

INCORPORATION OF  $^3\text{H}$ -URIDINE BY PIG BLASTOCYSTS *IN VITRO*

by

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## CHAPTER I

## INTRODUCTION

Development of the mammalian conceptus can be divided into three stages: the zygote, the embryo and the fetus. During pig embryogenesis, the zygotic period begins with fertilization and ends 10 days later, at which time the germ layers and fetal membranes are formed. The embryonic period then follows, extending to one month after fertilization, and is succeeded by the fetal period which terminates at parturition, an average of 114 days after mating. In practice, the term embryo is often used to describe the conceptus from the time of fertilization to the fetal period and even beyond. The zygotic period can be further subdivided into cleavage and blastocyst stages (figure 1). Cleavage of the fertilized egg occurs over the first 6 days of development, producing a round, compacted aggregate of 8 to 16 cells at 5 days (Hunter, 1974). This morula is enclosed within the zona pellucida. As the cells continue to divide, a central fluid-filled cavity is formed producing a blastocyst at 6 to 7 days (Heuser and Streeter, 1929). At about 7 days the zona pellucida is shed and the blastocyst continues to grow. At 10 to 12 days of gestation the embryo begins attaching to the endometrium of the uterus, and by day 18 the elongated embryo has implanted (Amoroso, 1952). At this time the trophoblast has begun differentiating into the placenta, and the inner cell mass (ICM), visible at 9 days, has initiated formation of most of the organ systems.

Many investigations involving a large number of animals indicate that not all embryos in a pregnancy survive to term. Embryonic mortality in pigs appears to be a general, but yet unexplained phenomenon. At 25 days of pregnancy between 30 and 40% of the potential embryos have been lost (Hanly,

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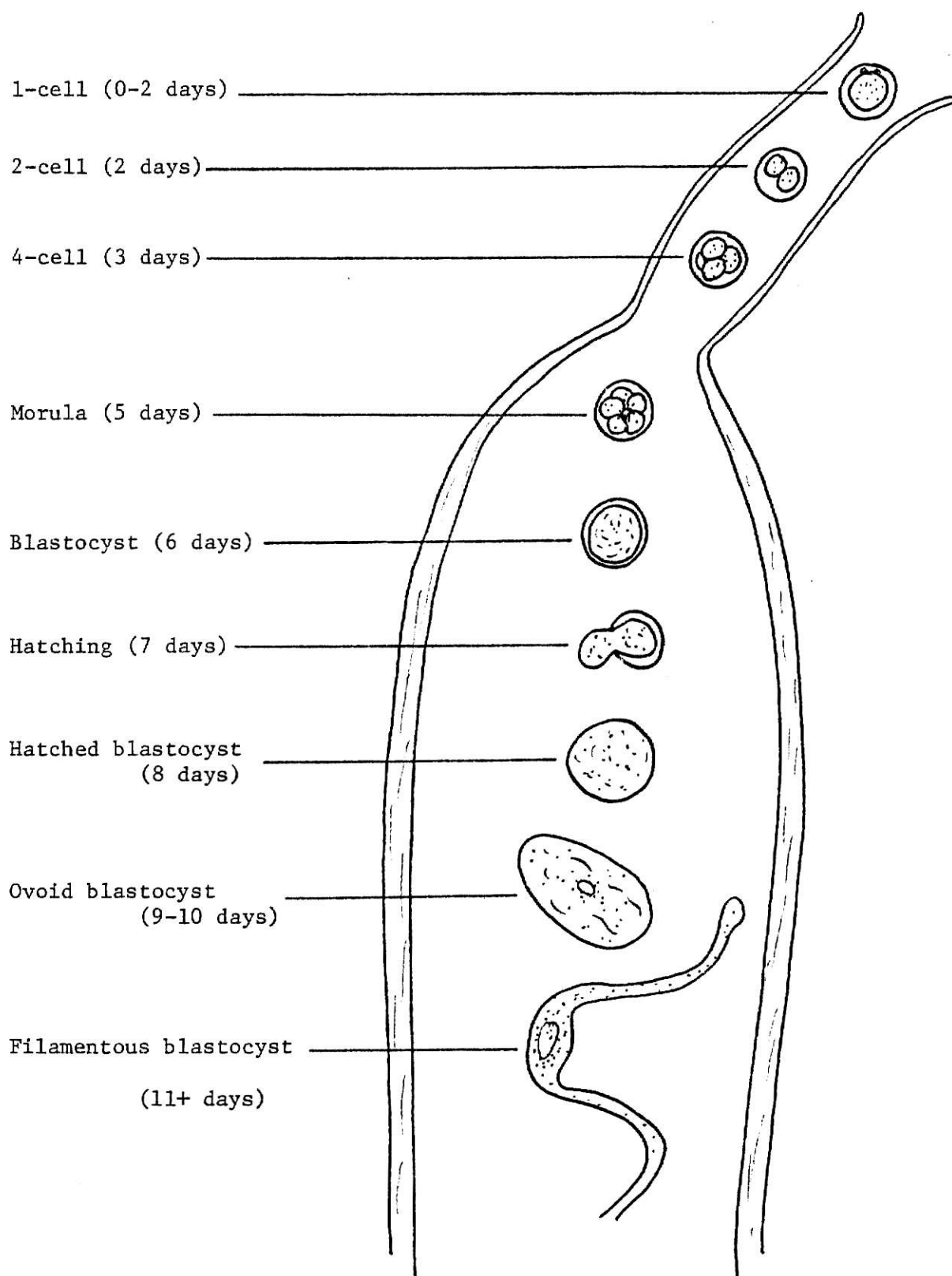


Figure 1. Schematic representation of the stages of pig embryo development timed in days from estrus.

