxidase enzyme system has been implicated in the decrease in progesterone levels. However, Parr et al (1987), Parr et al (1993) have suggested that progesterone clearance rate is increased mainly by an increase of blood flow through the liver rather than elevated enzyme activity. In the ewe, changes in the metabolic clearance rate of progesterone due to differences in feed intake have been suggested as indirectly affecting embryo survival (Parr et. al. 1987, Parr 1992, Parr et. al. 1993). Earlier reports (Toplis and Ginesi 1983, Prime et. al. 1988) questioned the existence of such a relationship in the pig, but recently Prime and Symonds (1993) showed that plane of nutrition affects portal blood flow and metabolic clearance rate of progesterone in the pig. These conflicting results may therefore be due to differences in the parity of the animals used and differences in the management regimes employed.

According to Prime and Symonds (1993) reduced progesterone concentration may alter the progesterone:oestrogen ratio. If the plasma progesterone is substantially reduced, ova viability may be compromised (McEvoy et. al. 1993) and the uterine environment may become out of synchrony with the development of the embryo (Kirkwood and Thatcher 1988). A high proportion of embryonic mortality occurs during the period of rapid conceptus elongation and adequate progesterone secretion is essential during this period of maternal recognition of pregnancy (Wallace, Aitken and Cheyne 1994). Furthermore progesterone induces secretion of a retinol binding protein in the pig uterus (Adams, Bazer and Roberts 1981) and other specific proteins which are beneficial to the survival of the embryos.

c) Are there specific nutrients that affect embryo survival?

Most of the studies on nutrition-reproduction usually consider only the effects of plane of nutrition. According to Robinson (1993) correction of deficiencies of minor minerals; Cu, Mn, Zn, Fe, Co and Se have been shown to improve conception and litter size. This is understandable because a severe deficiency of any micro-nutrient will have an adverse effect on general body function including reproduction (Kirkwood and Thatcher 1988). With regard to the effects of the trace elements and the effects on embryo survival per se as opposed to reproduction in general, it is only for Se that there is evidence for an effect.

Brief and Chew (1985) reported an improved litter size when gilts were given supplementary retinol and/or B-carotene by injection.

How vitamin A improves litter size is not known, but Adams et al (1981) noted the presence of retinol binding protein in the pig uterus. Another vitamin that might affect embryo development is folic acid. It has been found that intracellular mRNA concentrations for the synthesis of embryos protein is detectable by days 12-13 in both the sheep and cow (Farin et. al. 1990, Nephew et. al. 1993) and it is well recognised that folic acid is essential for DNA and RNA synthesis.

If protein has a biochemical role in embryo survival, the likely mediators must be the amino acids or other protein metabolites

2.2.0 Protein and Nitrogen Metabolism

2.2.1 Requirements and utilisation of protein and non-protein nitrogen

Animals need protein for the synthesis of lean tissue, foetal growth, milk, wool and fibre production. This need is met by amino acids absorbed from the small intestine (ARC 1980). In ruminants these amino acids are partly supplied by microbial protein synthesised in the rumen and partly by dietary protein that has escaped fermentation in the rumen. The value of urea and other non-protein nitrogen (NPN) is established (Wohlt and Clark 1978) and the degree of its degradation in the rumen to ammonia and synthesis into microbial protein depends on the availability of energy (ARC 1980). This unique feature of rumen microorganisms has conferred on ruminants the ability to utilise low quality roughages, provided a source of rumen degradable nitrogen is

present. An additional component of this system involves the ability of the ruminant animal to recycle urea to the rumen via saliva and blood (Φrskov 1982, Rémond et. al. 1993)

Depending on the degradability of the protein, the fraction that is not degraded in the rumen will be digested in the small intestine and used for productive purposes.

2.2.2 Urea and the Liver

Microbial protein is synthesised from the ammonia produced in the rumen, but in many instances not all of the ammonia is used. Normally, if the intake of rumen degradable protein exceeds the requirements of the rumen microorganisms, ammonia produced in such large amounts can be detoxified by the liver to urea (Jordan et. al. 1983, Canfield, Sniffen and Bulter 1990). However some escapes through the rumen wall (Rémond et. al. 1993). The immediate rise of ammonia concentration in intestinal and caecal veins implies that ammonia crosses to the veins via peritoneal fluid. The peritoneal fluid drains into the thoracic duct and the thoracic duct empties into a large vein (i.e. jugular, anterior vena cava), therefore ammonia can arrive into the peripheral circulation blood without passing through the liver (Chalmers, Jaffray and White 1971)

Ammonia is a weak electrolyte which equilibrates between its ionic (NH_4^+) and non-ionic (NH_3) states (Visek 1984). Symonds, Mather and Collis (1981) found that the maximum capacity of the liver to remove NH_3 during its first pass was on average 1.84

mmol/min/kg wet weight. The hepatic urea cycle reactions a enzymes involved in converting ammonia to urea are indicat below.

1) Carbamyl phosphate synthetase I:

$$NH_4^+ + HCO_3^- + 2ATP \xrightarrow[\text{N-acetylglutamate}]{Mg^{+2}} \text{carbamyl phosphate} + 2ADP + P_i$$

2) Ornithine carbamyltransferase:

$$\text{Carbamyl phosphate} + \text{L-ornithine} \xrightarrow{Mg^{+2}} \text{L-citrulline} + I$$

3) Argininosuccinate synthetase:

$$\text{L-aspartate} + \text{L-citrulline} + ATP \xrightarrow{Mg^{+2}} \text{L-argininosuccinate}$$

AMP + PP

4) Argininosuccinate lyase:

$$\text{L-argininosuccinate} \longrightarrow \text{fumarate} + \text{L-arginine}$$

5) Arginase:

$$\text{L-arginine} \xrightarrow[H_2O + Mn^{+2}]{} \text{urea} + \text{L-ornithine}$$

(Source: Visek.W.J (1984) J Dairy Sci 67:481-498)

Jordon et al (1983) and Visek (1984) reported that when anima

are fed excess dietary protein, the activity of the urea cyc enzymes increase.

2.2.3 Blood ammonia concentrations

After infusing a solution of NH_4Ac (approx 2.0mmol/ml) into t mesenteric vein of the dairy cow Symonds et al (1981) fou elevated amounts of ammonia in the hepatic vein. Amounts of ur were also elevated (mean value of 7.0 mmol/l) at the intoxicati point for NH_4Ac. This value is similar to the range of valu (5.6 to 8.2 mmol/l) found by Bishonga (1993) in the peripher circulation after feeding a high level of urea (30g/kg) to ewe Studies with dairy cows (Jordan et. al. 1983) and sheep (Bishor 1993) showed that plasma ammonia concentrations of high-prote or high-urea fed animals can reach about twice the levels of lc fed animals.

The increase in blood ammonia concentrations may reflect t inability of the hepatic enzyme system to convert all the ammon into urea (Jordon et. al. 1983, Bishonga 1993). It may al indicate a temporary inability of the hepatic enzyme systems adjust to high protein intakes, hence a need to allow anima time to gradually adapt to urea containing diets (Symonds et. a 1981). Besides the possible reasons given above, the elevat plasma ammonia may be reflecting that ammonia has diffused acro the peritoneal cavity to the peripheral circulation witho passing through the liver. This would mean that the rise peripheral ammonia could still occur even in situations where t capacity of the liver to convert ammonia to urea is n

overloaded (Chalmers et. al. 1971).

2.3.0 Influences of protein levels on fertility

The effects of excessive protein and non-protein nitrogen feedi on reproduction have been assessed in dairy animals (Jordon a Swanson 1979, Carroll et. al. 1988, Canfield et. al. 1990, Elr and Butler 1993) and sheep (Bishonga 1993). However, the resul are equivocal. Though there is a general trend towards reduction in fertility, Carroll et al (1988) did not find a effects of increasing dietary crude protein intake on any grc measure of reproductive performance.

2.3.1 Effects of highly degradable nitrogen

The readily degradable component of dietary protein and N accounts for the elevation of plasma ammonia, hence high rum degradable protein (RDP) levels have been linked to a decrea in the fertility of dairy cows (Canfield et. al. 1990, Elrod a Bulter 1993). This was illustrated by Ferguson and Chalupa (198 who used logistic regression analysis and found an inver relationship between RDP and the probability of pregnancy (s Figure 1a). However the theory regarding the role of RDP conception and pregnancy is equivocal. The initial thinking w that RDP is responsible for reduction in fertility since it the dietary component which is readily converted to ammonia. E Ferguson and Chalupa (1989) produced evidence for an effect an interaction between RDP and UDP on the probability pregnancy (see Figure 1b). This would seem understandable sir

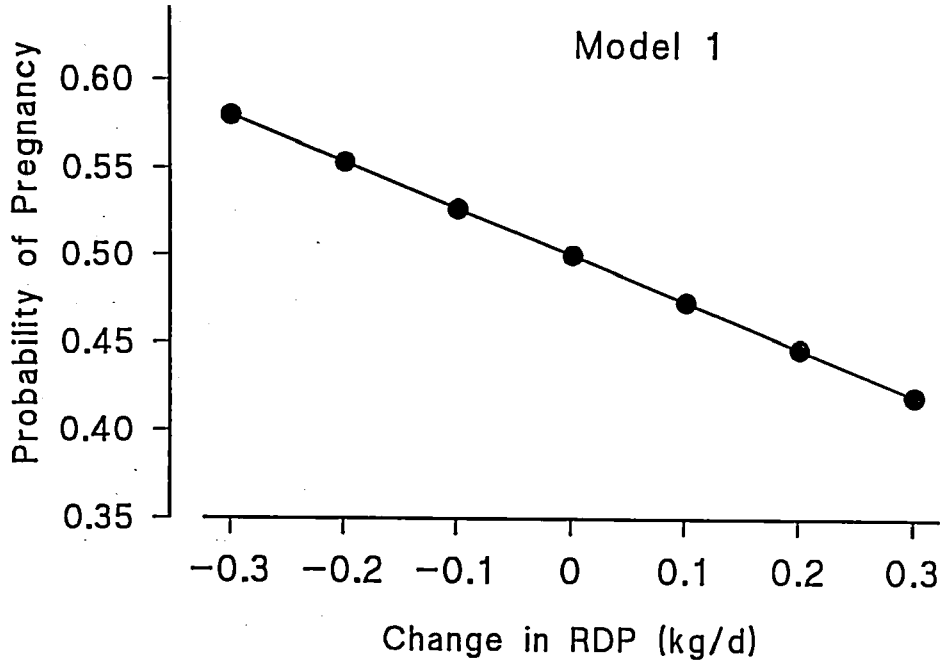


Figure 1a. The impact of the difference between the requirement and intake of RDP on the probability of pregnancy.

(Source: Ferguson & Chalupa, 1989)

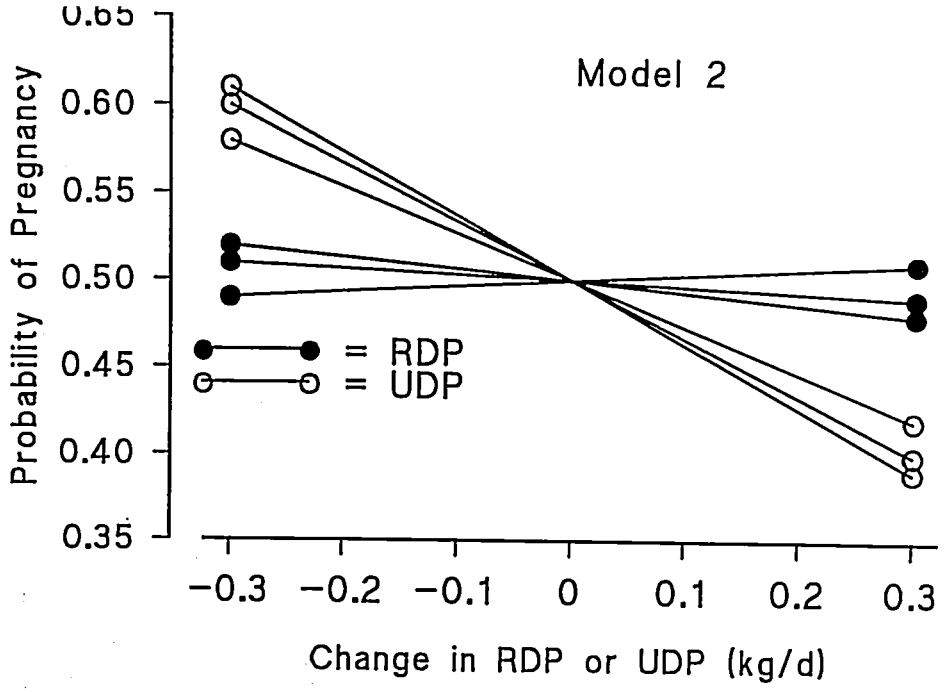


Figure 1b. The impact of the difference between the requirement and intake of RDP or UDP on the probability of pregnancy.

(Source: Ferguson & Chalupa, 1989)

in a recent report, Elrod, Amburgh and Butler (1993) note detrimental effects of excess protein regardless of source o degradability.

2.4.0 Excess protein-energy interaction

Oldham (1982) identified two aspects of the inter-relationshi between protein and energy-yielding nutrients in dairy cows Firstly a change in protein level can change overall plane o nutrition since at high levels of feeding rumen microbial deman for nitrogen per unit fermentable organic matter is high Secondly, a change in protein supply to tissues can alter th pattern and efficiency of absorbed nutrient use. Together wit this shift in energy utilisation, the extra energy required t deaminate ammonia and amino acids at high protein feeding level may cause negative energy balance. This potential energy defici has been suggested as a cause of the effects of high protei diets on reproductive performance (Kaim et. al. 1983, Carroll et al. 1988, Canfield et. al. 1990).

2.5.0 Effects of urea nitrogen and ammonia in biological system

The negative influence of excess protein on fertility could b a result of toxic effects of ammonia and urea nitrogen on sperm ovum or the developing embryo (Jordon and Swanson 1979, Chalup 1984). Ammonia serves essential functions but because it can als be detrimental by inhibiting essential metabolic reactions, i may elicit biphasic responses (Visek 1984). For instanc Dasgupta, Kar and Dhar (1971) suggested that above the critica

value of 72mg/100ml, urea level seems to be fatal to human spermatozoa.

2.5.1 Ammonia and the endocrine system.

Progesterone is a hormone of pregnancy. Any event that might disrupt production of progesterone during pregnancy may lead to embryo loss. According to Carroll et al (1988) high urea nitrogen or ammonia concentrations may reduce LH binding receptors leading to a decrease in serum progesterone concentration. A study with rats, (Saitoh and Takahashi 1977), showed that progesterone synthesis was maximum when a 30% protein diet was given and was minimum at 39% protein in the diet. These effects of dietary protein on progesterone synthesis implies that increased end-products of protein metabolism (amino acids, urea) may be involved in reduced progesterone synthesis. When urea was used as rumen degradable nitrogen, Bishonga (1993) did not find any effect of dietary treatment on plasma progesterone concentrations in ewes primed with a standard dose of progesterone. Neither was the timing nor the magnitude of the LH surge affected by urea feeding. Thus the adverse effects of high plasma urea and ammonia on early embryo survival may not arise from an alteration in the concentration of peri-ovulatory and early pregnancy hormones.

Ammonia elevates insulin levels (Visek 1984) and this will certainly lead to altered glucose metabolism. Visek (1984) hypothesised that arginine might be involved in insulin metabolism, however in the liver arginine is used to detoxify ammonia. So, during high levels of ammonia, arginine may not be

available for insulin metabolism and other cell functions.

2.5.2 Effects of ammonia on the reproductive tract

a) The uterus

Ammonia in the peripheral blood tends to rise in parallel with its concentration in portal blood, probably because it can diffuse from the intestine across the peritoneal cavity without passing through the liver (Chalmers, et. al. 1971). Therefore it is conceivable that during high levels of rumen ammonia, some would be found in the reproductive tract. It has been found that the ionic forms of the elements Ca, Mg, P, K and Zn in the uterus are altered as ammonia levels increase (Jordan et. al. 1983). This could lead to a disruption of the integrity and normal metabolic functions of the cells of the uterine endometrium. Jordon et al (1983) found that zinc concentration was increased by a high protein diet and according to Canfield et al (1990) high levels of zinc decrease progesterone binding in the human uterus.

Changes in the uterine ionic composition caused by ammonia are likely to alter uterine pH due to a change in the buffering system. Elrod et al (1993) and Elrod and Bulter (1993) found that uterine pH was different for high protein fed cows than low protein ones (6.79 and 7.09 respectively), at day 7 after oestrus. This decrease in pH is consistent with the decrease in uterine Mg, K and P (Jordon et. al. 1983). The buffering molecules are eventual acceptors of the hydrogen ions generated by the equation below:

6) $CO_2 + H_2O \dashleftarrow\dashrightarrow HCO_3^- + H^+$ (Rowlett et. al. 1991).

The decrease in concentration of buffering ions will cause high hydrogen ions and low pH. Another mechanism suggested by Elrod et al (1993) for low uterine pH is the inhibition of endometrial carbonic anhydrase. This enzyme system which according to Rowlett et al (1991) catalyses the preceding reversible reaction, works within a narrow range of ionic concentrations.

The optimum development of the embryo depends on the secretion by the uterus of proteins and growth factors which communicate with and nourish the embryo (Ashworth 1992, Wales 1992, Simmen, Ko and Simmen 1993). Messenger RNA for maternally derived insulin and insulin-like growth factor 1 (IGF1) have been detected in mouse (Wales 1992) pig, cow and sheep embryos (Simmen et. al. 1993). Visek (1984) suggested that during ammonia intoxication, insulin concentration is altered. It is most likely therefore that IGF's produced in the uterus will be affected as well. Furthermore, the stimulation of protein synthesis by IGF-1 operates through the insulin receptor rather than the IGF-1 receptor (Wales 1992) and ammonia would probably interfere with this reaction.