1961; Wrathall, 1971). By birth losses have only increased to 45% (Perry, 1954; Hanly, 1961). The rate of mortality is determined by the percentage of corpora lutea not represented by ova or embryos at a particular stage of development. Perry and Rowlands (1962) found that , on the average, only 4.5% of the ova were not fertilized so that a low fertilization rate could not account for the discrepancy between ovulated eggs and the embryos recovered. They also observed that 22% of the embryos appeared morphologically abnormal between 6 and 9 days of gestation, and that the major proportion of losses occurred before day 18. It would therefore seem that the second week of gestation is critical to survival of pig embryos.

In the past two decades several microtechniques have been successfully used to study the metabolism of mammalian embryos *in vitro*. Originally only laboratory animals, such as mice, rabbits and rats were used for such studies due to the need for large sample volumes. However certain techniques are now sensitive enough to allow analyses of individual embryos from larger farm animals where it is often difficult to obtain many ova.

Amino acid uptake, incorporation and even absolute rates of protein synthesis have been measured in mouse embryos (Brinster, 1971; Epstein and Smith, 1973; Schultz *et al.*, 1979). Uptake and incorporation of amino acids have also been quantitated in rabbit (Manes and Daniel, 1969), hamster (Weitlauf, 1971) and pig embryos (Jones *et al.*, 1976). In addition, ribonucleic acid uptake and incorporation have been measured in mouse (Mintz, 1964c; Monesi and Salfi, 1967), rabbit (Manes, 1969), rat (Austin and Braden, 1953) and hamster embryos (Utakoji, 1969). Absolute rates of RNA synthesis in embryos was first successfully measured by Clegg and Pikó (1977) in mice.

*In vitro* measurements of embryos are generally used to make inferences of function *in vivo*. This approach is only warranted if the culture condi-

tions used to measure metabolic parameters mimic the maternal environment. Great strides have been made in the culture of embryos of some species. Hsu *et al.* (1974) were able to culture mouse embryos from the 2-cell to the 10-somite stage in Eagle's minimal essential medium (MEM) supplemented with serum. Embryos that progressed that far appeared microscopically normal. Rabbit embryos have been cultured from 1-cell to hatched blastocysts in Ham's F10 medium plus 1.5% bovine serum albumin (BSA) (Kane, 1972). Embryos recovered as hatched blastocysts and cultured in Ham's F10 supplemented with 20 to 25% rabbit serum, or rabbit and fetal calf serum can develop 18 to 20 somites (Daniel, 1970). Cultured embryos not only appear grossly normal but Van Blerkom and Manes (1974) showed that patterns of protein synthesis were virtually identical for rabbit embryos grown *in vivo* or *in vitro* up to mid-blastocyst stage (day 4). This gives us more confidence that *in vitro* culture of embryos can be used to measure certain metabolic phenomena.

Pig embryos have been successfully cultured from the 4-cell to the blastocyst stage in a modified Krebs-Ringer bicarbonate medium (mKRB) supplemented with BSA (Davis and Day, 1978). Culture of 8 to 10 day blastocysts for 48 hours was accomplished in the same medium with 10% fetal calf or sheep serum (Robl, 1979). It should therefore be possible to culture pig blastocysts for the short periods necessary to measure various metabolic rates.

In the research reported here the relative rate of RNA synthesis in 8, 9 and 10 day pig blastocysts was estimated by measuring the incorporation of <sup>3</sup>H-uridine into high molecular weight RNA. Rates of transcription can be sensitive indicators of developmental activity in amphibian embryos according to Brachet (1974). He wrote that, "RNA synthesis always runs parallel with development. If the development stops early, at the blastula

stage, RNA synthesis will be almost negligible; if abnormal embryos develop, there is a close relationship between deficiencies in morphogenesis and decreased RNA synthesis." Measurements of  $^3\text{H}$ -uridine incorporation in pig blastocysts were combined with morphological observations to estimate the timing and extent of embryonic mortality in the second week of pregnancy in the pig.

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## CHAPTER II

## LITERATURE REVIEW

Embryonic Mortality

Swine production enjoys an economic advantage over production of other domesticated species in reproductive efficiency. Pigs produce litters of 4 to 18 offspring and have a gestation averaging only 114 days. However, the number of young actually born represents only 50 to 70% of the eggs released at ovulation (Hanly, 1961). In one of the earliest studies, Corner (1923) determined embryonic losses at various stages of gestation. He observed that 28.2% of the corpora lutea (CL) were not represented by fetuses at term in one group of sows. Another group of sows, slaughtered between 13 and 21 days of pregnancy had 26.6% unaccounted for ova, and of 9 pregnancies examined between days 5 and 12, only 53% of the CL were represented by morphologically normal blastocysts. In addition, the oldest group of embryos contained about 2% abnormal fetuses, the 13 to 21 day embryos included 4.7% with abnormalities, and the youngest group contained 14.4% abnormal blastocysts, 9.6% unfertilized eggs and 23% of the CL were not accounted for. Overall, a total of 47% of the ova were either missing, abnormal or unfertilized in the earliest group. Corner explained that this final value (47%) would be higher than the 30 or 31% loss plus abnormalities in the older groups since it included whole litters lost from the earliest group.

Perry and Rowlands (1962) determined a 4.5% fertilization failure, 28.4% loss of embryos by days 13 to 18, and 34.8% loss by days 25 to 40. They also noted 22% morphologically abnormal embryos between days 6 and 9. Corner (1923) classified abnormal embryos as those that were either opaque and degenerating or wrinkled and collapsed. Perry and Rowlands (1962) des-

cribed abnormal embryos as being "fragmented, or shrunken and misshapen and much darker in color than is normal". Since the latter authors used spermatozoa in the zona pellucida as evidence of fertilization, it is possible that many of the abnormal "embryos" were in fact fragmenting unfertilized eggs. However, it is obvious that much of the embryonic loss occurs by completion of attachment (day 18) and considerable losses are likely early in the second week of gestation.

Why embryonic losses occur is unknown but has been a subject of much speculation. Corner (1923) grouped the possible reasons for embryonic abnormality and mortality into three categories: defects of "zygosis", defects of the maternal environment, and defects within the gametes and zygotes themselves. Category one losses are the result of fertilization failure and irregular fertilization. As previously discussed, 4.5 to 9.6% of the eggs ovulated are not fertilized and an additional 1% are polyspermic or dygynic (Hancock, 1959; Hunter, 1967). The second two categories may then account for 20 to 25% of ovum wastage.

There are several ways that the maternal environment (category two) can be defective. Corner (1923) suggested that mechanical disorders of the uterus, such as myomatous tumors could contribute to losses. Other possibilities include uteri which are unable to respond adequately to ovarian and/or embryonic stimuli. Recently more information has become available concerning endometrial products secreted during early pregnancy. In 1967, Krishnan and Daniel isolated a uterine specific product from pregnant rabbits which they called "blastokinin". They suggested that this substance induced and regulated blastocyst formation. However Kane (1972) demonstrated that rabbit ova could be cultured *in vitro* from 1-cell to the blastocyst stage without the influence of any maternal proteins, thereby refuting the

requirement for blastokinin. Blastokinin, or other uterine proteins, may however be important for blastocyst growth.

There is also some evidence that uterine secretions may be critical to the survival of pig embryos. Murray *et al.* (1971) restricted pig ova to the oviduct and observed that only 4- to 32-cell embryos developed. This was interpreted as an indication of the need for uterine stimulation. However, Pope and Day (1972) showed that blastocysts could be formed when ova were maintained in the ampulla of the oviduct, and 4-cell pig embryos formed blastocysts *in vitro* (Pope, 1972). Therefore uterine factors are not required for blastocyst formation in the pig. However, uterine secretions may have important roles in further embryonic development and implantation. Quantitative and qualitative changes in the proteins secreted by the uteri of gilts occur throughout the estrous cycle (Murray *et al.*, 1972). These changes are temporally related to ovarian hormones during the cycle; during the luteal phase, at maximal levels of plasma progesterone, the greatest changes in protein secretion take place. These changes also occur during the rapid phase of blastocyst growth in pregnant animals (days 12 to 15). Knight *et al.* (1973) have shown that progesterone is the hormone primarily responsible for changes in uterine protein profiles. After summarizing a series of experiments using passive immunization against uterine specific proteins and progesterone/estrogen treatment of pregnant gilts, Bazer (1975) suggested that the main role of porcine uterine protein secretions is related to placental development.

Uterine proteins may also serve as carriers of steroid hormones. El-Banna and Daniel (1972) demonstrated that day 5 rabbit blastocysts developed best in an *in vitro* environment containing both blastokinin and progesterone as opposed to either molecule alone. The authors suggested that progesterone may mediate metabolic activity when available to blastocysts and that the

role of blastokinin is to make progesterone available to the blastocyst. Arthur *et al.* (1972) demonstrated that blastokinin bound both progesterone and estrogen, and suggested that it could function as a carrier molecule.

The pig endometrium has been shown to secrete a substance which stimulates protein synthesis in day 16 cultured pig trophoblast (Heap *et al.*, 1979). However, neither estrogen nor progesterone affected the capacity of the porcine endometrium to synthesize proteins *in vitro* (Basha *et al.*, 1979). Protein synthesis in cultured bovine chorion and endometrium was actually depressed by the addition of progesterone or estradiol-17 $\beta$  (Wathes, 1980). Wathes suggested that perhaps the inhibition of glucose membrane transport caused by addition of progesterone *in vitro* (Flint, 1970) resulted in an overall reduced rate of metabolism, and therefore protein synthesis. One needs to be cautious, therefore, when comparing *in vitro* and *in vivo* phenomena.

An *in vitro* system of stimulated lymphocytes is commonly used to test potential immune suppressive agents. Murray *et al.* (1978) have shown that a certain fraction of uterine proteins suppresses stimulated pig lymphocytes. The authors suggested that the function of these proteins is to protect implanting embryos from immune rejection by the mother. This protein fraction is synthesized between days 6 and 16 of the estrous cycle (Squire *et al.*, 1972) and can be found from day 16 on, in pregnant animals (F. A. Murray, unpublished data cited in Murray *et al.*, 1978).