Unfertilised sea Urchin eggs exposed to ammonia showed unusual activities including switching on of DNA synthesis, changes in RNA and increased protein synthesis (Visek 1978). Hence any change in the production of insulin, IGF's and other proteins due

to ammonia could be attributed to the abnormal switching on of DNA synthesis.

b) Effects of ammonia on the embryo

It is now a fundamental fact that mammalian cells can tolerate only modest concentrations of ammonia due to disruptions in intracellular pH and intermediary metabolism (Visek 1978). The adverse effects of ammonia on the embryo may be mediated through changes in the reproductive tract or directly on the embryo. A study with sheep (Bishonga 1993) showed that mean ovulation rate was decreased when animals were fed diets containing urea; 3.56±0.71, 3.2±0.55 and 2.4±0.37 for controls, low and high urea respectively. This would probably imply that ammonia affects the development of the ova in the ovaries. Ovulations, as represented by recovered ova on the low and high urea diets, were 18% and 49% lower, respectively, than for the controls, implying a detrimental effect of the urea treatment on the successful capture of the ova by the fimbriated infundibulum (Bishonga, 1993) or damage of the ova along the oviduct (Wallace, Aitken and Cheyne 1994).

In the same study, recovery rate of Day 11 embryos for the high urea treatment was low, with only 10% of the corpora lutea resulting in recovered embryos, thereby implying a further detrimental effect of treatment on the embryo between Days 4 and 11. These animals were given the diets one week before the start of the experiment to accustom them. It would be interesting to see how much of the effect is directed to the embryo and how much

to oocyte maturation.

Like the uterus, the conceptus produces a variety of proteins and growth factors; embryo-derived platelet-activating factor (Gandolfi et. al. 1992), trophoblast protein-1 (Ashworth and Bazer 1989, Farin et. al. 1990, Ashworth 1992, Schalue-Francis et. al. 1992) and insulin like growth factor II (Wales 1992). High ammonia levels are likely to affect production of these proteins, probably through their effects on mRNA (see section 2.5.2 a). An in vitro study (Bishonga 1993) showed that rate of protein synthesis by the embryo was seriously compromised, as indicated by ^{3}H-phenylalanine incorporation which was 87±34.0 and 39±15.1 c.p.m. for embryos from the control and high urea diets respectively.

2.6.0 Embryo metabolism

Metabolism of cells, in terms of oxygen uptake, glycolysis and the by-products of intermediary metabolism can be modified by ammonia (Visek 1978). How this modification occurs is not well understood, but there are a few possibilities:

a) by affecting ionic composition of cells, ammonia may alter the role of mineral coenzymes leading to inhibition or stimulation of certain enzyme activities;
b) altering production of hormones concerned with metabolism;
c) shifting the metabolism of energy-yielding pathways.

2.6.1 Glucose

The growth and development of the early embryo requires considerable metabolic activity for the production of energy and for the synthesis of a variety of complex molecules (Tiffin *et. al.* 1991). So any disruption of specific physical conditions and chemical factors necessary for early embryonic development will also affect the production and expenditure of cellular energy.

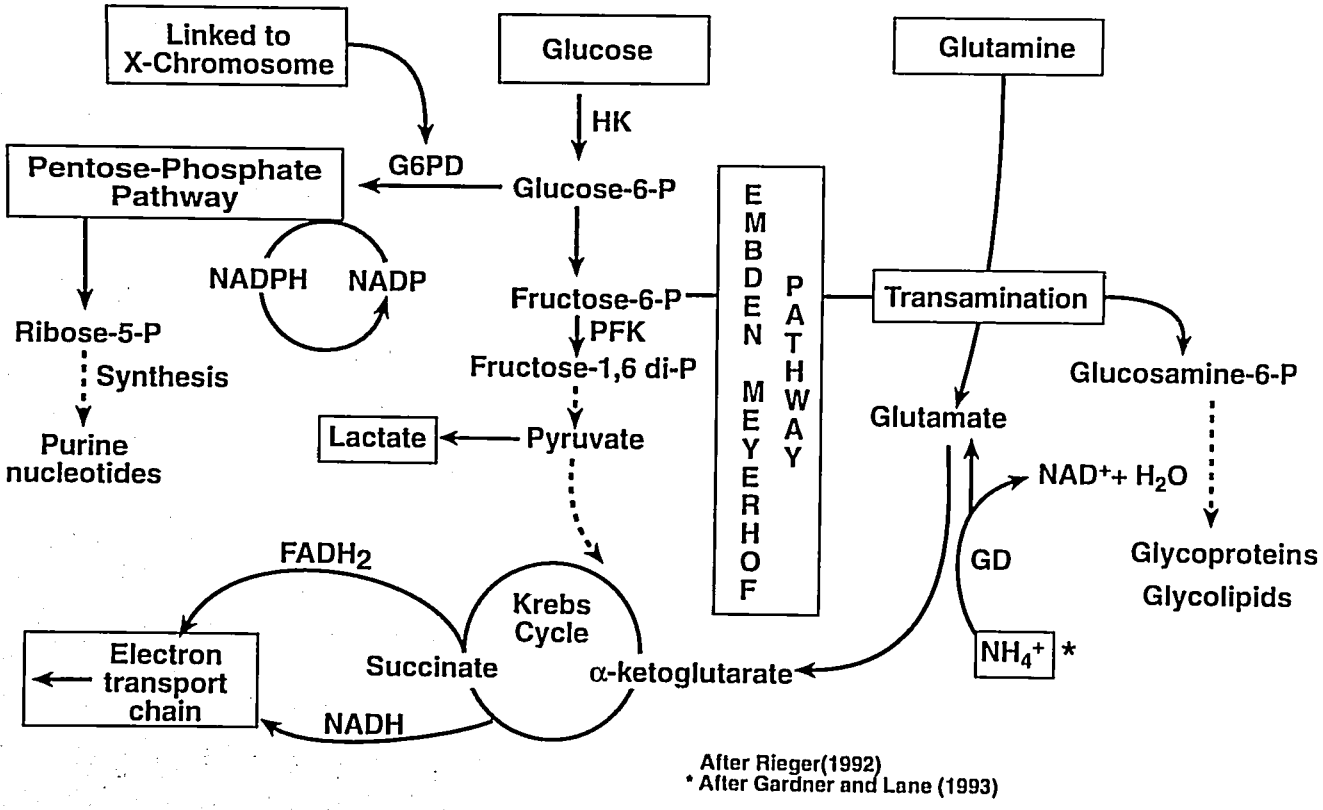
Central to the issue of energy is glucose. Embryos use glucose as a source of energy (Liggins 1982) and *in vitro* studies with cattle (Renard, Phillippon and Ménézo 1980) and mice (Gardner and Leese 1987) have shown that glucose uptake is related to viability after autotransfer. From the two-cell to blastocyst stage, radiolabelled glucose metabolism increases markedly in the sheep (Thompson *et. al.* 1991).

It is suggested by Rieger (1992) that this increase in glucose metabolism occurs at or about the time of activation of the embryonic genome. This probably coincides with the synthesis of important glycolytic enzymes or co-enzymes. The cessation of sheep embryo development *in vitro* at the 8-16-cell stage occurs at the same time as the pre-implantation embryo switches from maternal to embryonic genome control (Gardner *et. al.* 1994). If high levels of ammonia in urea-fed ewes create conditions that would interfere with the activation of the embryonic genome *in vivo*, it is likely that a developmental block will occur.

A recent study by Bishonga *et al* (1994) showed that for the low

proportion of embryos that survived from high-urea-fed ewes, single lamb birth weight was high (7.3 to 10.1 kg compared with 5.3 to 7.5 kg for controls). This may reflect the ammonia-induced alteration of the metabolic activity of the early embryo. Walker, Heard and Seamark (1992) made observations which indicate an association between *in vitro* culture and an increase in mean gestation length, mean lamb birth weight and lamb mortality. In a study by Bishonga *et al* (1994), ammonia (*in vivo*) is possibly mimicking effects similar to those evoked by *in vitro* culture. Ammonia is supposed to have a biphasic role in cells (Visek 1984) and could initially stimulate embryo development, perhaps leading to the subsequent increase in fetal growth observed by Bishonga *et al* (1994). Alternatively, the difference in development may be related to the different pathways by which embryos can utilise glucose. For example, Tiffin *et al* (1991) found that although total glucose metabolism was greater in male embryos, the proportion of glucose metabolised through the pentose phosphate pathway (PPP) was greater in females. High levels of ammonia may increase the activity of the Embden-Meyerhof pathway (EMP) or that of PPP. The increase in the activity of the PPP may be affected through elevated activity of the fluxing generating enzyme, glucose-6 phosphate dehydrogenase, (Gardner *et. al.* 1994). The EMP activity may be increased by stimulating the activity of phosphofructokinase (Gardner and Lane 1993) (see Figure 2 for the pathways). The first scenario is likely to rob the embryo of the essential substrates like pyruvate and lactate which are produced through the EMP. In the second, observations similar to those noted by Visek (1978) may occur in which ammonia

Figure 2: Pathways for Energy Production and Synthesis of Complex Molecules



After Rieger(1992)
* After Gardner and Lane (1993)

stimulates glycolysis and the Krebs cycle metabolism and shortens the metabolic life span of intestinal cell. Alternatively, ammonia may perhaps be delaying or advancing the inactivation of the X-chromosome control over glucose metabolism via the pentose phosphate pathway (PPP).

Javed and Wright (1991) noted that PPP activity in good quality embryos was greatest at the 6-cell stage and it decreased with increasing embryo development. Poor quality embryos on the other hand had a lower total glucose metabolism and higher PPP activity. This is similar to the observations made by Tiffin et al (1991) whereby rate of development was different for embryos of different sexes. The implication here may be that glucose at high concentrations influences embryo development, possibly by altering the activity of Krebs cycle (Thompson et. al. 1992).

2.6.2 Pyruvate

As well as undergoing morphological changes the mammalian embryo goes through a series of alterations in energy metabolism. In addition to glucose the embryo also utilises pyruvate, glutamine and lactate (see Figure 2) in different proportions throughout the different developmental stages. Except for a minor increase at the one-cell stage, the uptake of pyruvate by sheep embryos is relatively constant but increases through the blastocyst and expanded blastocyst stages (Rieger, Loskutoff and Betteridge 1992). Uptake of pyruvate by cattle embryos in vitro at Day 7 is inhibited by lactate and glucose (Dorland, Kruip and van der Donk 1991).

2.6.3 Amino acids

Glutamine metabolism is high at the early cleavage stages, decreases at the blastocyst stage and then increases at the expanded blastocyst stage in cattle (Javed and Wright 1991) and sheep (Thompson *et. al.* 1991). In the presence of glucose, cultured mouse embryos utilize less glutamine (Chatot, Tasca and Ziomet 1990). Glucose metabolism is not critical for ruminant embryonic development *in vitro*, (Thompson *et. al.* 1992) and the ruminant blastocyst can utilise amino acids as key energy sources (Gardner *et. al.* 1994). The beneficial non essential amino acids are similar to those present in the mammalian oviduct and endogenous pool of the early embryo (Gardner and Lane 1993). However it has been shown that the rate of ammonia production was increased when mouse embryos were cultured *in vitro* in the presence of amino acids (Gardner and Lane 1993). Ammonia can react with α-ketoglutarate to produce glutamate (see Figure 2 for reaction). The generation of energy in the form of ATP in the Krebs cycle may be depleted if high levels of ammonia convert α-ketoglutarate to glutamate. High levels of ammonia *in vivo* due to high degradable nitrogen in the form of urea are likely to cause similar effects.

2.7.0 *In Vitro* embryo culture

The use of culture systems to study embryo development *in vitro* facilitates the application of techniques such as *in vitro* fertilization, microinjection of individual spermatozoa and gene

transfer. The use of culture medium also provides the opportunit to determine the viability of *in vivo* fertilised embryos and t gauge the effects of various treatments imposed *in vivo* According to Gardner *et al* (1994) the introduction of procedure such as cloning and transgenesis is currently being hampered b suboptimal culture conditions. Embryos are exposed *in vivo* to a continually changing environment in terms of nutritional, hormonal and physio-chemical factors as they pass through the female reproductive tract (Leese 1988). This dynamic state of the reproductive tract is necessitated by the alteration in nutrient needs of the embryo which is accompanied by morphological changes. Because such conditions are very difficult to reproduce *in vitro* (McCaffrey 1993), the success of a culture medium is determined by its ability to keep the embryo alive. To achieve this, biophysical and biochemical parameters derived from *in vivo* studies are used to try to achieve a culture medium that works (Heyman *et. al.* 1987).

2.7.1 Physical environment *in vitro*

In the oviduct, the "medium:embryo volume" ratio is very low as the embryo is normally surrounded by picolitre volumes of fluid. The large volume of medium which surrounds the embryo in vitro may cause nutrient leakage into the medium (Kane 1987, Kane *et. al.* 1992) or represent adverse physical conditions. A study by Walker *et al* (1988) showed that pronuclear sheep embryos can tolerate changes in osmolarity ranging from 260 to 300 mOs. A relative humidity of 93% (Walker *et. al.* 1988, Walker, Lampe and Seamark 1989,) or at least more that 90%, is maintained by

keeping a water tray inside the incubator. In some instances embryos are cultured under mineral or paraffin oil (Walker et. al. 1988, Eyestone and First 1991, Wales and Du 1993) to prevent evaporation.

The temperature in vitro is adjusted to reflect that of the donor female body. However different studies reported slightly different temperatures for incubation of sheep embryos (37°C Wales and Du 1993, 38°C Walker et. al. 1988, 39°C Thompson, et. al. 1991). The inability of these studies to demonstrate an effect of changing the temperature of the culture system does not mean that it is not important, but probably reflects the differences in the entire culture systems used. Alternatively it could be indicating the temperatures which are within a tolerable range for sheep embryo development in vitro.

The pH range that supports optimum cellular multiplication is generally narrow for all types of cells (McCaffrey 1993). To achieve near physiological values, a buffering system and gaseous atmosphere is usually used to maintain culture medium pH at around 7.4 (Walker et. al. 1988). However mammalian embryos appear to develop over a wide range of values (6.0 to 7.8) (Walker et. al. 1989, Bavister 1988). The buffering agents which are employed include phosphate in a buffered saline (PBS) medium (McLaughlin et. al. 1990, Chatot et. al. 1989) and bicarbonate (Kane 1975, Walker et. al. 1988). Unlike bicarbonate, phosphate or n-2-hydroxy-ethyl-piperazine-N-ethane-sulphonic acid (Hepes) buffered media do not require a CO_2 controlled gas phase to

maintain a relatively constant pH (Kane 1987). Replacing bicarbonate with Hepes as a buffer was associated with reduced embryo development (Walker et. al. 1989). However the use of Hepes as opposed to bicarbonate as a buffer results in a greater stability of pH (Walker et. al. 1992). Since it appears that there is a need for bicarbonate in embryo development (see paragraph below), the best compromise would be to mix the two buffers in the culture medium.

Gaseous phases used for pH maintenance are generally 5% CO_2 in air or 5% CO_2: 5% O_2: 90% N in conjunction with bicarbonate (Quinn and Wales 1973, Kane 1975). The dissociation of sodium bicarbonate is shown in reaction equation 7 (ICN catalogue 1994). The artificially supplied CO_2 to the atmosphere prevents the CO_2 in the medium escaping thus reducing the OH^- concentration and maintaining the correct pH. A study by Quinn and Wales (1973) comparing the effects between a phosphate and a bicarbonate buffered medium on development of mouse embryos, showed that cleavage of 2-cell, 8-cell and morula-stage embryos cultured in phosphate buffered medium was limited to that which occurred during the first 24 hours of culture. High levels of ATP were recorded in embryos cultured in phosphate buffered medium, implying that there was a possible reduction in ATP utilization by the embryos. Kane (1975) observed that 78% of 1-cell rabbit ova developed into blastocysts when a bicarbonate (5% CO_2) system was used as a buffer while no ova developed a blastocoele cavity when Hepes was used. Besides regulating pH, the CO_2/bicarbonate system may have other functions in vitro (Kane 1987), as

demonstrated by the studies of Quinn and Wales 1973, and Kane 1975. The general increase in embryo mass taking place at the time of blastocyst formation certainly necessitates a great increase in the supply of Krebs cycle intermediates (Kane 1975). The functions of CO_2 and bicarbonate probably include 1-carbon metabolism and the conversion of pyruvate to oxaloacetate for replenishment of Krebs cycle intermediates (Kane 1987).

7) $NaHCO_3 + H_2O \leftrightarrow Na^+ + HCO_3^- + H_2O \leftrightarrow Na^+ + H_2CO_3 + OH^- \leftrightarrow Na^+ + H_2O + CO_2$

Depending on its concentration, oxygen may either have beneficial or detrimental effects on embryo development in culture. The mechanism by which oxygen concentration in the atmosphere affects development of embryos *in vitro* is not clear (Thompson *et. al.* 1990). However it has been shown that low oxygen concentrations (5-10%) enhance development of sheep (Thompson *et. al.* 1990), goat (Batt, Gardner and Cameron 1991) and cow embryos (Thompson *et. al.* 1990, Nagao *et. al.* 1994). The oxygen tension within the female reproductive tract is approximately 5% (Rieger 1992), hence embryo development *in vitro* without co-culture was observed to be better under low rather than high oxygen concentrations (Tervit *et. al.* 1972, Batt *et. al.* 1991, Nagao *et. al.* 1994, Watson *et. al.* 1994). High oxygen concentrations generate free radical formation which damage embryonic cells (Thompson *et. al.* 1990, Batt *et. al.* 1991, Rieger 1992, Walker *et. al.* 1992, Waston *et. al.* 1994).