Another uterine protein that may be important during pig blastocyst implantation is an inhibitor of plasminogen activator (PA). Mouse blastocysts invade the uterus when they implant, and have been shown to produce PA (Sherman *et al.* 1976). Plasminogen activator converts plasminogen into an active proteolytic enzyme, and may play a key role in cell migration and

invasion. Pig blastocysts do not implant by invasion, rather by attachment. However, they do produce high levels of PA at day 12 when attachment begins (Mullins *et al.*, 1980). The latter group has also shown that the uterine flushings contain an inhibitor of PA, causing PA activity to be low during the luteal phase of the estrous cycle and from days 12 through 18 of pregnancy. They have also shown that the PA inhibitor is induced by progesterone in ovariectomized gilts. Furthermore, early pig blastocysts transplanted to ectopic sites in the uterus form syncytia which interact with the uterine tissue (Samuel and Perry, 1972). This inhibitor may be a necessary product of the uterus to prevent invasion by pig blastocysts.

There are other uterine secretory products that do not have obvious potential functions. Murray *et al.* (1980) found that riboflavin was secreted by the uterus in greatest quantity between days 6 and 8 in pregnant gilts and was also high on day 8 of the estrous cycle. Riboflavin secretion could also be induced in ovariectomized gilts by estrogen and/or progesterone treatment. The significance of this peak of riboflavin is unknown. However, it appears at about the time of cavitation and may therefore be involved in blastocyst formation or development.

Nonspecific infections in the reproductive tract could also have deleterious effects on embryos either directly, or indirectly through stimulation of lymphocyte infiltration. The latter can result in production of embryo-toxic substances (Anderson and Alexander, 1979). Scofield *et al.* (1974) found almost twice as many missing or abnormal ova from infected gilt uteri compared to sterile uteri at 9 and 13 days after mating. Overall, 21.4% of the ova were lost at 9 days whereas a 52.4% loss was recorded at 13 days, again emphasizing the vulnerability of embryos during the second week. In a preliminary study, 8, 9, 10 and 12 day pig embryos were recovered, morpho-

logically scored, and the uterine flushings cultured for evidence of infectious organisms (D. L. Davis, O. I. Dawydiak, C. Y. Lee and D. Y. C. Fung, unpublished observations). Uteri were classified as having both horns infected, one horn infected or neither infected (sterile). When both uterine horns were infected, a higher percentage of unfertilized and abnormal eggs were obtained than when only one horn was infected. Gilts with one infected horn had fewer normal embryos than gilts producing sterile cultures from both horns. The percent unrecovered eggs was also higher in the most infected group (25% versus 4% and 14%, respectively). More data is required to verify these trends. In addition to local infections, any systemic infection which raises the mother's temperature above  $40.6^{\circ}\text{C}$  for several days will also cause embryonic death and abortions (Done, 1968). The ambient temperature is critical during the first 2 weeks of gestation (Warnick *et al.*, 1965) and exposure to temperatures above  $31^{\circ}\text{C}$  for as little as 24 hours can reduce embryo survival.

The nutrition of the mother may also affect embryonic survival. Severe malnutrition can result in anatomical disturbances which prevent normal implantation and pregnancy (Corner, 1923). Overfeeding can also adversely affect porcine embryos (Robertson *et al.*, 1951; Bazer *et al.*, 1968) although the mechanism is not understood.

Ovulation rate must also be considered in its relation to embryonic mortality. Summarizing work from a number of sources, Wrathall (1971) concluded that by days 25 to 30 of gestation, there is a 1.08% decrease in embryonic survival for each additional egg shed over 13. Perry (1959) reported a similar value (1.2%) for litters where some embryonic loss had occurred, and suggested that the death of one embryo affects the survival of others in the litter.

Maternal age is also an important variable in reproduction (Warnick *et al.*, 1949). Sows tend to have twice as many whole litter losses as gilts. Gilts, on the other hand, tend to have twice as many complete fertilization failures as sows. The latter is due mainly to bilateral tubal abnormalities, missing tract segments or cystic follicles. In older sows the problem of prenatal losses may be related to degeneration of the endometrium.

The hormonal conditions to which the uterus and embryos are exposed may have an effect on embryo survival. Progesterone, combined with serum or uterine proteins, has been shown to stimulate growth in mouse and rabbit embryos *in vitro* (Roblero and Izquierdo, 1976; El-Banna and Daniel, 1972). However, Warner and Tollefson (1978) found that neither progesterone nor progesterone plus estrogen had any effect on RNA synthesis in mouse blastocysts, but translational stimulation was not investigated. Sub-optimal hormone levels have not been conclusively shown to be responsible for embryonic mortality in pigs (Wrathall, 1971). In some experiments hormone therapy has reduced the number of animals experiencing very high prenatal losses, but results have been inconsistent (Day *et al.*, 1963).

After summarizing a number of *in vitro* implantation studies, Sherman and Wudl (1976) suggested that it is primarily the uterus, not the blastocyst, that is the target of steroid hormones during implantation. They pointed out that in ovariectomized animals, or during lactation in some species, the uterus is not receptive to blastocysts unless steroids are administered. It is possible, however, that certain maternal hormones could be metabolized by the embryos which in turn affect the uterus. Progesterone can be utilized by pig blastocysts to form other steroids, such as estrogen (Perry *et al.*, 1973) which may be required for further development. Embryonic steroids may also be involved in maternal recognition of



pregnancy and implantation (Cook and Hunter, 1978). This argument is discussed in more detail in the next section.

Defects in the maternal environment consisting of inappropriate temperatures, uterine secretions, hormonal levels or a variety of other conditions are likely to affect all the embryos unless microenvironments exist in the uterus. Thus uterine defects are probably not the sole cause of mortality. However these factors may contribute to embryonic death in that different embryos may have different susceptibilities to fluctuations in uterine products and hormone levels. These differences may result from the varying genetic make-up of individual embryos. The interaction between maternal environment and genotypic expression of each embryo could then become the major determinant of embryo survival.

The final category proposed by Corner (1923) to affect embryo mortality involves genetic and non-genetic defects of the gametes and the zygote. In a cytogenetic analysis of 7 litters of 10 day pig blastocysts, McFeely (1967) found that 10% of the embryos had gross chromosomal abnormalities including trisomies and polyploids. Some of the polyploid embryos probably resulted from polyspermy. Shaver and Carr (1967) found a similar trend in rabbit blastocysts.

Lethal and semi-lethal genes which affect developing embryos may also be responsible for some of the observed losses. In mice, several such genes are known to exist in the T-locus of the genome (Mintz, 1964a,b; Bennett, 1975). These deleterious genes are maintained in the population by unequal sperm transmission. Although similar genes have not been reported for swine, the critical searches necessary to detect them have not been conducted. If they do exist, it may be possible to select a genetic line that has very few or none of these genes, which may be represented by crosses resulting in 95 to 100% survival of embryos to term.



By testing for certain gene products, it may eventually be possible to identify defective embryos by the enzymes or carrier proteins they fail to secrete adequately. Perry *et al.*, (1973) demonstrated that pig blastocysts were capable of producing estrogens from neutral steroids, progesterone and conjugated steroids. Estrogen biosynthesis occurs in trophoblastic tissue by day 12 (Heap *et al.*, 1977), in time for it to possibly be involved in signaling pregnancy to the uterus (Dhindsa and Dziuk, 1968). This adds significance to the observation that administering estrogens to intact, unmated gilts, starting on day 11 of the estrous cycle, results in maintenance of the corpora lutea (Gardner *et al.*, 1963). It raises the possibility that embryonic estrogens act as a signal necessary for CL maintenance. Rabbit blastocysts are also capable of synthesizing estrogens on days 5 and 6 of pregnancy (George and Wilson, 1978). Since synthesis decreases after implantation and anti-estrogens prevent implantation (Dey *et al.*, 1976) it is possible that embryonic estrogens are necessary for implantation. Another proposed function of embryonic estrogens is stimulation of uterine blood flow (Dickson *et al.*, 1969). Ford and Christenson (1979) suggested that early pig embryos could unilaterally control uterine blood flow, this effect possibly being mediated by estrogens. Moeliono *et al.* (1977) felt that uterine secretion of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>), a luteolytic agent, was decreased by pig blastocysts. Ford and Christenson (1979) reasoned that the increased blood flow to the uterus at days 12 and 13 after mating could be a mechanism for removal of PGF<sub>2α</sub>. Another possibility is that estrogen may inhibit the release of PGF<sub>2α</sub> into the bloodstream by redirecting its secretion into the uterine lumen (Frank *et al.*, 1977).

Another candidate for hormone regulation is embryonic histamine. Histamine has been implicated in implantation in mice (Brandon, 1977) and

rabbits (Dey *et al.*, 1979a). The latter group has demonstrated that prior to implantation, rabbit blastocysts have an increased enzymatic capacity to synthesize histamine. Both blastocysts and the endometrium have histamine receptors (Dey *et al.*, 1979b) but the function of histamine during implantation is unknown. It is possible that histamine synthesis by blastocysts could stimulate phospholipase A<sub>2</sub> activity and secondarily increase synthesis of prostaglandins by the embryo or the endometrium. Prostaglandin synthesis has been demonstrated by Dey *et al.*, (1980) in day 6 rabbit blastocysts and endometrium. The suggestion was made that prostaglandins, histamine or both could affect cyclic nucleotides which may have a role in mouse and rabbit implantation (Dey *et al.*, 1978). Phospholipase A<sub>2</sub> activity has also been demonstrated in preimplantation swine blastocysts (D. L. Davis, C. L. Cox and S. K. Dey, unpublished observations).

Another embryonic product that may be involved in embryo survival is alpha-fetoprotein. This protein has been shown to suppress immune responses of mouse and human lymphocytes (Murgita and Tomasi, 1975; Yachnin, 1976). However, alpha-fetoprotein has only been detected after implantation in mouse embryos (Dziadek and Adamson, 1978) so it may have no relevance to early blastocyst development and implantation.