The cessation of sheep embryo development *in vitro* at the 8-16-cell stage may indicate that the activation of a particular gene is not occurring normally when embryos are cultured *in vitro*. Culture methods could be modified until the appearance of this marker mimics what is observed for *in vivo* embryos (Peters 1992). However a defined medium for domesticated animals has not yet been arrived at. Only laboratory animal embryos can be cultured successfully in chemically defined media such as CZB (a modified medium containing an increased lactate/pyruvate ratio, but no glucose; Chatot *et. al.* 1989) and hamster embryo culture medium (HECM) (McCaffrey 1993). Simple media such as Synthetic Oviduct Fluid Medium (SOFM; Tervit *et. al.* 1972) supplemented with serum is used to culture embryos from domesticated animals. However there is always a developmental block at the 8-16-cell stage. Co-culture systems are increasingly being used since no one culture medium is able to sustain the growth of preimplantation domestic animal embryos through the *in vitro* developmental block (Kane *et. al.* 1992). After culturing pronucleate eggs with oviductal epithelial cells, Gandolfi and Moor (1987) found that 30% of blastocysts were viable after transfer. Oviductal cells are beneficial to embryonic development through the secretion of polypeptides, glycoproteins and non-specific growth factors. They also supposedly detoxify the culture medium by metabolising or neutralising harmful compounds (Kane *et. al.* 1992, McCaffrey 1993), such as embryo toxic ammonia as hypothesised by Gardner and Lane (1993). Trophoblastic vesicles have also been used for co-culture and were found to enhance *in vitro* cleavage of early stage embryos of cattle and sheep (Heyman *et. al.* 1987).

2.7.2 Medium constituents

Culture media usually contain nutrients, antibiotics and serum. Lactate, pyruvate and glucose in the culture medium supply energy to the growing embryo. During the initial cleavage stage mammalian embryos prefer pyruvate, lactate and possibly amino acids. Glucose is not used to any great extent until the morula and blastocyst stage. During this time, the blastocyst undergoes both cell proliferation and expansion (Gardner et. al. (1994). Hence glucose is needed to supply energy in the form of ATP, glycoprotein and glycolipids necessary for cell function (McCaffrey 1993). However if the supply of glucose is insufficient, glutamine metabolism becomes significant in cattle blastocysts (Rieger et. al. 1992), indicating that amino acids can be utilised as energy sources (Gardner and Lane 1993). It has been found that the inclusion of amino acids, in particular, non-essential ones, enhances sheep (Thompson et. al. 1992) and mouse embryo development (Gardner and Lane 1993). Gardner and Lane (1993) proposed that this group of amino acids is similar to those present at relatively high levels in the mammalian oviduct and endogenous pool of the early embryo.

When serum is used to supply nutrients it is not possible to know with any degree of accuracy which nutrients or substances are present. Sheep serum, foetal calf serum, bovine serum albumin and human serum not only supply nutrients but probably also contribute other factors, some of which may not have been defined yet. Some reports claim that heat inactivated human serum (HIHS,

at 56°C for 30 min) is superior to other serum supplements (Walker et. al. 1988, Thompson et. al. 1992, Walker e.t al. 1992). However commercially available human serum is not a viable alternative (Walker et. al. 1992). Serum contains both essential and non-essential amino acids. Gardner et. al. (1994) demonstrated that Eagle's non-essential amino acids were beneficial to sheep embryo development up to the blastocyst stage. Therefore, essential amino acids in serum would probably not be beneficial to embryo development. In a review Bavister (1992) questioned the validity of using HIHS as a supplement. Heating human serum may adversely alter cell-growth-supporting properties or even render it toxic. A preliminary study (McEvoy et. al. 1994, unpublished data) showed that when SOFM was supplemented with Human Serum more ammonia (214μmol/l) was produced after 4 days than when BSA was used as a supplement. This value is higher than the lowest ammonia concentration (75μM) found by Gardner and Lane (1993) to have reduced blastocyst cell number of mouse embryos cultured in amino acid supplemented medium. Walker et al (1992) reported the successful culture of 1-cell sheep embryos to the blastocyst stage within 4 days in SOFM supplemented with 20% HIHS. According to McCaffrey (1993) blastocoel formation in 1-cell embryos cultured in oviducts of synchronised recipients does not occur until day 5 of culture. The study of Walker et al (1992) showed that SOFM/20% HIHS causes formation of the blastocoel to occur 1 cell cycle earlier or, as it is argued by McCaffrey (1993), a high proportion of blastocysts may have degenerated and formed vacuoles in a manner resembling blastocoel formation. A better way of using human

serum in culture which will confer its beneficial effects without any confounding problems (i.e. accumulation of toxic ammonia) would be to periodically renew the culture medium as proposed by Gardner et al (1994). Ideally it would be easy to use serum-free medium, but all the growth factors needed by embryos have not been fully characterised. So for the moment it would be appropriate to try to understand embryo requirements in vitro and to define essential factors in sera.

A study by Gandolfi and Moor (1987) caused an interest in the study of co-culture and growth factors which may be beneficial to embryos in vitro. Since embryo development in culture is inferior to that in vivo (Kane, Carney and Ellington 1992), exogenous growth factors may become a viable option in improving embryo development in vitro. Furthermore these molecules could be good candidates for experiments directed at clarifying the molecular basis of beneficial co-culture systems (Watson et. al. 1994). However the growth factors involved are many and are still being found. Walker et al (1992) reviewed some studies which showed that embryos from several livestock species can develop to the blastocyst stage in simple media supplemented with sera or BSA, probably implying that specific oviductal factors may not be required for embryo development up to this stage. Alternatively the benefits of such systems may be conferred by the serum, suggesting a need to fully characterise serum, in order to achieve better utilization of such supplements.

2.8.0 Assessment of viability of embryos.

2.8.1 Morphology

Embryos in culture are usually evaluated under microscopic examination by counting the number of normal blastomeres in the cleavage-stage embryos, or by recording morula and blastocyst formation and expansion (Kane 1987). Lindner and Wright (1983) also described parameters commonly used to evaluate embryo quality and one of the simple methods is to classify embryos into excellent, good, fair and poor on the basis of their morphology. According to the same authors this method of assessing embryos is useful in predicting pregnancy rates for a group of embryos, but is of limited value in determining survival of individual embryos. This is particularly true with early-stage embryos, because the evaluation is based on morphological development and is subjective (Bishonga 1993). However after the 8-16-cell stage developmental block (ie morula/blastocyst stage) embryo quality based on morphological development is a good predictor of viability. To strengthen and complement morphological assessment of embryos more objective methods are necessary.

2.8.2 Protein synthesis and glucose metabolism.

Although it is evident that morphologically good embryos have a much higher survival rate than poor embryos (Kennedy, Boland and Gordon 1983, Lindner and Wright 1983), viability of presumed good embryos is variable. This is because the assumption has always been that the more an *in vitro* cultured embryo resembles its *in vivo* counterpart, the more viable it would be (Peters 1992). Therefore the development of reliable methods of assessing embryo viability still remains a worthy challenge. According to Rieger

(1984) a possible and perhaps a promising alternative approach is the assessment of the metabolic activity of the embryo.

The assessment of embryo viability based on a biological activity is a useful approach as has been demonstrated by the studies below. Bishonga (1993) found that not only was there a positive correlation ($r=0.79$; $p<0.01$) between embryo developmental stage and rate of protein synthesis (^{3}H-phenylalanine incorporation), but also the rate of protein synthesis declined with increase (i.e., deterioration) in embryo grade (scale: 1=excellent to 5=dead). The uptake and metabolism of glucose and its metabolites is well documented (see section 2.6.1). It has been shown that there is a good correlation between glucose uptake *in vitro* and subsequent viability in utero after autotransfer (Gardner and Leese 1987, Renard *et. al.* 1980). Hence Rieger (1984) proposed monitoring glucose utilization by the embryo as an alternative method of assessing viability. In this approach, radio-labelled glycolytic and pentose phosphate pathway substrates are used and radioactive CO_2 and H_2O used as indicators of glucose utilization (Rieger 1984, O'Fallon and Wright 1985, Javed and Wright 1991).

2.9.0 Objective

The objectives of this study were to assess the effects of readily degradable nitrogen in the form of added urea on early development and survival of sheep embryos *in vitro* and to investigate the mechanisms involved.

3.0.0 MATERIALS AND METHODS

3.1.0 Introduction

The present experiment was designed to study the effects of readily degradable protein (RDP) in the form of urea on embryo survival in ewes. The experiment also investigated the mechanisms by which RDP influences embryo survival. Microscopic assessment of embryo morphology was done on Day 3 following insemination. Metabolic assays using [U-^{14}C]glucose/[5-^{3}H]glucose and ^{35}S-Methionine were carried out to estimate the metabolic activity of the embryos. Insulin was measured to find if it was involved in alterations of glucose metabolism by the embryo.

Oviductal fluid and cells were assayed to try and find if RDP had an effect on oviductal secretory proteins and protein synthesis.

Hormonal measurements of progesterone and LH were carried out to find if there is any relationship between RDP level and endocrine status during the preovulatory period (progesterone) and at oestrus (LH).

Urea was fed at a level of 48 g/day. The beneficial inclusion of urea for maximal microbial protein synthesis was enhanced by addition of sodium sulphate in the ratio of 8 parts urea to 1 of sodium sulphate to supply sulphur.

3.2.0 Animals

Twelve Greyface ewes aged between 3 and 6 years were used. The

animals were previously maintained on a Complete Diet (see Tables 1 and 2 for composition) and were shorn in early May just before the start of the experiment. Two ewes of similar liveweight were brought into the experimental pens each week for six weeks. The initial liveweights ranged from 66.8 to 95.5 kg with a mean weight of 75.3 kg. Within each pair, one ewe was allocated at random to the control diet and the other to the high urea diet.

Table 1: Composition of the Complete Diet

Ingredients	g/kg
Hay	500
Barley	299.5
Molasses	100
Fish meal	91
Salt	3
Mineral/Vitamins#	1.5

supplied per kg of diet: 0.9 mg retinol palmitate, 0.015mg cholecalciferol, 12 mg Dl-α-tocopherol acetate, 0.12 g MgO, 85 mg $ZnSO_4.7H_2O$, 40mg $MnSO_4.4H_2O$, 0.12 mg KIO_3 and 0.25 mg $CoSO_4.7H_2O$

3.3.0 Experimental design and diets

The experiment was a randomised design with two dietary treatments, replicated six times. The treatment layout is shown below;

(C) basal diet (1600g) + 2g urea to meet microbial needs..Control

(H) basal diet (1600g) + 48g urea ..... High urea

The diets were supplemented with minerals and vitamins and were

weighed separately for each ewe. The animals on the control diet had 2g urea added to their diet during its preparation. This was to meet the rumen degradable N requirements of the rumen bacteria for maximum microbial protein synthesis. Those on high urea treatment had the urea weighed separately and mixed with the basal diet just prior to feeding. Sulphur in the form of sodium sulphate was also weighed out at the same time as the urea and mixed at the ratio of 8 parts urea to 1 of sodium sulphate. This was to ensure that dietary sulphur did not limit microbial protein synthesis. The ingredients and composition of the basal diet and hay are given in Tables 2 and 3.

Table 2: Composition of the basal diet

Ingredients	g/kg*
Hay	832
Molasses	147
Dicalcium phosphate	10
Sodium chloride	10
Mineral/Vitamins#	1

*Based on fresh weights
see Table 1 for composition

Table 3: Chemical composition of the Complete Diet (CD) and of Hay.

Diets	Dry matter (%)	Nitrogen (%)
Complete diet	86.93	1.729
Hay	87.99	1.026

3.4.0 Housing and Management

The ewes were housed in individual concrete floored pens bedded with sawdust. The pens were cleaned daily and fresh sawdust bedding added each time. The experimental diets were introduced 15 hours after an intramuscular injection of 125μ of a prostaglandin $F_{2\alpha}$ analogue, (Cloprostenol, Estrumate, Coopers Animal Health, Crewe, UK). The animals were fed twic daily at 08.00 and 16.00 h. The amount of feed at each time was 800g. Clean water was available at all times. After embryo collection, urea supplementation was stopped and the ewes were returned to the complete diet at a level of 300g f the first feed, thereafter increasing by 100g/feed up to a final intake of 600g/feed.

3.5.0 Oestrous synchronization and superovulation

The ewes were given a single i.m. injection (125μg) of a prostaglandin analogue at 17.00 h. The prostaglandin was give in order to regress any corpus luteum that might still be present on the ovary and therefore would mask the actual concentrations of progesterone generated by the standard exogenous-administered source (CIDR). Oestrus was induced by priming the animals with exogenous progesterone provided by a Controlled Internal Drug Release (CIDR) device (Inter-Ag, Hamilton, NZ). The CIDR (containing 0.3g progesterone) was inserted into the vagina and left in place for 12 days.

Porcine follicle stimulating hormone (FSH, Sigma, UK) was

given intramuscularly at four times to induce superovulation. Spreading the administration over time was done in order to avoid high levels of FSH which might otherwise cause ovarian cysts and improper follicle development. The schedule for the administration of FSH is shown in Table 4.

Table 4: Timing of FSH administration relative to CIDR application and dosage used.

Injection No	Time relative to CIDR application	Dose (mg)
1	24 hours before CIDR withdrawal	6
2	12 hours before CIDR withdrawal	5
3	At the time of CIDR withdrawal	3
4	12 hours after CIDR withdrawal	2

To detect oestrus a vasectomised ram was allowed into the ewes pens at intervals of 2 hours starting 10 hours after the removal of the CIDR device. Ewes were taken to be in oestrus if they stood to be mounted by the ram.

3.6.0 Intrauterine Insemination

The day before the insemination, water was withheld from 17.00 h until after the insemination the following day. On the day of the insemination the 08.00 h feeding was also withheld. This was done to reduce rumen volume and to avoid the risk of regurgitation at laparoscopic insemination. Approximately fifty minutes before insemination each ewe was given an intramuscular injection (0.7ml) of a tranquillizer, = 5mg Acepromazine Maleate (C-Vet Ltd, Bury, St Edmunds, UK).

The ewes were inseminated using a laparoscopic intrauterine insemination method. Intrauterine insemination using laparoscopy results in consistently high fertilization rates even after synchronization of oestrus and stimulation of superovulation by the administration of exogenous gonadotrophin (McKelvey et al 1985). Insemination was carried out using fresh semen approximately 52 hours after removal of the CIDR device. This method (see McKelvey et al 1985 and Bishonga 1993 for details) involves restraining the animal in a supine position on a cradle. The area cranial to the udder was shorn and then cleaned using hibitane solution (15% water, 75% alcohol, 10% v/v hibitane concentrate; ICI Pharmaceuticals, Macclesfield, Cheshire, UK). The two areas at which cannulas were to be inserted were injected with a local anaesthetic, 5ml of Lignavet-plus (Lignocaine and adrenaline, C-Vet Ltd, Bury St Edmunds, UK). The insemination was carried out with the animal reclining at an angle of 45° to the horizontal. To assist the view of the uterine horns the abdomen was insufflated with CO_2 gas. Semen was introduced through a 5mm cannula inserted on the right side of the udder. With the aid of a 7mm laparoscope inserted via a trochar on the left side of the udder, 0.2ml diluted semen (see later for dilution) was injected into each uterine horn using a fine glass pipette attached to a 1ml syringe. An intramuscular injection (4ml) of an antibiotic (Duphapen-strep, 200mg procaine penicillin + 250mg/ml dihydro-streptomycin sulphate) was given after the insemination.

All semen was collected from one Suffolk ram of proven fertility using an artificial vagina. It was diluted with phosphate buffered saline (PBS, pH 7.4) in the ratio of 1:1. The prepared semen was held briefly in an incubator (37°C) until it was required for insemination.

The withheld feed and water were given to the animals immediately after insemination.

3.7.0 Embryo and cell collection, and autotransfer

a) embryo collection

Embryo collection was done on Day 3 following insemination. Animals were denied water starting from 17.00 hours the day prior to embryo collection. The usual morning feed was also withheld on the day of embryo collection. These were later reintroduced after the surgery.

After restraining the animal on the cradle, a general anaesthesia was induced (by a face mask) and maintained (by intra tracheal tube) using halothane, nitrous oxide and oxygen. The embryos were collected using a two phase procedure (see Figure 3a for illustration). Following surgical exteriorisation via mid-ventral laparotomy, a sterile siliconised glass tube was inserted near the infundibulum to collect the oviductal flush. Ten ml of phosphate buffered saline solution (PBS, pH 7.38, Sigma Diagnostics, USA) filtered with 0.2μm low protein, non pyrogenic filter (Acrodisc, Gelman Sciences, Michigan USA) was introduced near

the utero-tubal junction using a 10 ml syringe and blunted 18 gauge needle. The flushing was collected through a siliconised tube into a sterile 15ml polystrene centrifuge tube (Corning Incorporation, NY, USA). The second phase involved the introduction of another 10ml PBS at the tip of the uterine horn. This flush was collected via a 10 FG Foley self-retaining catheter inserted at a point coincident with the external bifurcation of the uterine horns (see Figure 3a for illustration). The procedures were repeated for the other oviduct and uterine horn. However in the fourth week the procedure was changed due to loss of flush fluid during the recovery procedure. Instead a single phase flushing technique was employed whereby a siliconised tube was inserted near the infundibulum and a syringe containing PBS introduced at the end of the uterine horn (see Figure 3b)

b) autotransfer

Immediately after flushing the pH of the fluid was determined and the embryos were located using a stereomicroscope (40x magnification). The embryos were picked up in 50μl PBS and transferred to a collecting dish containing 5ml Ovum Culture Medium (OCM, ICN Biomedicals Ltd, Oxfordshire, UK) plus 10% Foetal Calf Serum (FCS, ICN, Biomedicals Ltd, Oxfordshire, UK). The embryos were picked up with about 70μl OCM and placed in a four well dish containing OCM + 10% FCS.

A preliminary assessment of the stage of development of the embryos was made. Usually when more than one embryo was

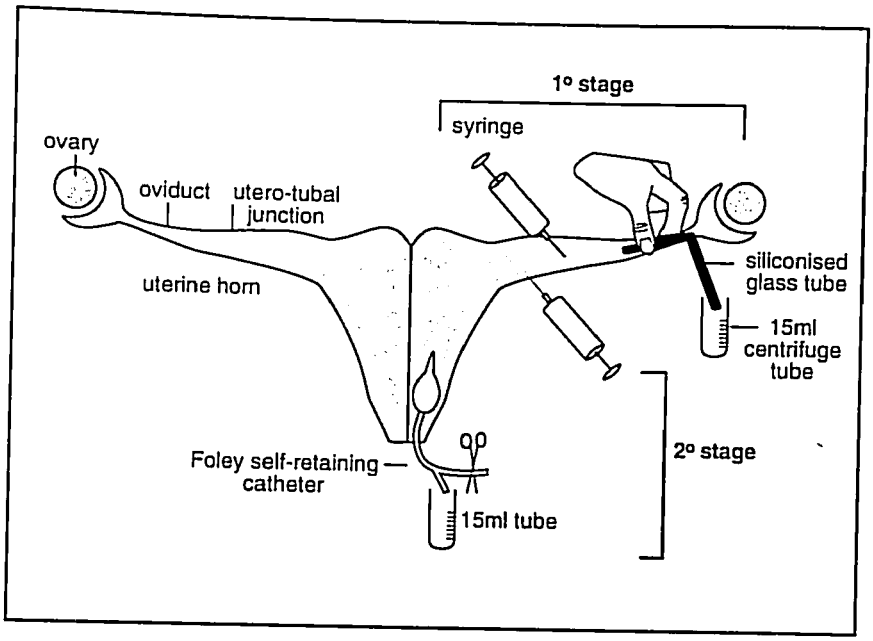


Figure 3a: The two-stage flushing technique used to recover embryos at Day 3 after insemination.

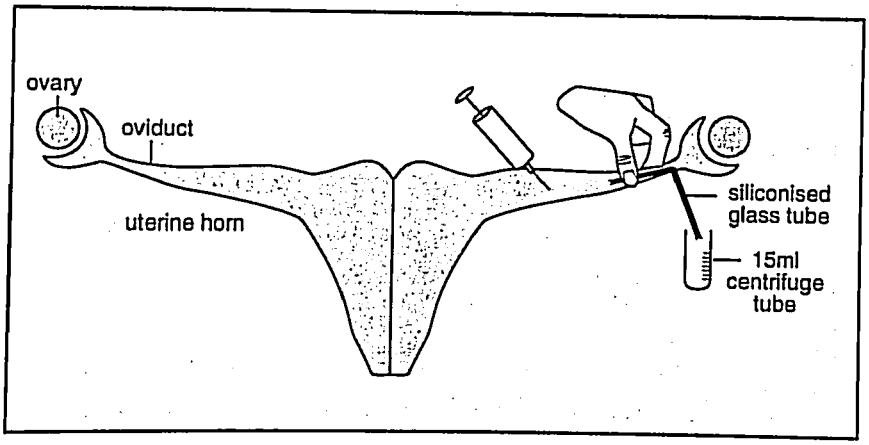


Figure 3b: One-stage flushing technique used to recover embryos at Day 3

recovered, one was returned to the donor. A fine glass pipette was used to insert back the embryo (in 70μl OCM + 10% FCS) into the tip of the uterine horn.

c) collection and preparation of the cells.

The total fluids collected from each oviduct and uterine horn, including any dislodged cells during flushing, were centrifuged (500 rpm; 5 minutes at 25°C) separately before aliquots of the resulting supernatant were taken for subsequent analysis of insulin, glucose, urea and ammonia. This supernatant was centrifuged again (200 rpm; 20 minutes) before being frozen (-20°C) for later analysis of proteins secreted *in vivo*. The supernatant was then aspirated, leaving about 1.5 ml of fluid and cells in each tube. The cells from the oviductal flushing were transferred to 1.5 microtubes and washed repeatedly, twice in OCM + 10% FCS and 4 times in Tissue Culture Medium 199 (M199 with Earle's salts) supplemented with heat treated FCS, L-Glutamine (200mM solution) and Penicillin-Streptomycin (5000IU Pen/5mg Strept/ml) at a volume ratio of 100:10:1:1 (all supplied by ICN Biomedicals Ltd, Oxfordshire, UK). At each washing step, fluid was added followed by vortexing and allowing the cells to sediment for 5 minutes. The supernatant was then replaced and the procedure repeated. This method of washing was preferred to the serial washing in a 4 well dish because it is quicker and the cells are not lost. The washed cells were either placed into a 4 well dish for *in vitro* culture or incubated in the presence of ^{35}S-Methionine to determine

protein synthesis.

When no embryos were expected, (i.e. no corpora lutea present on the ovary) some or all of the dislodged cells from the relevant oviductal flushing were preserved in liquid nitrogen for RNA analysis at a later date.

3.8.0 Embryo assessment and assays

After placing the embryos in a 4 well dish containing OCM +10% FCS, they were taken to the laboratory for further assessment. Each day at about 13.00 hours embryo development was assessed until Day 4 after embryo recovery. The assessment was done by one operator in order to maintain a reasonable degree of objectivity.

a) morphology

The stage of development of each embryo was determined at 200x magnification using an inverted microscope. Parameters described by Lindner and Wright (1983) which included shape, colour, number and compactness of cells and size of the perivitelline space, were used to categorise and grade the embryos. Grades from 1 to 5 (Scudamore 1991) were allocated within the stage of development as follows;

Grade 1 = Excellent; a perfect embryo, symmetrical with cells of uniform size, colour and texture.

Grade 2 = Good; an embryo with few imperfections such as few extruded blastomeres, irregular shape and few vesicles.

Grade 3 = Fair; definite but not serious abnormalities, presence of extruded blastomeres, few cells degenerate.

Grade 4 = Poor; increasing level of abnormality, numerous extruded blastomeres, lack of symmetry, high proportion of dark and degenerated cells.

Grade 5 = Degenerate; completely lysed embryo.

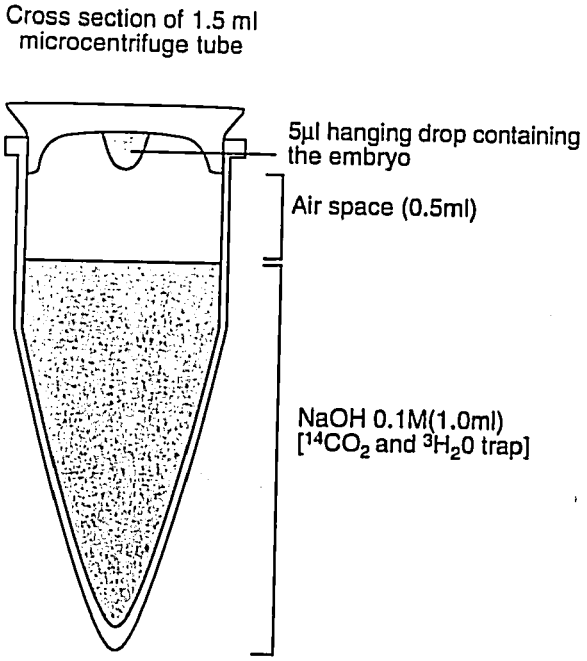
b) culture and assays

i) glucose utilization determination

Determination of glucose utilization was carried out using a modification of the methods of Thompson et al (1991). Immediately after assessing the embryos, they were put through a series of 4 x 1.0ml washing steps in Synthetic Oviductal Fluid Medium (SOFM Tervit et al 1972). HEPES-buffered (25mM, Sigma Chemical Co. St Louis, USA) SOFM, containing 32mg/ml of BSA (Sigma Chemical Co. St Louis USA), was prepared the previous day and stored at 38.5°C. At each washing step the embryo was transferred in 3μl of medium. From the last wash each embryo (in 3μl SOFM) was added into a 2μl droplet of radioactively-labelled SOFM solution on a previously detached cap of a 1.5 ml microtube (Sarstedt, Germany; see Figure 4). The radioactive SOFM solution was prepared by adding 1850Bq [5-^{3}H]glucose (1.7μl; specific activity = 5.9Ci/mmol) and 1850Bq [U-^{14}C]glucose (8.4μl; specific activity = 283mCi/mmol) to 30μl of SOFM. The cap was then placed on its tube, which contained 1.0 ml of 0.1M NaOH as a trap for $^{3}H_2O$ and $^{14}CO_2$. The 5μl drop was incubated at 38.5°C for three hours. Thereafter

Figure 4: Metabolic incubation tube

{ conditions; 3 hours incubation at 38.5°C
droplet content; SOFM + 1850Bq [U-^{14}C]glucose + 1850Bq [5-^{3}H]glucose }



the embryo was removed and its morphological development assessed. The NaOH "trap" was transferred into vials containing 8ml of scintillation solution (Emulsifier-Safe, Packard, A Canberra Co. The Netherlands). Radioactivity of $^{3}H_2O$ and $^{14}CO_2$ was measured on a ß-counter for 10 minutes. By simultaneous measurement of radioactive H_2O and CO_2 levels isolated in the NaOH trap, the activities of the pentose phosphate pathway and the glycolytic pathway during the 3 hour incubation period could be determined for each embryo (Thompson et. al. 1991)

After assessment embryos were cultured in 4 well dishes for 72 hours with oviductal cells. These cells were previously washed in M199 and when plating the 4 well dish (containing M199) care was taken to place equal amounts of cells (approx 2-3 clumps) in each well. Though this method of allocating the oviductal cells to the wells is subjective, the allocation was done by one operator in order to maintain consistency. Embryos were randomly allocated to wells in each dish. Following co-culture another glucose utilization determination was carried out. On completion of the second incubation in radiolabelled glucose the embryos were co-cultured with oviductal cells for 24 hours before they were analysed for incorporation of radio-labelled amino acid (^{35}S-Methionine) into de novo synthesised proteins.

iii) co-culture

Previously washed (see section 3.7.c) cells from control animals were left standing in uncapped 1.5 ml microtubes

containing Medium 199 (with supplements) at 38.5°C under 5% CO_2 in air. This was done to make the contents of the microtube adjust to, and equilibrate with conditions in the incubator (i.e. pH). A 1.0 ml aliquot of filtered Medium 199 (with supplements) was pipetted into each well of a 4 well dish. Aliquot samples were taken from the 4th well and preserved for analysis of ammonia, urea and glucose concentrations. These were to be used as reference blanks for subsequent determination of ammonia, urea and glucose during in vitro co-culture. A packed cell aliquot (5 µl) was removed from the microtube and some of these cells were shared equally between each well of the 4 well dish used for co-culture. The embryos from the 3 hour "glucose assay" incubation were washed four times in Medium 199. After the 4th wash each embryo was transferred in a 1.0 µl aliquot of supplemented Medium 199 into a previously randomly chosen well containing 1.0 ml supplemented Medium 199 and oviductal cells. The 4 well dish was incubated for 72 hours at 38.5° under 5% CO_2 in air. Cells for co-culture were supplied only by control animals. This was to avoid extending the possible effects of high urea and ammonia from "high urea" fed animals which might otherwise mask the developmental potential of the embryos.

After 72 hours incubation in co-culture the embryos were removed and aliquot samples were taken from the co-culture medium for analysis of ammonia and urea and glucose at a later date. The embryos were washed four times in HEPES-buffered SOFM supplemented with 32mg/ml BSA. Morphological assessments

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After 72 hours incubation in co-culture the embryos were removed and aliquot samples were taken from the co-culture medium for analysis of ammonia and urea and glucose at a later date. The embryos were washed four times in HEPES-buffered SOFM supplemented with 32mg/ml BSA. Morphological assessments

followed to measure amino acid incorporation.

T.C.A. Precipitation Procedure:

The buffer used was a 20% (w/v) solution of Trichloroacetic acid (Sigma, U.K.; #T-4885; FW=163.4) in distilled water. This was chilled (4°C) prior to use. Squares of 3 mm Whatman paper (1 cm^2) were soaked for at least 2 h in the 20% TCA solution, then dried thoroughly. After this time, gloves were worn when handling the papers to avoid transfer of proteins from bare hands. Prior to use, papers were labelled with pencil and placed on aluminium foil. One paper per test sample was used, plus two untouched papers as background "blanks", while 4 "wet" papers served as drying indicators (n=2) and as controls (n=2). Using a micropipettor, a 1 μl aliquot of sample was spotted onto the paper. Papers were left to dry thoroughly under a lamp, with some of the "wet" samples used to manually check that complete drying had occurred before proceeding to the wash stages.

Anhydrous dicalcium pyrophosphate (Sigma, U.K.) was added to the 20% TCA solution at the rate of 5.08 g/l. This solution of 20% TCA with 20 mM pyrophosphate was the first wash used, while the second and third washes were produced by diluting the first at the rate of 1 volume in 3 volumes of distilled water (e.g 20 ml + 60 ml), yielding a solution of 5% TCA with 5 mM pyrophosphate. The fourth and fifth washes were in 95% ethanol. All solutions were chilled to 4°C before use, as follows:

10 minutes 20% TCA with 20 mM pyrophosphate;

20 minutes 5% TCA with 5 mM pyrophosphate;

10 minutes 5% TCA with 5 mM pyrophosphate;

10 minutes chilled 95% ethanol;

5 minutes chilled 95% ethanol.

After washing, papers were again dried thoroughly. Using sterile forceps, dried papers were folded once and individually introduced into separate scintillation vials. A 4.50 ml volume of scintillation fluid was then added to each vial, and samples were counted for 3 minutes. Each sample's c.p.m. was calculated by subtracting the background c.p.m..

v) nuclei counting

Instead of analysing the embryos from animals of the last two groups for amino acid incorporation, nuclei counting was carried out. This would give information on the utilization of glucose per cell. After the second incubation in radiolabelled glucose the embryos were washed in 4 x 2 µl aliquots of PBS before placing them in fixative. PBS was used in order to minimise the amount of protein material adhering to the embryo which would otherwise precipitate when it comes in contact with fixative. After washing the embryo in PBS it was placed in fixative (ethanol:acetic acid 3:1 v/v) which stopped growth and dissolved the zona pellucida. The embryo was then removed from the fixative with as small a volume as possible and placed in a glass slide. A coverslip was placed over the embryo and a few drops of aceto-orcein stain are allowed to

run under the coverslip and bathe the embryo. The stain is taken by the nuclei, nucleoli and chromosomes while the cytoplasm remains pale pink. This makes the nuclei and their contents visible for counting. The nuclei are counted by using a microscope (400x magnification) linked to a computer. The image of the embryo from under the microscope is transferred to the screen on the computer. After marking the embryo on the computer screen with the cursor, the nuclei are counted automatically.

3.9.0 Cell culture, cell and fluid assays

a) Cells

i) co-culture (see section 3.8 (a))

ii) glucose concentration

The determination of glucose concentration is based on the oxidation reaction in which 4-amino phenazone acts as an oxygen acceptor. Glucose in the sample reacts with glucose oxidase in anaerobic conditions and yields gluconic acid and hydrogen peroxide. The peroxide is then reduced by peroxidase and the colour produced by the simultaneous oxidation of an oxygen acceptor (4-amino phenazone) is read at 510nm using an autoanalyser.

iii) protein synthesis (^{35}S-Methionine incorporation)

Half of the oviductal cells which were not used for co-culture were incubated in the presence of ^{35}S-Methionine to determine incorporation of label and secretion of <u>de novo</u> synthesised

proteins. The incubation medium was prepared by supplementing 10ml MEM (Methionine- and glutamine-free, ICN Biochemicals Ltd, Oxfordshire, UK), with 10.5mg Polyvinyl alcohol (PVA; Sigma Chemical Co., St Louis, USA) and 0.1ml of 200mM L-Glutamine (ICN Biochemicals Ltd, Oxfordshire, UK). A 248μl aliquot of this medium was pipetted into a 1.5ml microtube and 2μl ^{35}S-Methionine was then added. The cells were washed 3 times in MEM/PVA/L-Glut medium by serial vortexing (10 to 20 sec) and settling (5 minutes). Cells were aspirated in 50μl aliquots of the third wash and placed in the same incubation tube left uncapped for 10 minutes at 38.5°C in 5% CO_2 in air and then capped. After two hours, the tubes were removed from the incubator and centrifuged at 5000rpm for 10 minutes. Five 50μl aliquot samples of the supernatant were collected in separate 1.5ml tubes and stored -70°C for subsequent analysis of de novo secreted proteins. The remaining cells were mixed with 500 μl lysis buffer and the contents heated at 95°C for 10 minutes before freezing at -70°C to be analysed later for incorporation of the radio-labelled amino acid.

b) Gel electrophoresis of proteins from ova and oviductal cells.

Following removal of 1 μl of each sample for estimation of ^{35}S-Methionine incorporation into acid-insoluble protein in individual ova (see Section 3.8.0.b.iv), the remainder (9 μl) was loaded along with 20 μl sample buffer and 20 μl water onto separate lanes of vertical (20 cm x 20 cm) 7.5% polyacrylamide gels in order to electrophoretically separate the labelled polypeptides on the basis of their relative molecular mass. Using separate gels, 30 μl radio-labelled samples of sheep oviduct epithelial cells (SOEC) were similarly loaded, but in this case with 10 μl sample buffer and 10 μl water added per lane. Likewise, supernatant incubation media samples containing de novo synthesised and secreted oviductal proteins were loaded on other gels. These 50 μl samples were loaded without any additional water or sample buffer.

Two sets of molecular weight standards, ranging between 14,200 and 66,000 (MW-SDS-70L kit; Sigma UK Ltd) and between 29,000 and 205,000 (MW-SDS-200 kit; Sigma UK Ltd), respectively, were loaded on either side of each gel. The gel itself consisted of a separating gel overlaid with a stacking gel, with slots formed in the latter by means of a comb placed in position when casting the gel and removed prior to loading the samples. After loading all the samples, a running buffer (3 g Tris, 14.4 g glycine, and 1 g Sodium dodecyl sulphate (SDS) per litre) was added and the apparatus connected to a power pack. During the first phase in the separation procedure, a current

of 13 mA per gel (i.e., 26 mA if two gels were run simultaneously) was applied. During this period (approx. 1.5 to 2 h) protein from the samples migrated through the stacking gel. Thereafter the current was increased to 26 mA per gel until separation was complete. This second phase, during which a visible dye front moved to the end of the gel plates, lasted about 4 to 5 hrs.