New gene products, as will be discussed later, appear at precise times during development. Some of these are polypeptides that are probably involved in cell surface phenomena associated with embryonic development and embryo-uterine interactions. Stage specific changes in membrane-associated enzymes (Vorbrodt *et al.*, 1977) and surface glycoproteins (Pinsker and Mintz, 1973; Johnson and Calarco, 1980b) have been found in early mouse embryos. Johnson and Calarco (1980a) suggested six possible roles for the embryonic cell surface glycoproteins. The first, cell recognition, would

be important at fertilization (egg and sperm), at differentiation (trophoblast and ICM) and at implantation (trophoblast and endometrium). The second role, cell adhesion, would also be important at the three stages mentioned above. Surani (1979) has shown that surface glycoproteins play an important role in compaction and trophoblast adhesion of mouse embryos in culture. The third role, cell communication, is now being actively investigated. At the time of differentiation of the outside cells to trophoblast, which Gardner and Rossant (1976) have shown occurs at the 8-cell stage in the mouse, Ducibella *et al.* (1975) found both tight junctions and gap junctions between embryonic cells. Tight junctions could provide an intercellular barrier in the trophoblast cells, separating the external environment from the ICM. This potentially would help to establish and maintain a new milieu for the ICM, different from that around the trophoblast cells. Lo and Gilula (1979) suggest that gap junctions may be responsible for the ionic coupling between all the cells observed at this time. Such communication between cells could further serve to expedite differentiation via molecular gradients. Cell motility is the fourth role suggested by Johnson and Calarco (1980a) and it would be involved in blastocyst attachment during implantation, and blastocyst invasion of the endometrium in the case of rodent embryos. The fifth role of the cell surface is to function to protect the embryo from the mother's immune system. This is another very active area of research, particularly in relation to methods of contraception. Johnson and Calarco (1980a) have summarized various experiments on embryonic expression of histocompatibility antigens by proposing that a lack of, or decrease in, trophoblast antigenicity at the appropriate time may allow successful implantation. Therefore a failure to suppress antigen expression could result in destruction of embryos. The sixth role suggested for the

cell surface involves cell surface receptors which might trigger mitosis, changes in gene expression and/or cell metabolism. Dey *et al.* (1979b) have shown the presence of histamine receptors, and others have yet to be determined. The absence of or defect in receptors at critical times in development could potentially result in embryonic death.

Inbreeding may also contribute to increased embryo losses (Heuser *et al.*, 1952; Young, 1969). Genetic abnormalities that are not grossly visible could express themselves as deficiencies in certain protein products, as previously discussed. Mutations which affect any of the above mentioned embryonic products may suppress a variety of developmental events and cause embryonic mortality. It has been shown that each 10% increase in the inbreeding coefficient results in 0.55 less ovulations and 0.33 fewer embryos surviving (Squiers *et al.*, 1952).

It is possible that each of the factors influencing the maternal environment contributes a little to account for most of the embryonic losses. However, the basal loss seems relatively constant (Hanly, 1961) which argues against the highly variable environmental factors. The ability of embryos to survive may be determined to a large degree by their genetic make-up, which can be further influenced by the uterine environment. The onset of embryonic death is likely to be characterized by changes in overall RNA and protein synthesis. Analyses of such synthetic activity may pinpoint precise times of lethality, and indicate possible abnormalities in developmental processes which could cause mortality. Studies on RNA and protein synthesis during embryogenesis are reviewed in the subsequent sections.

#### RNA Metabolism

Gross (1968) summarized the importance of early gene expression in sea urchin embryos by saying that without normal RNA synthesis, "early embryo-

genesis becomes abnormal, and later development and differentiation fail altogether". Molecular hybridization studies have shown that during development different parts of the embryonic genome are being transcribed. This suggests that new gene products become available in the form of RNA, some of which will eventually be involved in protein synthesis. The relevant questions are: what is the pattern of RNA synthesis during development; what forms of RNA are synthesized at specific stages of development and in what amounts; what are the control factors in this pattern of synthesis? In addition, how does embryonic protein synthesis correlate with embryonic transcription?

In amphibians and sea urchins, there is considerable evidence for the accumulation of ribosomal (rRNA), transfer (tRNA) and messenger (mRNA) RNA's during oogenesis (Gross, 1968). Upon fertilization or activation of the egg the maternal mRNA is translated and development proceeds to gastrulation without any embryonic RNA synthesis (Brown, 1965; Brown and Littna, 1965). Only at this point can further development be blocked by inhibitors of transcription, such as actinomycin D.

The mouse oocyte also contains a large store of maternal RNA, estimated to be about 100X that of adult cells (Reamer, 1963). By the 16-cell stage, each cell contains approximately one tenth of the original amount of RNA (Ellem and Gwatkin, 1968; Olds *et al.*, 1973) and RNA synthesis becomes a necessity for cell maintenance. Ellem and Gwatkin (1968) detected a rapid increase in rRNA synthesis in the 8-cell embryo. Nucleoli, the site of rRNA transcription in the nucleus, were first labelled with  $^3\text{H}$ -uridine at the 4-cell stage (Mintz, 1964c). Woodland and Graham (1969) then found that  $^3\text{H}$ -uridine was incorporated into rRNA and tRNA at the same stage, and suggested that since mouse ova did not have the large stores of ribosomes

found in amphibians, rRNA synthesis was essential at an earlier stage. Nucleoli and some nuclear chromatin of Syrian hamster ova were labelled in the late 2-cell stage, but no label was found in the cytoplasm (Utakoji, 1969). More recently, Clegg and Pikó (1975) detected synthesis of 18s, 28s (rRNA) and 4s (tRNA) by the mid 2-cell stage in mouse embryos. These species of RNA formed the bulk of stable RNA's synthesized from the 4-cell stage on.