Each gel was then removed, nicked at the top left corner to aid subsequent identification, and placed into a fixing solution (70 ml glacial acetic acid, 400 ml ethanol, and 530 ml water per litre) and left overnight on a rocking platform. Next day, the gel was placed into a Coomassie staining solution, prepared by dissolving 1.25 g Coomassie Brilliant Blue R (Sigma UK Ltd) in 1 litre of the aforementioned fixing solution. This was removed 2.5 h later (1.5 h in the case of SOEC gels) and replaced with destaining solution. The destaining solution (70 ml glacial acetic acid, 100 ml ethanol, and 830 ml water per litre), used to remove any stain not bound to proteins in the gel, was replaced with fresh solution after 6 to 8 h, and thereafter at approximately 24 h intervals for 2 to 3 days, by which time the gel had cleared to reveal the distinct banding pattern of the molecular weight marker lanes and, if sufficient protein was present, some bands in the sample lanes.

Subsequently, each gel was transferred to distilled water for at least 0.5 h, and if required, for indefinite storage,

before drying. Prior to drying, gels were soaked for 0.5 h in Salicylate solution, prepared by dissolving 160 g sodium salicylate (Merck, Darmstadt, Germany) in 1 litre of water. Gels were then placed on 3 mm Whatman chromatography paper and dried, singly or in pairs, at 60°C under vacuum, the process taking up to 4 h to complete.

After drying, gels were placed against pre-flashed XAR5 film (Kodak, Cambridge, UK) in autoradiography cassettes, stored (-70°C) for several days and subsequently developed (4 min), washed (1 min), fixed (1 min; then 4 min in a second tank), washed again (1 h in water) and dried (30 min).

c) Oviductal Fluid

i) glucose concentrations (see section 3.9 (a) ii)

ii) Urea, ammonia and insulin concentrations (see Section 3.10.0.)

3.10.0 Plasma sampling

Each ewe was bled by jugular vein puncture just before insertion of the CIDR device (08.00 hours) and then daily at 14.00 hours for 18 days after insemination. The first sample gives the basal concentration of progesterone. The heparinised blood samples were centrifuged at 2500rpm for 50 minutes at 4°C; aliquot of plasma was frozen for subsequent determination of progesterone. Ten hours after the CIDR device was removed, blood samples were taken every 2 hours for 40 hours. These

samples were preserved to be analysed on a later date for LH, urea, ammonia, insulin and glucose. Blood samples were also taken at the time of FSH injection and an hour before embryo recovery.

a) Analysis of urea concentration in plasma, oviductal fluid and co-culture medium.

Plasma, oviductal fluid and co-culture medium urea concentrations were determined by using an auto-analyser. The analysis is based on the hydrolysis of urea to yield ammonia and CO_2 by urease. Ammonia is then made to react with 2-oxo-glutarate in the presence of glutamate dehydrogenase to form L-glutamate. Accompanying these reactions is the oxidation of NADH to nicotinamide adenine dinucleotide (NAD) which results in a decrease in absorbance at 340nm. Urea level in the sample is proportional to the absorbance (Talke and Schubert 1965).

b) Analysis of ammonia concentration in plasma, oviductal fluid and co-culture medium.

Ammonia concentration was determined in plasma, oviductal fluid and daily samples of co-culture medium. The determination of ammonia concentration by the auto-analyser in the samples is based on the method described by Talke and Schubert (1965) whereby the reductive amination of 2-oxo-glutarate is catalysed by the enzyme glutamate dehydrogenase. The simultaneous oxidation of NADH to NAD results in the decrease in absorbance at 340nm and is proportional to plasma

ammonia concentration. Methylated amines do not react in this method (Bishonga 1993).

c) Plasma and oviductal fluid insulin determination

Insulin was analysed by radioimmunoassay method.The standards were prepared by adding six aliquots of 100μl of serial double diluted Porcine Insulin (263μIU/ml) (Sigma Chemical Co. Ltd, Poole, Dorset) into 6 tubes. Non Specific Binding (NSB) and zero standards were prepared by adding 100μl of assay buffer (Phosphate Buffer, pH 4, 0.05M, 0.5% BSA).

Aliquots (100μl) were pipetted from the stock sample into 4ml polystyrene tubes. A 500μl working anti-serum solution, (raised in guinea-pig, ICN Biomedicals Ltd, Thame Park Business Centre, Wenman road, Thame, Oxfordshire) was added to all except NSB tubes. Instead, NSB tubes were filled with 500μl of assay buffer. The tubes were shaken and incubated overnight at room temperature. The following day 100μl of working tracer solution ^{125}I-insulin, dilution; 15000cpm/100μl with assay buffer) was added to all the tubes. The tubes were then shaken and left standing for 4 hours at room temperature. 500μl of a second antibody mixture;335μl sheep anti-guinea-pig IgG serum and 100μl normal guinea-pig serum diluted with 50μl assay buffer, (SAPU, Law Hospital, Carluke, Lanarkshire) was added to all the tubes. The tubes were then shaken and incubated overnight at 4°C. The following day the tubes were centrifuged at 3500rpm at 4°C for 20 minutes. The supernatant was decanted and the tubes left inverted for a few minutes to

drain. The precipitated pellet was counted in a gamma counter.

d) Plasma progesterone

The determination of progesterone by radioimmunoassay was measured as described by Djahanbakhch et al (1981). After progesterone was extracted from plasma the samples were assayed in duplicate with at least 3 quality controls in quadruplicate. 50µl of ^{3}H-Progesterone recovery trace; 1000-1500cpm (Amersham International Plc., Amersham, Bucks,UK) made up in phosphate gelatin buffered saline (PGBS) was added to the 100µl of sample in glass tubes. 50µl of recovery trace was also added to two tubes containing 100µl of buffer (Buffer Blanks) or solvent (Solvent Blanks) and to four scintillation vials (total count vials). The tubes were then vortexed and allowed to stand on the bench for 30 minutes. 2.5mls diethyl ether (Rhone-Poulene Ltd., Manchester, UK) was added to each tube and the tubes were shaken for 40 minutes on a multi-tube vortexer (Luckham Ltd., Sussex, UK). The tubes were then immersed in a methanol bath to freeze the aqueous layer so that the organic layer could be decanted in glass tubes. The glass tubes were then placed on a dry-block set at 40-50°C and the organic layer was dried under air. After cooling the residue from the tubes was dissolved in 200µl of PGBS buffer. The tubes were vortexed and left overnight at 4°C.

The following day, 50µl of reconstituted sample was added to 3ml scintillation fluid, shaken and counted on a ß-counter for 3 minutes. This would provide the recovery of ^{3}H-Progesterone

and hence unknown progesterone in the original plasma sample. (Results were multiplied by 4 and divided by the mean total count result, then multiplied by 100 to give the percentage recovery rate.)

100 μl of the reconstituted sample or standard (Sigma Chemical Co. Ltd., Poole, Dorset, UK), 100μl of sheep antibody (initial dilution 1:20 000) and 100μl of ^{125}I-Progesterone tracer, approx. 15000cpm/tube (Amersham International Plc., Bucks, UK) were added to glass assay tubes. The tubes were vortexed and incubated for 3 hours on the bench. 100μl donkey anti-sheep serum (DASS, 1:20 dilution, SAPU, Carluke, Lanarkshire) and 100μl normal sheep serum (NSS, 1:700 dilution) were added to each tube. The tubes were vortexed again and left overnight at 4°C. One ml of 0.9% saline was added to each tube to aid the separation of the bound from the free antibody. The tubes were centrifuged at 2000rpm at 4°C for 30 minutes, descanted and the residue allowed to dry. A gamma counter (Cobra Auto-gamma, Canberra, Packard) was used to count radioactivity for 60s/tube (the results were multiplied by 2 and the individual extraction recovery applied to determine the actual amount of progesterone in the original sample.

e) Plasma luteinizing hormone

Duplicate samples were measured for plasma LH concentrations to determine the onset of the LH surge using a technique described by McNeilly et al (1986). 100μl sample or standard (NIH-oLH-S25, NIH, Bethesda, Maryland,USA), 200μl buffer and 100μl first antibody (R29 Rabbit anti-ovine LH, initial

dilution 1:120 000) were mixed. The tubes were incubated at 4°C for 2 hours after which 100µl of tracer (LER-1056-C2 iodinated using a chloramine-T iodination method approx 15000cpm/tube/60s) was added. The tubes were left to incubate at 4°C for 24 hours. A 100µl of second antibody of donkey anti-rabbit serum (DARS dilution 1:32) was added to the tubes and a further 100µl of normal immunised rabbit serum (NIRS dilution 1:200) before being incubated at 4°C overnight.

The following day 1ml of 0.9% saline was added and the tubes centrifuged at 2500rpm for 30 minutes at 4°C. The supernatant was decanted quickly and the residue counted on the gamma counter at 60s/tube. The analysis for LH was only carried out on samples taken at 2 hour intervals between 10 and 40 hours after the withdrawal of the CIDR device. Three tubes in duplicate were set up for quality control. The intra-assay coefficient of variation was 10.5%.

4.0.0 Statistical Analysis

The effects of treatment on body weight, progesterone, the onset of oestrus, the LH onset, levels of plasma insulin, glucose, urea and ammonia were tested by t-Test analysis. Correlations were computed between the times of onset of the LH and oestrous onset, the time of onset of the LH peak and ovulation rate. It was also tested if the levels of plasma insulin and glucose, plasma insulin and plasma urea were correlated.

Treatment effects on ovulation rate were tested using a t-

Test. A Chi-square test was used to find if there were any differences between groups in embryo recovery rates and in embryo viability. A two tailed t-test was carried out to identify if there was any difference in metabolism of embryos from the control and urea-fed ewes. Correlation between the amount of $^{3}H_2O$ and $^{14}CO_2$ generated by the embryos was calculated. Regression analysis was carried out on the rate of protein synthesis and the amount of radio-active H_2O and CO_2 generated by the embryos.

5.0.0 RESULTS

5.1.0 Diets

According to Robinson et al (1980) energy needs for body weight maintenance is 0.42 MJ of ME of body weight(BWT)$^{0.75}$ therefore ME requirements were; 0.42 x 77.0$^{0.75}$ = 10.9 MJ of ME/day. The Basal diet was supplying 7.5 MJ of ME/kg, hence total ME intake was; 1600 x.8871 g of DM x 7.5 = 10.6 MJ/day. Thus diet was just supplying enough energy for maintenance.

In order to meet rumen microbial needs for Degradable Protein (RDP) it was assumed that the diet requires 8g RDP/MJ of ME. Therefore 8.0g x 10.6 MJ = 84.5g RDP.

The Complete Diet was supplying 113.3g/day RDP, so RDP was satisfied. Average daily intake of the Basal diet by the animals was 82.0g/day RDP.
Hence the diet for the Control (Basal Diet) was deficient in RDP to the extent of 2.5g which is equivalent to 3.1 g/day of degradable protein (this would require the addition of 1.1g urea). After the addition of 2 g urea as a precaution that rumen degradable protein was not limited, the Basal diet now had an excess of degradable protein equivalent to 2.6 g. The animals getting 48g urea/day were receiving excess degradable protein to the extent of 136.0 g/day (see Table 5).

Table 5: RDP supplied by the basal and treatment diets in relation to microbial RDP requirements.

Diets	RDP (g/day)	RDP - microbial needs (g/day)
Complete diet	113.3	29
Basal diet	82	-3
Basal + 2g urea	88	3
Basal + 48g urea	220	136
Microbial needs	84.5	

5.2.0 Body weight

At the end of the experimental period (22 days) the mean weight of the animals was 74 ± 2.0 compared to 77 ± 2.45 kg at the beginning of the study. The mean initial and final weights and weight gains/22 days for the two treatments are shown in Table 6. The differences in weight gain/22 days was not significant ($p<0.45$) between the treatment groups.

Table 6: The effect of treatment on mean body weight and gain/22 days (kg; Mean ± SEM).

Treatment	No. of ewes	Initial Wt	Final Wt	Gain/22day
Control	6	76.4±4.13	72.5±3.25	-3.9±1.05
Urea	7	77.6±3.17	74.9±2.61	-2.5±1.04
Overall	13	76.3±2.45	73.8±1.99	-3.1±0.74

5.3.0 Progesterone

The results presented here were collected from a different experiment which had exactly the same protocol and was carried out under the same conditions as the present study.

Plasma progesterone concentrations for the two treatments before CIDR

device withdrawal were (Mean ± SEM) 1.91 ± 0.15 and 1.55 ± 0.12 ng/ml for the urea and control animals respectively. There were significant differences ($p<0.10$) between the treatments (see Table 7). Plasma progesterone profiles of six animals from the urea and control treatments prior to CIDR device withdrawal are shown in Figure 5.

5.4.0 Periovulatory LH

The plasma collected between 10 and 40 hours post CIDR removal was analysed for LH. The onset of the LH surge was 20.4 ± 2.48 hrs for the control (n=5) and 22.9 ± 1.68 hrs for the urea (n=7) animals. These values were not significantly different ($p>0.5$). There was a strong positive correlation between the times of onset of the LH peak and oestrous onset for urea ($r = 0.888$) and control ($r = 0.931$) animals (see Table 7). The correlation between time of onset of the LH peak and ovulation rate was negative for the urea ($r=-0.488$) and positive for the control ($r= 0.255$) animals (see Table 8).

Table 7: The effect of treatment on times of LH surge onset oestrus onset and progesterone conc. (Mean ± SEM)

Treatment	No. of ewes	P4 conc (ng/ml)*	Oestrous onset (hrs)	LH onset (hrs)
Control	5	1.55±0.15	18.4±2.93	20.4±2.48
Urea	7	1.91±0.12	18.3±1.82	22.9±1.68
Overall	12	1.73±0.07	18.3±1.53	21.8±1.40

*P4 = Progesterone (From a different study).

5.5.0 Plasma Insulin

The mean insulin concentrations for the urea and control animals between 10 and 40 hours after CIDR removal were 48.5 ± 1.97 and 34.9 ± 1.27 μU/ml respectively (see Table 9). These were

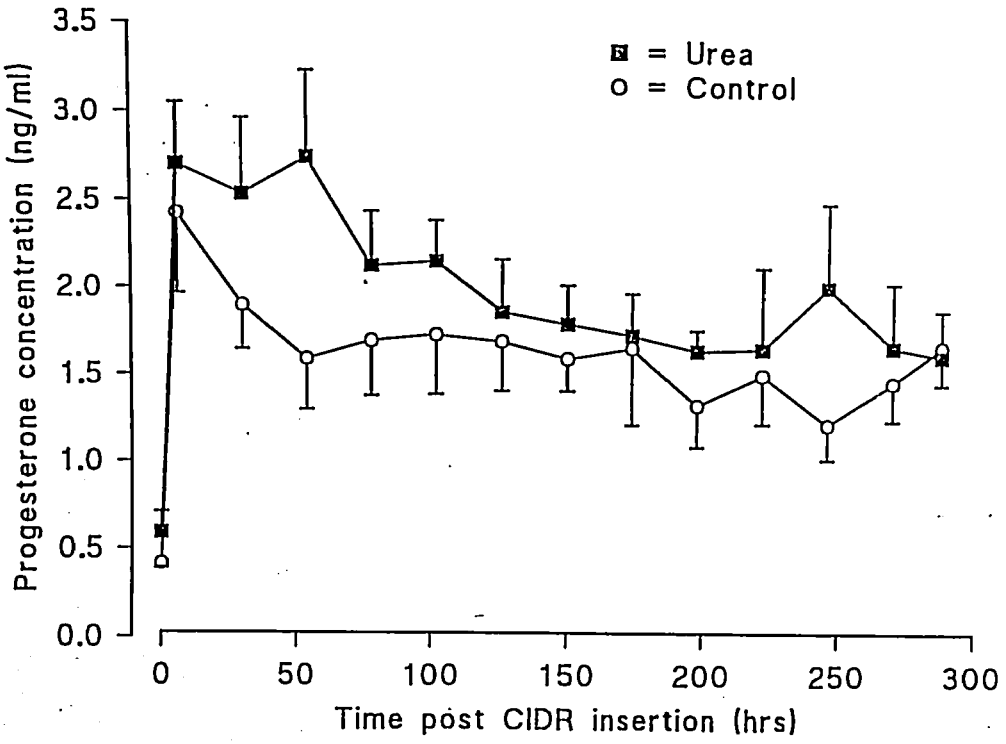

Figure 5. Progesterone profiles of urea and control fed ewes up to CIDR withdrawal.

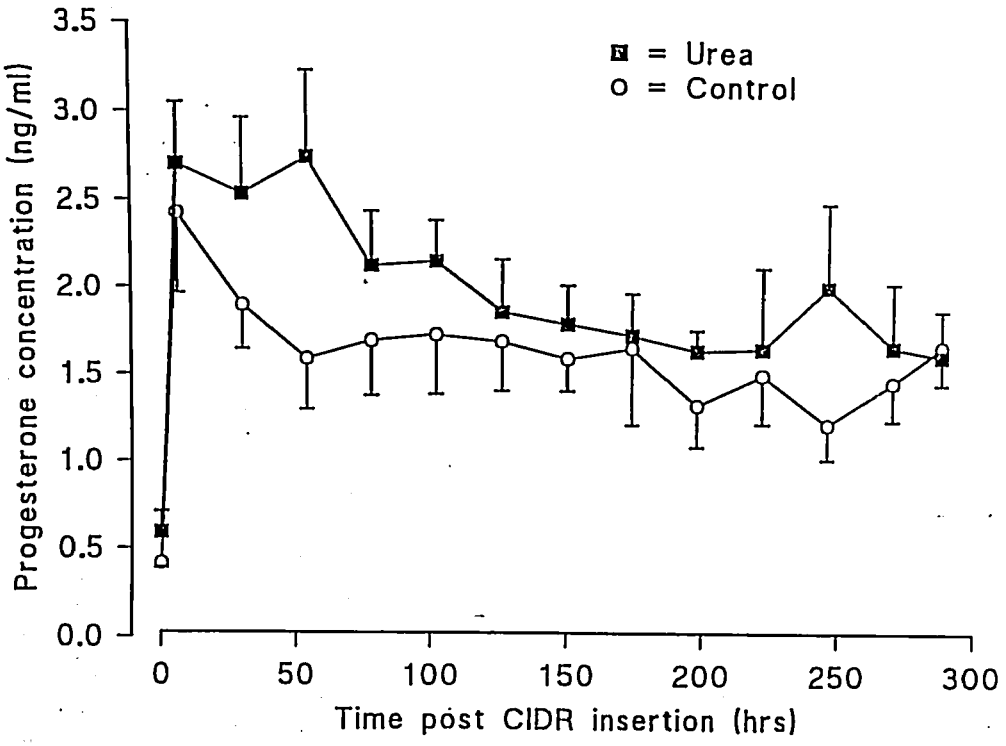

Figure 5. Progesterone profiles of urea and control fed ewes up to CIDR withdrawal.

significantly different ($p<0.001$). The insulin profiles are illustrated in Figure 6. There was a negative correlation ($r= -0.184$) albeit not significant between insulin levels and plasma glucose in control animals while in urea fed ewes it was positive ($r= 0.540$). For both treatments the correlation between insulin levels and urea concentration in the plasma during the priming period was small but positive ($r= 0.148$ and 0.106 for control and urea-fed ewes respectively (see Table 8).