Patterns of transcription in rabbit embryos have also been investigated. Ribonucleic acid synthesis peaks at day 3 (morula), decreases slightly at day 4 (early blastocyst), then drops substantially by days 5 and 6 (Manes, 1969). Transfer RNA was first detected at the 2-cell stage (1 day) and increased in synthesis up to day 3 (Manes, 1971). Ribosomal RNA was not detectable before day 3 and heterogeneous RNA (hnRNA), which presumably contains mRNA, was most prominent between 67 to 77 hours post coitum (Manes, 1971). The majority of new RNA synthesized was rRNA, however (Karp *et al.*, 1973).

Nuclear hnRNA is eventually processed and transported to the cytoplasm as mRNA (Darnell *et al.*, 1971; Rottman *et al.*, 1974). Both forms of RNA contain a polyadenylated (poly-A) sequence at the 3' end and a cap of methylated residues at the 5' end. One can supposedly identify mRNA by the poly-A tracts present. Synthesis of mRNA has been detected as early as the 2-cell stage in mouse embryos (Clegg and Pikó, 1975) and the 16-cell stage in rabbit embryos (Schultz, 1973). The ratio of mRNA to total RNA synthesis is about the same from the 16-cell to the blastocyst stage in rabbits (Schultz *et al.*, 1973). According to Warner and Hearn (1977a) the proportion of mRNA in mouse embryos remains at about 2.2% of total RNA from the 8-cell to blastocysts stages, while Levey and Brinster (1978) found about 3.4% mRNA. Warner and Hearn, (1977b) also determined the relative proportions of the other RNA classes, and found them to be approximately the same at the 8-cell

and blastocyst stages, rRNA being the predominant type at all times. To summarize then, RNA synthesis is first detected at the 2-cell stage in both mouse and rabbit embryos. Transcription increases more rapidly in mouse than in rabbit embryos, but the relative proportions of RNA's formed is the same in both.

By the blastocyst stage there are two tissue types in mammalian embryos--the trophoblast and inner cell mass (ICM). Karp *et al.* (1973) were able to differentiate the two cell types on the basis of amount and distribution of  $^3\text{H}$ -uridine. Trophoblast cells mainly took up label in the nucleoli, whereas ICM cells had both intense nucleolar labelling and more label over the nuclear chromatin. Since nucleoli are the site of rRNA transcription, the other RNA's are transcribed from extranucleolar chromatin. This indicates more mRNA and tRNA synthesis in the ICM than in the trophoblast cells. This would seem logical in light of the number of cell types which originate from the ICM in the morphogenetic events about to occur.

The absolute rate of RNA synthesis in mouse embryos was measured by Clegg and Pikó in 1977. All previous measurements were reported as incorporation of  $^3\text{H}$ -uridine into high molecular weight RNA, since the extent of the endogenous precursor pool was unknown. They reported that the UTP pool increased 10 fold from the 1-cell to blastocyst stages. This meant that the absolute rate of RNA synthesis per embryo increased 50X in the same span, and the rate of synthesis per cell only increased 4X from the 2-cell to the blastocyst stage.

Administration of hormones to normal embryos has resulted in inconsistent trends in RNA synthesis. Lau *et al.* (1973) found rapid increases in  $^3\text{H}$ -uridine uptake and incorporation by mouse blastocysts *in vitro* when physiological levels of estradiol were available. However, Warner and Tollefson



(1978) found no stimulatory effect on embryo transcription when they administered progesterone or progesterone plus estrogen. Zelenina *et al.* (1979) found that the appropriate concentration of  $\text{PGF}_{2\alpha}$  increased uridine incorporation in mouse and rat embryos. Autoradiographic studies further showed that  $\text{PGF}_{2\alpha}$ -treated embryos, particularly blastocysts, displayed labelling over the entire nucleus and a little in the cytoplasm, whereas the label was restricted primarily to nucleoli in control embryos. The authors suggested that  $\text{PGF}_{2\alpha}$  may increase not only the rate of transcription, but also the rate of RNA transport into the cytoplasm.

Inhibitors of RNA synthesis depress  $^3\text{H}$ -uridine incorporation to varying degrees depending on the type of inhibitor and the concentration used (Monesi *et al.*, 1970; Rowinski *et al.*, 1975). Actinomycin D retards mouse embryo development but does not arrest it completely before the blastocyst stage. Since protein synthesis was only reduced to 50% of original levels, it was concluded that the embryos contained enough long-lived maternal mRNA to continue development without new RNA transcripts. The authors have further suggested however, that RNA synthesis is necessary for implantation to occur.

The processing of RNA before it becomes available for translation can be one point of control during development. Methionyl tRNA ( $\text{tRNA}^{\text{Met}}$ ) must bind to a ribosome before mRNA can attach to initiate protein synthesis. Clandinin and Schultz (1975) found that although the amount of  $\text{tRNA}^{\text{Met}}$  decreases with embryo age in rabbits, the rate of methylation of tRNA and ribosome affinity of  $\text{tRNA}^{\text{Met}}$  increase after blastocyst formation. Other examples of processing changes in developing embryos may eventually be discovered with the increasing sensitivity of microtechniques. The next level of control during development occurs in protein synthesis, the final expression of the embryonic genome.