Table 8: **Within treatment correlation coefficients (r) for a selection of measurements recorded around the time of ovulation.**

Parameters	Urea (r)	Control (r)
Time of Oestrous onset vs Time of LH onset	0.888	0.931
Time of LH onset vs ovulation rate	-0.488	0.255
Plasma insulin conc vs plasma glucose conc	0.540	-0.184
Plasma insulin conc vs plasma urea conc	0.106	0.148

5.6.0 Plasma Glucose

Plasma glucose levels were significantly different ($p< 0.001$) between treatments (3.64 ± 0.045 and 3.95 ± 0.053 mmol/l) for the control and urea-fed ewes respectively (see Table 9). Figure 7 depicts these differences over time.

5.7.0 Plasma Urea

The mean plasma urea concentrations for the two treatments were 2.41 ± 0.099 and 5.47 ± 0.152 mmol/l for the control and urea-fed ewes. The differences in urea concentrations between the treatments

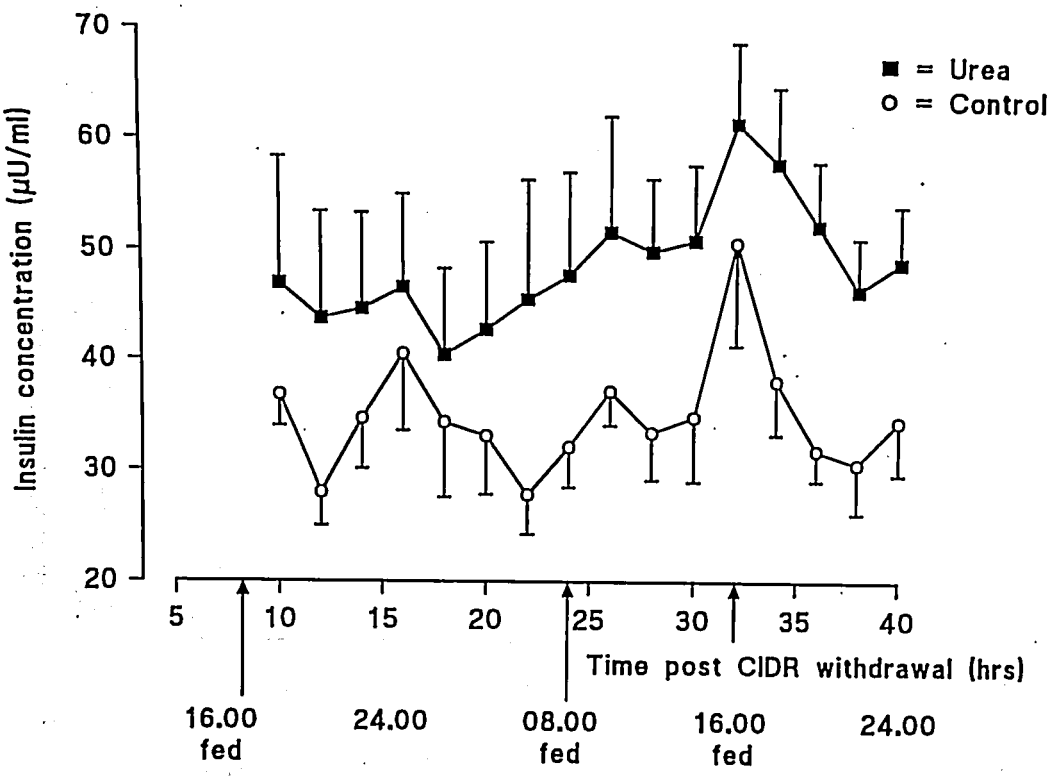

Figure 6. Plasma Insulin profiles of urea and control–fed ewes.

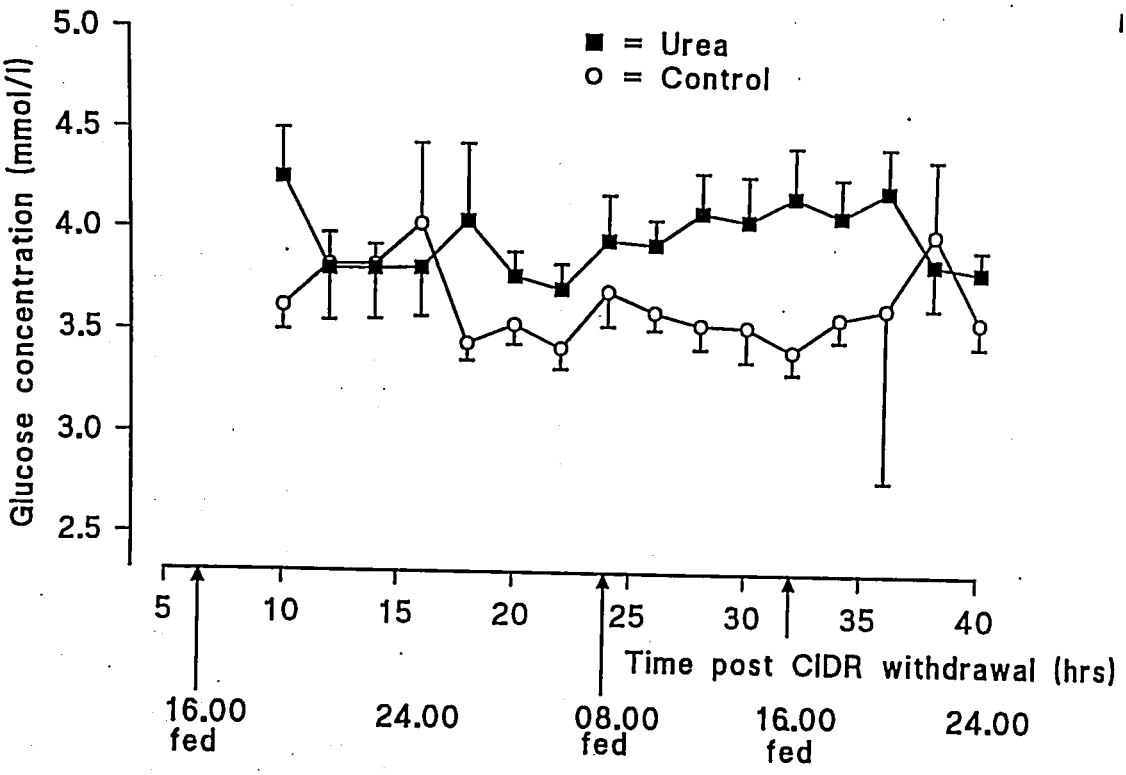

Figure 7. Plasma glucose profile of urea and control fed ewes.

were highly significant ($p<0.001$). The graph (Figure 8) shows the urea profiles over a 30-hour period and in relation to feeding times. The first sampling (16.00 hours) coincided with the urea levels on their decline for both the control and the urea-supplemented animals. However at this time the value was highest for the animals receiving urea, 7.12 ± 0.669 compared to 2.47 ± 0.459 mmol/l for the controls. The profiles for all the group exhibited a nadir after midnight, though this point came earlier (02.00 hours) and was smaller (1.59 ± 0.186) for the controls, compared to 04.00 hours and 4.66 ± 0.436 for the urea animals. Thereafter there was an increase in plasma urea levels for all the treatments which started a long time before the morning feed the following day. However the afternoon (16.00 hours) feed did not cause any obvious change in the decline of the graph as was seen in the 08.00 hours morning feed.

5.8.0 Plasma ammonia

The mean concentrations of plasma ammonia are shown in Table 9 and there was a significant difference ($p<0.01$) between urea-fed and control ewes. The ammonia levels before feeding were not different between the treatments but two hours post feeding the levels in the urea-supplemented animals had reached a peak of 145.2 ± 21.40 and 150.6 ± 21.30 μmol/l in the morning (08.00 h) and afternoon (16.00 h) respectively (see Figure 9).

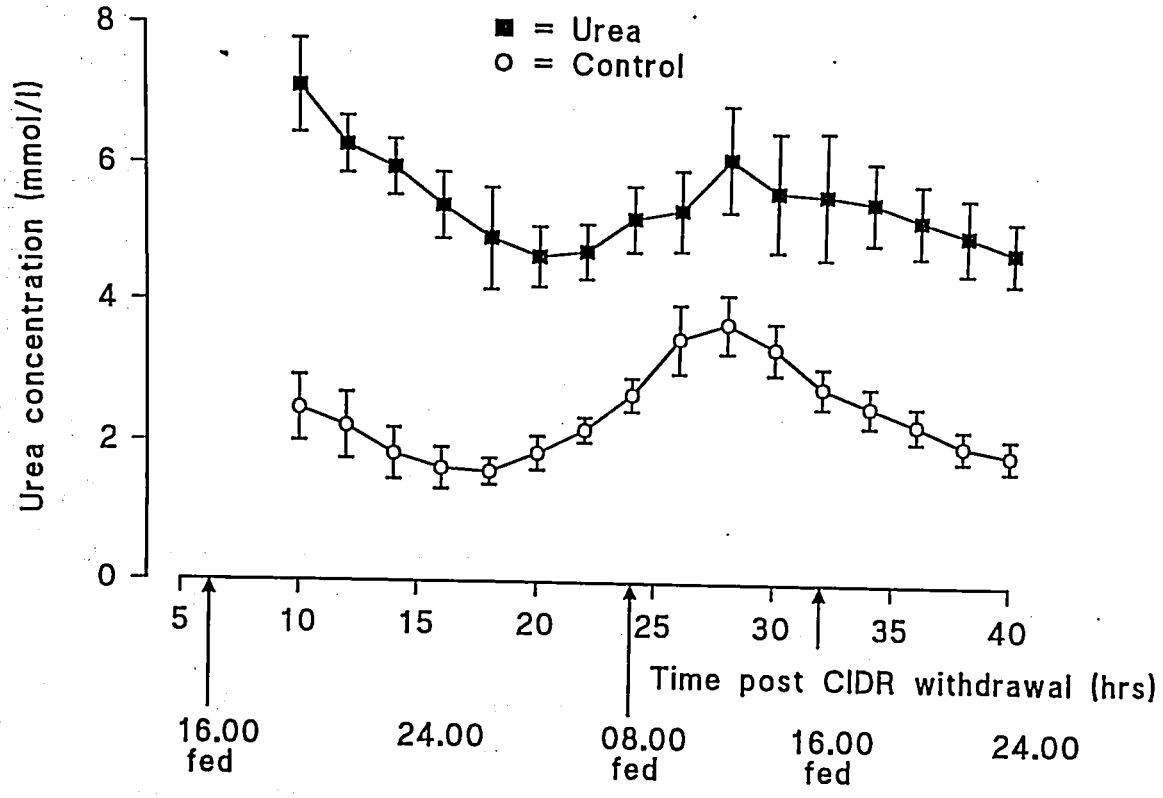

Figure 8. Plasma Urea profiles of urea and control fed ewes.

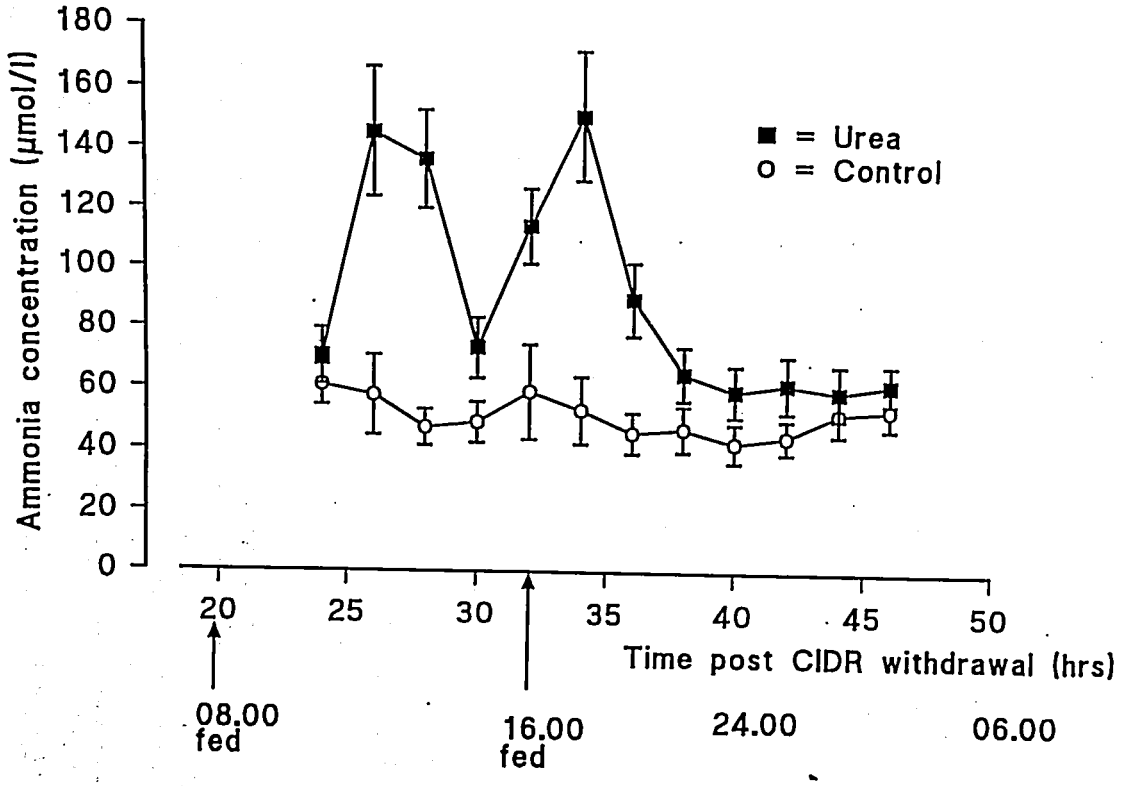

Figure 9. Plasma ammonia profiles of urea and control fed ewes.

Table 9: Treatment mean values (±SEM) for glucose, urea, ammonia and insulin.

Treatment	No.of ewes	glucose (mmol/l)	urea (mmol/l)	ammonia (μmol/l)	insulin (μU/ml)
Control	6	3.6±0.05	2.4±0.10	50.9±6.92	34.9±1.27
Urea	7	3.9±0.05	5.5±0.15	90.4±9.22	48.5±1.97
Overall	13	3.8±0.04	4.1±0.14	66.7±5.42	42.2±1.30

5.9.0 Oestrus onset

The mean time intervals between CIDR device removal and onset of oestrus were 18.4 ± 2.93 and 18.3 ± 1.82 hours for urea and control animals (see Table 7). There was no significant difference between the treatments ($p>0.5$). One animal in the control group did not exhibit oestrus and its LH level did not peak. This animal was omitted from the analysis.

5.10.0 Ovulation rate and embryo recovery rate

The ovulation rates were not significantly different ($p>0.5$) between the treatments. The mean numbers of embryos recovered were also not significantly different ($p>0.5$) between treatments (see Table 10).

Table 10: Effect of treatment on ovulation rate and the number of embryos collected at Day 3 after insemination.

Treatment	No.of ewes	Ovulation rate Mean±SEM	No. of embryos collected Mean±SEM	Recovery rate (%)
Control	5	6.5±1.41	4.3±1.20	68.1±13.50
Urea	7	7.0±1.09	4.4±1.19	57.6±14.30

5.11.0 Embryo developmental stages

The trend in the developmental stages of embryos over time (Time 0 = time of embryo recovery, Time 1 = 72 hours in co-culture and Time 2 = 96 hours in co-culture) shows that at the time of recovery embryos from the control ewes were less developed than those fed urea. However at Time 1 and 2 embryos from all the treatment groups were generally developed to the same extent. There was one embryo from a urea-fed animal that developed to the hatched blastocyst stage while the most developed from the control animals went only to an expanded blastocyst. Table 11 shows the percentage of embryos at specific stages of development for both the control and urea-fed ewes.

Table 11: Percentages of embryos at different stages of development at time of recovery and following 72 and 96 hours *in vitro* co-culture.

Stage	Control			Urea		
	time 0	72 hrs	96 hrs	time 0	72 hrs	96 hrs
<8 cell	21	13	15	10	7	9
8-32 cell	79	33	25	90	46	18
morula		50	45		43	50
Blastocyst		4	15		4	23
Total	100	100	100	100	100	100

5.12.0 Embryo quality and viability

a) Morphological assessment (stage and grade)

The system employed to categorise embryos into viable and non-viable was based on morphological development and the grade of the embryo. At Time 0, any embryo which had less than 8 cells or was degenerate was considered non-viable. Those embryos which were

below morula or degenerating at Time 1 (after 72 hours in co-culture) were taken as non-viable. At Time 2 (after 96 hours in co-culture) any embryo which had not passed the early-mid blastocyst stage was considered as non-viable. Empty zonas were not included in the analysis. Table 12 shows the proportions of non-viable and viable embryos at Time 0, 1 and 2 from the control and urea-fed ewes. There was a higher percentage of viable embryos from the urea (90%) than the control (81%) animals at Time 0. However, at Time 1 (72 h) there was a higher percentage of viable embryos from the control (59%) than from the urea-fed ewes (46%). After 96 hours (Time 2) in co-culture a higher percentage of embryos from the urea-fed animals were viable (23%) than from the control animals (17%). All these differences were however not significant ($p>0.5$) probably because the numbers of observations were small.

Table 12: Percentages of viable and non-viable embryos at the time of recovery and after 72 and 96 hours in co-culture.

Treatment	Viability	Time 0 (%)	72 hrs (%)	96 hrs (%)
Control	non-viable	18	41	83
	viable	81	59	17
Urea	non-viable	10	54	77
	viable	90	46	23

b) Glucose utilization

The mean values of $^{3}H_2O$ and $^{14}CO_2$ production show that the more advanced the embryo the higher its rate of metabolism. This is clearly indicated by the increasing amounts of metabolised $^{3}H_2O$ and $^{14}CO_2$ by an 8-16-cell embryo compared to empty zona (EZ) during the

first incubation (Table 13). The correlation between the amounts of $^{3}H_2O$ generated and stage of development was positive and significant ($r=0.80$, $p<0.05$). There was also a positive and significant correlation ($r=0.89$, $p<0.01$) between the amounts of metabolised $^{14}CO_2$ and the stage of development of the embryos. This trend showed up again after the embryos had been incubated for 72 hours in co-culture (Table 14). The corresponding correlations were $r=0.86$ and 0.90 and all significant at $p=0.01$. Interestingly the amounts of $^{3}H_2O$ and $^{14}CO_2$ generated by the 8-cell and 8-16-cell embryos from the urea-fed ewes were higher than those from the control ewes.

Table 13: Amounts of $^{3}H_2O$ and $^{14}CO_2$ generated by embryos of different stages of development immediately following recovery.

Stage	Treatment	n	$^{3}H_2O$ pmol	$^{14}CO_2$ pmol
EZ	control	2	0.0±0.00	0.0081±0.0081
<8 cell	control	3	0.70±0.351	0.022±0.0114
	urea	2	0.22±0.223	0.00±0.00
8 cell	control	5	1.05±0.356	0.039±0.0167
	urea	8	2.11±0.313	0.081±0.0136
8-16 cell	control	13	1.18±0.254	0.088±0.0495
	urea	14	2.15±0.281	0.083±0.0168

Statistical analysis based on a two tailed t-test with a pooled variance estimate was carried out to identify if there was any difference in metabolism of the viable and non-viable embryos from the control and urea-fed ewes.

Table 14: Amounts of $^{3}H_2O$ and $^{14}CO_2$ generated by embryos of different stages of development after 72 hours in co-culture

Stage	Treatment	n	$^{3}H_2O$(pmol)	$^{14}CO_2$ (pmol)
EZ	control	2	0.0±0.0	0.0±0.0
<8 cell	control	1	0.0±0.0	0.0±0.0
	urea	1	0.40	0.0±0.0
Degenerate 8-16 cell	control	1	8.32	0.19
	urea	1	0.00	0.0051
8-16 cell	control	4	7.7±2.92	0.21±0.071
	urea	2	4.3±4.21	0.14±0.136
16-32 cell	control	2	6.4±0.62	0.17±0.059
	urea	7	10.1±2.86	0.26±0.053
Morula	control	12	14.7±1.88	0.31±0.040
	urea	12	14.4±2.19	0.39±0.054
Late morula	urea	2	21.6±9.78	0.68±0.410
Early-mid blastocyst	control	1	36.5	0.87
expanded blastocyst	urea	1	61.1	1.25

The assays carried out on the day of embryo collection show a difference in the amount of $^{3}H_2O$ and $^{14}CO_2$ produced by viable embryos; ($p<0.05$ and $p<0.09$, respectively). Embryos from urea-fed ewes were more metabolically active than those from the controls. This was also the case with embryos classified as non-viable (see Table 15). There was a positive correlation between the amounts of $^{3}H_2O$ and the amounts of $^{14}CO_2$ produced by viable embryos ($n=28, r=0.470, p=0.012$). A highly significant positive correlation also existed between $^{3}H_2O$ and $^{14}CO_2$ production by the retarded non-viable embryos ($n=20$ $r=0.864, p<0.001$). Combining data from the two types of embryo (viable and non-viable) gave the correlation coefficient, $r=0.61$ ($p<0.001$). On the third day of *in vitro* culture there were no significant differences in the amounts of $^{3}H_2O$ and

$^{14}CO_2$ produced by embryos from the control and urea-supplemented ewes (viable and non-viable). The results are summarised in Table 16. However, there was a positive correlation between the amounts of $^{3}H_2O$ and $^{14}CO_2$ produced by the embryos; for viable, non-viable and total embryos, correlation coefficients were $r=0.917$ ($n = 28$, $p<0.001$); $r=0.953$ ($n = 22$, $p<0.001$) and $r=0.942$ ($n = 50$, $p<0.001$) respectively.

Table 15. Summary of metabolic assays carried out on the day of embryo collection.

	Source of Ova				
	Control Group		Urea Group		P
	n	pmol/ovum	n	pmol/ovum	
Viable ova					
$^{3}H_2O$ production	13	1.18±0.25	15	2.01±0.30	0.046
$^{14}CO_2$ production	13	0.042±0.009	15	0.078±0.017	0.087
Non-viable ova					
$^{3}H_2O$ production	10	0.733±0.232	10	1.729±0.354	0.030
$^{14}CO_2$ production	10	0.028±0.009	10	0.065±0.015	0.055
Total ova					
$^{3}H_2O$ production	23	0.98±0.18	25	1.90±0.23	0.003
$^{14}CO_2$ production	23	0.036±0.007	25	0.072±0.012	0.010

Table 16. Summary of metabolic assays carried out on the third day of *in vitro* culture.

	Source of Ova				
	Control Group		Urea Group		p
	n	pmol/ovum	n	pmol/ovum	
Viable ova					
^3H_2O production	13	16.4±2.41	15	18.48±3.69	0.65
$^{14}CO_2$ production	13	0.356±0.057	15	0.482±0.084	0.24
Non-viable ova					
^3H_2O production	10	5.20±1.57	12	6.62±2.13	0.61
$^{14}CO_2$ production	10	0.141±0.042	12	0.172±0.047	0.63
Total ova					
^3H_2O production	23	11.53±1.91	27	13.21±2.50	0.61
$^{14}CO_2$ production	23	0.262±0.043	27	0.344±0.059	0.28

Data reflect the production of ^3H_2O and $^{14}CO_2$ due to metabolism of radio-labelled glucose. Statistics are based on two-tailed t-test with pooled variance estimate.

The embryos from the last two donor groups were fixed, stained and their nuclei counted. The amounts of ^3H_2O and $^{14}CO_2$ produced by these embryos were then expressed per cell. Table 18 gives the number of cells/embryo and glucose metabolism products per cell. Regression analysis of "^{3}H/cell" versus the number of cells ($y=2.88 - 0.170x$; y = ^{3}H/cell, x = no. of nuclei) showed that the number of nuclei did not affect metabolism per cell ($r^2 =0.259$; $p>0.1$). The analysis of ^{14}C/cell versus the number of cells ($y = 0.0753 - 0.00451x$; y = ^{14}C/cell, x =no. of nuclei) also did not show any significant influence of the number of nuclei on the metabolism of ^{14}C/cell ($r^2 =0.347$; $p>0.1$).

When comparing treatments it was found that there was no difference between cells of embryos from control and urea-fed ewes in their capacity to generate $^{3}H_2O$ ($p>0.75$) and $^{14}CO_2$ ($p>0.85$ see Table 17 for Mean ±SEM).

Table 17. Mean values of ^{3}H/cell and ^{14}C/cell of embryos fixed 24 hours after the glucose utilization test.

Treatment	No. of embryos	^{3}H/cell	^{14}C/cell
Control	3	1.78±0.675	0.044±0.0204
Urea	5	1.43±0.779	0.039±0.0164

Table 18. Number of nuclei/embryo and the amount of $^{3}H_2O$ and $^{14}CO_2$ generated per cell.

Egg no.	Trt	No. of cells	$^{3}H_2O$ (pmol)	^{3}H/cell	$^{14}CO_2$(pmol)	^{14}C/cell
49		16	12.74	0.796	0.311	0.0194
50		13	5.06	0.389	0.119	0.0091
51	Urea	4	18.12	4.591	0.399	0.0999
52		5	2.88	0.577	0.221	0.0442
53		6	5.20	0.866	0.119	0.0198
45		5	5.82	1.163	0.113	0.0226
46	Control	8	8.32	1.040	0.188	0.0235
47		5	15.62	3.123	0.422	0.0843

c) Protein synthesis

The rate of protein synthesis (^{35}S-Methionine incorporation) by the embryos was assessed and counts per minute (CPM) due to amino acid incorporation into the acid insoluble protein fraction of the viable embryos are presented in Table 19. The rate of protein synthesis by viable embryos was not different for the two treatment groups ($p>0.78$). The same was true for the non-viable embryos.

Table 19. Summary of the protein synthesis assay carried out on the fourth day of *in vitro* culture.

	Source of Ova				
	Control Group		Urea Group		
	n	c.p.m./ovum	n	c.p.m./ovum	p
Viable ova ^{35}S-Met incorporation	12	9280±4150	11	11820±8030	0.78
Non-viable ova ^{35}S-Met incorporation	7	2170±1090	6	802±292	0.29
Total ova ^{35}S-Met incorporation	19	6660±2730	17	7930±5270	0.83

Data represent counts per minute (c.p.m). due to the incorporation of ^{35}S-Methionine into the acid-insoluble protein fraction of each ovum. Counts were based on 10% of the total sample, with remaining material used for 1-D gel electro-phoresis. Statistics based on two-tailed t-test with pooled variance estimate.

Regression analysis was carried out on the rate of protein synthesis (96 hrs following *in vitro* culture) and the amount of radioactive H_2O and CO_2 generated during the second metabolic assay. The analysis was carried out separately for viable and non-viable embryos then combined for all the embryos. The equations are given below and in general metabolic activity was positively correlated with protein synthesis.

^{35}S-Methionine incorporation = y, $^{3}H_2O$ =x_1 and $^{14}CO_2$ = x_2

Viable embryos; $y = -13410 + 1350x_1$ (r^2 =0.590, p<0.001)

(n=23) $y = -9060 + 45600x_2$ (r^2 =0.369, p=0.002)

Non-viable embryos; $y = 325 + 333x_1$ ($r^2 = 0.331$, $p = 0.040$)

(n=13) $y = 248 + 11980x_2$ ($r^2 = 0.369$, $p = 0.028$)

All embryos; $y = -6280 + 1070x_1$ ($r^2 = 0.552$, $p < 0.001$)

$y = -4640 + 38030x_2$ ($r^2 = 0.382$, $p < 0.001$)

d) 1-D gel electrophoresis

Plate No 2 shows the one-dimensional image of ^{35}S-labelled polypeptides of embryos derived either from control (lanes 5 and 8) and urea (lanes 1, 2, 3, 4, 6 and 7) diet. Quantitative differences between the lanes (i.e, in the overall intensity of the different profiles) are due mainly to differences in the amounts of radio-activity originally present in the samples (range = 0 to 82,000 c.p.m.). Samples 1 and 2 contained less than 1,000 c.p.m. per lane, and only a very faint band is visible (M_r= 47,000 approx.). Samples 3 and 6 contained no radio-active material and are blank. Lanes 5 and 7, with 47,600 and 81,700 c.p.m., respectively, show the strongest banding patterns but no treatment-related differences are detectable. Lane 8 contains 14,250 c.p.m. but no bands are detectable. Overall, there is no indication that diet influenced the polypeptide profiles. Note that the gel was originally exposed to film for just 3 days and this preliminary illustration is therefore quite faint. Longer exposures are now being used to enhance the image.

5.13.0 Oviductal fluid

a) Glucose concentration

There was no detectable amounts of glucose in oviductal fluid of all treatment groups.

b) pH

The pH of the oviductal fluid was assessed in the first 8 animals in the experiment. For both treatments the pH was 7.5 for the flushing derived from the oviduct or horn.

A small experiment was set up to investigate the buffering capacity of PBS since it was suspected that lack of effects could have been caused by PBS. Ammonium Chloride (NH_4Cl) was weighed out (53.49 mg) and dissolved in either one litre distilled water or one litre PBS. Serial dilutions were then made to give solutions with NH_4Cl concentrations as follows; 0, 75, 300, 600, 900, 1000 μM. Table 13 shows the change in pH and the effect of PBS as NH_4Cl concentration increases. From these results it seems likely that PBS was able to buffer the effects of ammonium chloride and this would explain the lack of any treatment effect on pH.

Table 20. pH values of NH_4Cl of different concentrations either in water or PBS

Concentration (μM)	Water (pH)	PBS (pH)
0	6.62	7.44
75	6.96	7.44
300	6.13	7.45
600	5.86	7.44
900	5.63	7.44
1000	5.78	7.47

c) Urea Concentration

The mean urea levels in the oviductal fluid of the left side of the uterus were 0.174 ± 0.049 and 0.543 ± 0.107 for the control and urea animals. These were significantly different ($p<0.025$). The

difference in the right side was less significant ($p<0.5$), 0.195 ± 0.055 and 0.286 ± 0.091 mmol/l for the control and urea animals. The overall uterus urea concentrations for the urea-supplemented and the control ewes were significantly different ($p<0.05$) (see Table 14)

d) Ammonia Concentration

The mean oviductal fluid ammonia levels for the treatments were significantly different ($0.1>p>0.05$), 32.72 ± 6.49 for the control as compared to 65.4 ± 13.5µmol/l for the urea treatment for the left side of the uterus. For the right side there were 30.37 ± 6.56 and 61.7 ± 15.4 µmol/l for the control and the urea fed animals respectively. Again there was a significant differences ($0.1>p>0.05$). The overall ammonia concentrations from the uterus were significantly different ($p<0.025$), as shown by the mean values, 35.7 ± 4.30 µmol/l and 63.6 ± 7.50µmol/l for the control and urea-fed ewes (see table 14) In all cases the ammonia values were variable.

Table 21. Mean ± SEM oviductal fluid urea and ammonia concentrations

Treatment	No. of ewes	Left/Right	Urea (mmol/l)	Ammonia (µmol/l)
Control	6	left	0.17±0.049	32.3±6.49
		right	0.10±0.055	30.4±6.59
		left+right	0.19±0.044	35.7±4.30
Urea	7	left	0.54±0.107	65.4±13.50
		right	0.29±0.913	61.7±15.40
		left+right	0.41±0.084	63.6±7.50

Left/Right* = side of the uterus

e) Insulin concentration

Insulin was not detected in oviductal fluid, probably due to the minute amounts that were below the detection limits of the assay.

5.14.0 Somatic oviductal epithelial cells (SOEC)

a) Protein synthesis (^{35}S-Methionine incorporation)

The results from the gels showed that although there were qualitative differences (i.e, changes in the banding patterns) between the lanes (urea-fed versus control), there was no indication that diet consistently influenced the polypeptide profiles. Quantitative differences between the lanes (i.e in the overall intensity of the different profiles) were due mainly to differences in the amounts of radio-activity originally present in the samples (range = 28 000 to 120 000 c.p.m. (see Plate 1)

b) Secretory proteins (1-D gel electrophoresis)

At time the of writing, gels containing secreted proteins had not yet emitted a sufficient strong signal to provide a clear banding pattern on autoradiography films. Exposure time will be extended as necessary.

5.15.0 Co-culture medium (M199 + cells)

Samples from co-culture medium used to incubate embryos were analysed for ammonia, urea and glucose. Data for the amounts of ammonia, urea and glucose following 72- and 96-hour co-culture are given in Tables 22 and 23. There were no differences in the amounts of ammonia, urea and glucose in the media following the culture of

an embryo from a urea-fed ewe as opposed to a control ewe. In contrast the well that was used as reference (ie medium never replaced up to the end of incubation) had high levels of ammonia and urea. This observation is consistent with the results of a recent study by Gardner and Lane (1993) and indicates that long term co- culture without frequent media changes leads to a build up of the end-products of amino acid breakdown, presumably as the result of metabolism by the oviductal epithelial cells.

Table 22: Urea, ammonia and glucose concentrations in culture medium following 72-hour co-culture (1 ovum/well)

Treatment	No. of embryo	Stage	ammonia (μmol/l)	Urea (mmol/l)	Glucose (μU/ml)
	2	EZ or<8c	495±3.4	0.8±0.12	6.1±0.02
	4	8-16c	403±22.6	0.9±0.06	6.6±0.26
	8	16-32c	371±17.6	0.9±0.07	6.9±0.19
Urea	11	morula	373±43.8	0.8±0.06	5.9±0.13
	2	l.morula	272±193	0.7±0.07	6.5±0.38
	1	ex.blast	452	0.8	5.5
	2	EZ or<8c	443±20.4	0.9±0.02	6.1±0.12
	3	8-16c	393±3.6	0.9±0.00	6.8±0.23
	5	16-32c	463±38.2	0.9±0.59*	7.0±0.06*
Control	12	morula	404±143	0.8±0.05	6.0±0.15
	1	E-M blast.	385	1.0	7.2
Well 4 (0 hrs)	0 (n=18)		126±4.21	0.6±0.03	6.4±0.18
Well 4 (72hrs)	0 (n=18)		426±13.7	0.9±0.3	6.4±0.16

*no. of observations = 4
EZ = empty zona pellucida
E-M = early-mid
c = cell

Table 23. Urea, ammonia and glucose concentrations in culture medium following 96-hour co-culture (Except for Well 4 samples, medium was replaced at 72 hrs)

Treatment	No of embryos	Stage	ammonia (μmol/l)	urea (mmol/l)	glucose (mmol/l)
Urea	2	EZ or<8c	524±271	0.9±0.38	5.9±3.4
	2	8-16cell	272±41.6	0.8±0.19	6.5±0.66
	3	16-32c	290±2.5	0.7±0.06	7.1±0.07
	9	morula	310±17.7	0.8±0.04	6.1±0.12
	1	late morula	388	0.8	6.3
	1	E-M blast.	267	0.6	5.9
	1	hatched blast.	841	1.2	6.1
Control	2	EZ or<8c	281±14.4	0.8±0.03	6.2±0.77
	2	8-16c	269±4.9	0.8±0.00	6.9±0.02
	10	morula	348±59.5	0.8±0.06	6.1±0.19
	1	late morula	409	0.9	5.7
	2	E-M blast.	333±61.5	0.9±0.23	5.9±0.18
	1	expanded blast.	348	1.11	5.8
Well 4 (96hrs)	0 (n=14)		556±100	0.9±0.08*	5.9±0.22*

EZ= empty zona pellucida
E-M blast. = early mid blastocyst
* no of observations = 13

6.0.0 DISCUSSION

6.1.0 Diets

The energy intakes were estimated to maintain the liveweights of the ewes. Rumen degradable protein (RDP) was however in excess of microbial needs for both the control and the urea-supplemented ewes. The excess RDP in the control diet was so small (3 g/feed) as compared to the urea-supplemented diet (136 g/feed) that it was highly unlikely to adversely affect the parameters investigated. The complete diet given to the ewes before the start of the study had an excess RDP to the extent of 29 g/day. Previous studies at the Rowett Research Institute showed no evidence of a detrimental effect on embryo survival of this diet. Anyway in the present study the reproductive parameters were measured 12 days after the animals were taken off the complete diet, and it is highly improbable that effects, if they did occur at the time of feeding, would have carried over to ovulation, fertilization and early embryonic development.

6.2.0 Body weights

Both groups of ewes lost some weight during the experimental period. This loss was small and was not significant between the control and treatment groups.

6.3.0 Progesterone

Central to the maintenance of pregnancy in animals is the hormone progesterone. According to Ashworth et. al.(1989) embryo survival is associated with the rate at which progesterone concentration

increases and the time at which the concentration changes from periovulatory to luteal values following ovulation time. Even after conception progesterone is still critical to the embryo survival, especially during the period of conceptus elongation. At this time progesterone is needed for maintenance of a uterine environment conducive to embryo development (Wallace et. al.1994). Dietary effects have been found to interfere with the natural production of progesterone (Williams and Cumming 1982, McKelvey and Robinson 1986 and Parr et. al. 1994) and with artificially supplied progesterone (McEvoy et. al. 1993) in the ewe. The present study shows a difference in the plasma progesterone concentrations between treatments. Although the mean values, (1.55±0.15 and 1.91±0.12 ng/ml for the control and urea-fed ewes respectively) were close to the threshold for normal pregnancy maintenance, those from the urea-supplemented animals were consistently higher.

There has been speculation that abnormal development of follicles could result in corpora lutea which are inadequate. These in turn produce low amounts of progesterone (Ashworth et. al 1989). In the present study, the urea-supplemented diet had high levels of RDP but it seems it did not compromise circulating progesterone. In contrast high plane feeding suppresses progesterone concentration (McKelvey and Robinson 1986, McEvoy et. al 1993). Although Jordon et al (1983) observed differences between progesterone levels of animals fed high (23%) and those fed a low (12%) crude protein diet during the oestrous cycle, the differences were not significant. In contrast to the results of

the present study it was the cows given the low crude protein diet that had high progesterone levels. Carroll et al (1988) and Elrod and Butler (1993) did not find any difference in the progesterone levels after dairy cows were fed diets having low or high protein contents. In the present study progesterone levels at the time of CIDR device withdrawal were 1.65 and 1.60 ng/ml for the control and urea-supplemented ewes. This may imply that at this critical period, preceding ovulation, urea may not compromise fertility through progesterone production. If urea and its metabolites do have an effect on progesterone then the effects may be manifested at the time when progesterone concentration change from periovulatory to luteal values. According to Ashworth et al (1989) this period is critical for the changes in the uterus that is required for embryo development and survival. The data from the present study do not cover this period, hence it can not be verified if there were dietary-induced alterations in progesterone at this time.

6.4.0 Periovulatory LH

The onset of the LH peak was not different for the two treatments but was positively correlated to oestrous onset time in both groups. It has been proposed that high levels of urea or ammonia in animals fed high levels of protein may interfere with LH binding to its receptors (Jordon et. al. 1983); thus similar concentrations may create different physiological responses. Based on his experimental findings, Bishonga (1993) proposed that high circulating urea and ammonia concentrations do not have an effect on the timing of the activation of the gonadotrophin

releasing hormone (GnRH) pulse generator or the levels of LH released from the pituitary. There was no significant correlation between the onset of the LH peak and ovulation rate for either the control or urea-supplemented ewes in the present experiment thus indicating that the periovulatory events were not affected by the high urea supplement; neither was the subsequent ovulation rate. The lack of an effect on ovulation rate contrasts with the findings of Bishonga (1993) that high urea tended to suppress ovulation rate. The reason for the difference may be that in the present study the animals were given a superovulatory dose of p-FSH whereas those in the Bishonga (1993) study received only mild ovarian stimulus in the form of PMSG. This created ovulation rates of 2.4±0.37 and 4.4±0.72 for "urea" and "control" ewes in the Bishonga experiment compared with corresponding values of 7.0±1.09 and 6.5±1.41 in the present study.

6.5.0 Treatment effects on plasma insulin and glucose

Major shifts in energy metabolism occur when there is a short supply of energy-yielding nutrients. Insulin is the principal hormone involved in these shifts (Brockman and Laarveld 1986). There was a treatment effect on both insulin and glucose in the plasma between 10 and 40 hours after CIDR device removal. The concentrations of insulin and glucose were high in urea-supplemented ewes compared to the controls. The concentrations of insulin and glucose from the "urea" ewes were positively correlated ($r=0.54$) though not significantly so ($p>0.5$) For the control animals, insulin and glucose levels were negatively correlated, albeit not significantly. This relationship between

insulin and glucose levels in urea-supplemented ewes is out of line with normal trends. Insulin has hypoglycaemic effects and in ruminants an increment in plasma insulin causes a reduction in plasma glucose (Weekes et.al. 1983, Brockman and Laarveld 1986). It appears that in this study glucose was less responsive to insulin in urea-fed ewes. In the control animals the increase in insulin concentrations was always lagging behind those for glucose concentrations, reaching the highest values 1 to 2 hours after feeding when glucose production was high.

The correlations between urea concentrations and insulin were positive for both the control and urea-supplemented ewes. According to Visek (1984) ammonia elevates insulin levels and thus observations recorded in the present are in line with the elevation in plasma ammonia that occurred immediately following feeding in the urea-supplemented ewes. Prior and Visek (1972) found that an increase in peripheral blood ammonia of rats given an injection of urease closely parallelled the rise in blood glucose. The difference in the relationship between insulin and glucose levels between the two groups even when urea levels were still positively correlated to insulin levels in the control animals, was due to the fact that urea levels in the controls were about half those of the urea-fed ewes. This may imply that the mechanism for controlling glucose concentrations in plasma may have been impaired by urea and ammonia.

6.6.0 Treatment effects on plasma urea and ammonia

Excess ammonia produced in the reticulo-rumen is absorbed

directly and its levels in the plasma reflects the status of the RDP in the diets in relation to microbial needs. The amount of ammonia converted to urea by the liver also reflects the nature of the diet entering the rumen. Since ammonia can diffuse across the peritoneal cavity to the peripheral circulation without passing through the liver (Chalmers et. al. 1971) its levels in the plasma may not necessarily mean that the protein in the diet is highly fermentable. In the present study urea and ammonia levels were significantly higher in "urea-fed" than "control" animals (2.41 versus 5.47 mmol/l urea and 50.9 versus 90.4 µmol/l ammonia). This is understandable since excess RDP from the urea-supplemented diet was in the order of 136 g/day compared to 3 g/day in the control diet. The profiles of plasma urea clearly show this trend and the only time the urea levels of control animals rose was after feeding; otherwise there were consistently lower than for the "urea-fed" animals. This trend in the plasma urea profiles is similar to that found by Bishonga (1993) whereby the inadequacy of RDP in the control diet caused a continuous decline in urea concentration from the morning feeding through to the afternoon feeding, implying urea recycling to the rumen where it would be used to supply nitrogen for the rumen microbes.

The plasma ammonia concentration for the "urea-supplemented" ewes reached a peak 2 hours after feeding and remained elevated for about 5 hours before falling. Since the feeding times were consistent throughout the experimental period, it is assumed that these profiles represent what happened during the experimental

period. If that is the case it would suggest that urea and ammonia will eventually reach the reproductive tract of the ewes. Elrod and Butler (1993) attributed low fertility in dairy cows fed high levels of RDP to toxic effects of ammonia due to its alteration of the uterine pH. In the present study follicle maturation, egg and sperm transport along the oviduct and subsequently the conceptuses could be exposed to a deleterious environment. Mammalian cells can tolerate only a modest level of ammonia because of its disruption of intracellular pH (Visek 1978). The mechanism(s) by which urea or ammonia influence embryo survival is only speculative and may include other factors not investigated in this study such as growth factors from the oviducts and uterine endometrium.

6.7.0 Ovulation rate and embryo recovery rate

Ovulation rate can be stimulated in ewes by an increase in the level of nutrition (Robinson 1990). However on the basis of the results of the present study this does not hold true for RDP as was also the case in the study by Bishonga (1993), where ewes fed on low and high levels of urea had ovulations rates that were 27% and 45% respectively lower than those of the controls. In contrast to the detrimental effect of urea on ovulation rate (Bishonga 1993), there was no difference in ovulation rate between the "urea-supplemented" and control ewes; this may reflect the obliterating effect on ovarian response of the superovulatory regimes used in the present experiment. Other studies (Creed 1993, McEvoy et. al 1993) have shown that the normal plane-of-nutrition response on ovulation rate that is

observed for spontaneously-ovulating ewes, is obliterated by the administration of the superovulatory amounts of exogenous gonadotrophins used in the present experiment.

The numbers of embryo recovered on Day 3 after insemination were not significantly different between the two groups ($p>0.5$). In this study it seems the urea-supplemented diet did not affect the function of the fimbriated infundibulum as was suggested by Bishonga (1993). It appears that neither did it affect oviductal function and sperm transport. Rather, if the high-urea treatment had any effect on the ova then it must have occurred during oocyte maturation and was not of the nature that would impair ova transport or fertilization. According to Visek (1984) ammonia may have biphasic functions in biological systems. It may initially increase the rate of certain biochemical reactions. These may be detrimental because of exaggerated growth and there is no reason why the embryo should be isolated from this phenomenon. This implies that detrimental effects of urea or ammonia on the embryo should not be taken only to mean slowing down of chemical reactions or retarded growth but rather accelerated growth leading to disruption of the normal temporal expression of biochemical reactions. At certain concentrations of ammonia some embryos may be selectively "encouraged" by urea/ammonia to grow faster than normal and end up as viable and highly metabolically active embryos at recovery time (Day 3). This may include those embryos that would be retarded under normal conditions. This would explain the tendency for embryos from ewes receiving the "urea-supplemented" diet to be more developed than those from

ewes in the control diet when recovered on Day 3 following insemination.

6.8.0 Effects of treatment on developmental stages and viability at the time of embryo recovery and after 72 and 96 hours of *in vitro* co-culture.

The embryos were assessed and categorised according to stages of development (Scudamore 1991) and grades were assigned based on a 1-5 scale (Lindner and Wright 1983). Although this system is subjective, if consistency is maintained and assessment is made without any knowledge of which treatments the embryo emanate from, as was the case in the present study, it gives important information. In the present study it was found that at the time of embryo recovery 79% of the embryos from the control ewes fell in the class 8-32-cell as opposed to 90.1% from the urea-fed animals. This may be indicative of an initial selection for rapid growth by those subjected to a high urea/ammonia environment. After 72 and 96 hours of *in vitro* co-culture with oviductal cells from control ewes the same trend was observed; ie higher proportions of embryos which were more developed originated from the urea-supplemented ewes. It seems therefore that the initial stimulus to development, which in the longer term would have had a detrimental effect on a high proportion of embryos if they had remained for a longer time (to Day 4) in their *in vitro* environment (Bishonga 1993), remained as a permanent feature when they were transferred to the *in vitro* co-culture environment. This is particularly interesting in view of the findings of

Bishonga (1993) that although a high proportion of embryos from ewes receiving the same "high-urea" diet as those in the present experiment failed to survive following 4 days of exposure (ie 24 h longer than in the present study) to the high "urea/ammonia" environment of the oviduct, those that did went on to produce over-sized lambs at birth (Bishonga et. al. 1994). There is therefore strong evidence in the in vitro culture studies to support the idea that a transitory exposure to a "high ammonia" in vivo environment may enhance the metabolic activity of the embryo and provided it is recovered from this environment before any permanent damage is caused, it may continue to express increased levels of metabolism. In the present study it was found that the amounts of urea and ammonia from the uterus of urea-supplemented ewes were about double those from the control animals; 0.414±0.084 mmol/l and 63.6±7.50 μmol/l compared to 0.185±0.044 mmol/l and 35.7±4.30 μmol/l). This shows that indeed the embryos from urea-fed ewes were exposed to a "high urea / ammonia" environment.

6.9.0 Treatment effects on glucose metabolism and protein synthesis by the embryos.

Kane (1987) highlighted the need for more objective yet non-injurious methods of evaluating embryos. At that time it was thought that there was no routine biochemical assay that could be carried out on either the embryo or its culture medium to assess its viability. Since then Rieger (1992) suggested that measurement of embryonic metabolic activity would be a reasonable approach. More recently a study by Bishonga (1993) showed that

there was a positive correlation between the stage of development of the embryo and its rate of protein synthesis. In that study, (Bishonga 1993), it was also shown that the viability of embryos (based on grade scale of 1-5, with increased degeneration from 1 to 5) was negatively correlated to ^{3}H-phenylalanine incorporation. In the present study it was also found that the stage of development of the embryo was correlated to its metabolic activity (ie glucose metabolism and protein synthesis). For example there was a positive correlation between stage of development and ^{3}H (r= 0.804) and ^{14}C (r=0.892) metabolized by the embryos on the day of embryo collection. The corresponding values for the second metabolic assays were r=0.864 and 0.901. Regression analysis also showed that the rate of protein synthesis by the embryo was correlated with metabolic activity. These results confirm the idea that the metabolic activity of embryos can be used to assess their quality and viability.

As well as investigating the possible usefulness of metabolism as an index of embryo quality, the present study also assessed the effects of ammonia on embryonic development and biochemical activity. From the morphological assessment of the embryos at the time of recovery (Day 4) and following in vitro culture it had been observed previously that ammonia influences embryo development (Bishonga et. al. 1994). It was thus hypothesised that high "ammonia" alters embryonic metabolism. After their study with rats, Prior and Visek (1972) suggested that ammonia alters the TCA cycle metabolism through increased flux of glucose through the glycolytic pathway. In the present study it was found

that plasma glucose levels in urea-fed ewes were higher (3.95±0.053 versus 3.64±0.045) than those of the control ewes. Rieger (1992) suggested that the increase in glucose metabolism by the embryo occurs at or about the time of activation of the embryonic genome (8-16-cell). Prior to this stage high concentrations of glucose are detrimental to embryo development in that they lead to free radical formation and for this reason early cleavage-stage embryos rely on pyruvate as their energy source.

In the present study it was observed that there was a small number of embryos from the "urea-supplemented" ewes that were well advanced in their development. Overall, embryos from this treatment were more developed than those from the control ewes on the day of recovery (Day 3). There was one particular embryo from a urea-fed ewe that reached a hatched blastocyst following 96 hours in culture. This embryo had previously (following 72 hours in culture) generated the highest amounts of $^{3}H_2O$ and $^{14}CO_2$, (61.1 and 1.25 pmol) compared to the most developed embryo from the control ewe which gave corresponding values of 36.5 and 0.87 pmol. This observation may be pointing to the same phenomenon as was observed by Bishonga et al(1994) in which the small number of embryos that survived the detrimental effects of a "high ammonia" environment went on to produce over-sized lambs at birth.

In the present study the close association between $^{3}H_2O$ and $^{14}CO_2$ metabolism would appear to indicate that urea did not enhance the

activity of one pathway (pentose phosphate versus the glycolytic pathway) over the other (see Figure 2, Section 2.3.1). Since this is the first attempt to test the effect of a dietary-induced alteration to the environment of early-cleavage stage embryo on their metabolism there are no other observations with which to compare the present findings. However, in whole animal (rat) studies a high circulating concentration of ammonia has been shown to enhance the metabolism of glucose-6-^{14}C to $^{14}C_2O$ (Prior and Visek 1972). Clearly additional research on the possible influence of both in vivo and in vitro ammonia concentrations on the relative contributions of the pentose phosphate and glycolytic pathways to the energy metabolism of early-cleavage-stage embryos is warranted, as it may well be linked to other key features (eg protein synthesis) of the embryo.

7.0.0 Conclusion

1. The high correlation between metabolic activity of embryos and their stage of development implies that biochemical activities may be a useful objective method for assessing their viability.
2. In vitro culture medium for embryos exposed to long-term culture (IVM and IVF embryos) and destined for embryo transfer may need to be replaced frequently to avoid toxicity resulting from an increase in the concentration of ammonia. This is particularly interesting in view of the fact that "IVM/IVF" embryos can lead to foetal oversize, an observation that is not inconsistent with the initial stimulatory effect of ammonia on embryonic metabolism in the present study and with the oversized

lambs produced from the small number of embryos that survived the "high-urea" treatment imposed by Bishonga et al (1994).

3. Urea/ammonia do not influence embryo survival through an alteration in either progesterone or LH activity.

4. The alterations in plasma insulin and glucose however may mean that the effects of high-urea feeding may be manifested through changes in energy metabolism. Although there was no evidence of altered rates of $^{3}H_2O$ and $^{14}CO_2$ generation relative to each other by the embryos at their time of recovery (Day 3 after insemination) it is possible that the shifts in glucose flux through the TCA cycle occurred earlier than Day 3.

5. The metabolic changes induced by "high-urea feeding" may be a function not only of ammonia concentrations in plasma, the oviduct and uterus but duration of exposure may be critical with the initial period of exposure being stimulatory and longer term exposure leading to toxic effects on development.

7. The fact that ammonia can diffuse to other parts of the body, including the reproductive tract, without passing through the liver, may mean that urea levels in diets which otherwise would be considered moderate, may alter embryonic development without creating any clinical symptoms in the mother.

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