### Protein Synthesis

One of the most obvious and easily measured results of protein synthesis in embryos is their increase in size. During cleavage, however, there is very little, if any, change in the outside dimensions of mammalian embryos, even as cell numbers increase. Brinster (1967) measured the amount of protein found at various stages of mouse embryo development. He reported that between the 1-cell and morula stages there was a 25% decrease in total embryo protein content, and thereafter it remained approximately constant through the late blastocyst stage. Schiffner and Spielmann (1976) found that the total protein content in mouse embryos remains about the same until the late blastocyst stage, at which time it increases by 40%. However their measurements of rat embryos through the same stages showed a gradual decline to 73% of the original protein content. Weitlauf (1973) reported that the protein content of mouse embryos just prior to implantation increased by 52% in a 10 hour period on day 5 of pregnancy. In contrast, delayed implanting embryos maintained a constant amount of protein during the same times.

At the time of implantation, (day 5) mouse blastocysts consist of 60 to 70 cells. Rabbit embryos, which are twice the size of mouse ova to begin with, contain 80,000 cells when they implant on day 7. Pig embryos are unique in that they begin with an intermediate sized egg and implant at 18 days when they are 60 to 100  $\mu$ m in length. Between the late morula and early blastocyst stage (day 6) they already contain 25 times the total protein found in murine late blastocysts (Wright and Grammer, 1980). This amount gradually increases through day 8, at which time the authors noted a dramatic increase in protein (2.15 mg to 57.84 mg per embryo). Anderson (1978) measured total protein in pig blastocysts from days 9 through 18, and reported a four fold increase between days 9 and 10. His observations

for day 9 indicated only 22% of the protein found by Wright and Grammer (1980). A great deal of variation in embryo size can be found between litters at the same stage of pregnancy (personal observations), and even within litters (Wright and Grammer, 1980). This may or may not be significant, since the latter authors also found no correlation between the overall embryo size and total protein content.

Brinster (1971) measured uptake and incorporation of 11 different amino acids by mouse embryos in culture. He reported an overall five fold increase in uptake and a ten fold increase in incorporation between the 1-cell and morula stages. The largest changes occurred at the 8-cell stage. Compared to the other amino acids measured, more glutamine was taken up, and leucine showed the highest rate of incorporation. Epstein and Smith (1973) demonstrated amino acid uptake and cyclohexamide inhibitable incorporation into protein by follicular ova, unfertilized eggs and all stages up to the blastocyst in mouse embryos. These authors suggested that between the 1- and 16-cell stages embryos do not increase protein synthesis in proportion to the increase in cell numbers, and that mainly maternal mRNA is being translated. Between the 8- to 16-cell and early blastocyst stages, however, protein synthesis per cell increases, then drops sharply by the late blastocyst. The authors suggest this represents a specific developmental event.

Protein synthesis at all stages of rabbit development has also been demonstrated, peak protein synthesis per embryo occurring at 108 hours after mating (Manes and Daniel, 1969). On a per cell basis, amino acid incorporation decreases 100 fold between days one and four, and then increases ten fold between days four and six.

Specific enzymes have been studied in relation to levels of activity during embryonic development. Hypoxanthine, guanine and adenine phospho-

ribosyltransferases all increased steadily from the unfertilized mouse egg through the blastocyst stages (Epstein, 1970). Interestingly, the activity levels of enzymes remained the same, whether the embryos were measured upon recovery or after varying periods in culture. In addition, the control of activity did not seem to be a function of the number of cells in the embryo.

Two dimensional gel electrophoresis has been used to study qualitative patterns of protein synthesis in embryos. In mouse embryos it has been demonstrated that different proteins are synthesized at different times during development (Van Blerkom and Brockway, 1975; Howe and Solter, 1979). The same proteins were synthesized by unfertilized ova, fertilized ova and day one embryos; late into day one, and in early day two, new proteins became apparent. By day three (4- to 8-cell embryos) most of the changes had occurred, and only a few new polypeptides were synthesized by blastocysts.

The same pattern of changes occur in rabbit embryos, except the events are delayed (Van Blerkom and Manes, 1974; Van Blerkom and McGaughey, 1978). Between fertilization and the 8-cell stage there was a gradual replacement of 'old' proteins with new ones and the majority of qualitative changes took place between the 8-cell stage and early cavitation. The expanding blastocyst displayed a uniform and constant synthesis of polypeptides which, the authors suggested, were probably related to the requirements for growth, expansion and accelerated metabolism. The little data that is available for pig embryo protein synthesis indicates that leucine incorporation is 15 times greater between days 9 to 13 than between days 14 to 18 (Haumschild and Anderson, 1978). The significance of this finding is not readily apparent.

The first visible sign of differentiation into specific cell types occurs at blastocyst formation. The question of interest then, is when do

the trophoblast and ICM cells become 'determined'? One indication that determination has occurred is the production of tissue-specific cell products. Tissue-specific proteins have been identified for the trophoblast and the ICM of mouse blastocysts (Van Blerkom *et al.*, 1976). When the inside cells from the morula were compared to ICM of early blastocysts, the protein patterns were similar, indicating that molecular differentiation was well underway before blastocyst formation (Handyside and Johnson, 1978). Recently, stage-specific cell surface antigens in mouse embryos have been identified and characterized (Johnson and Calarco, 1980c,d). These antigens consist of a pair of glycoproteins which are synthesized only between the 2-cell and morula stages, but their significance remains unknown.

It is now possible to measure the absolute rate of protein synthesis in small embryonic samples. Brinster *et al.* (1976) estimated that turnover of about 10% of the proteins in 2-cell and blastocyst mouse embryos occurred at 18.3 and 11.2 hours, respectively. The other 90% were thought to turn over much more slowly. The absolute rate of protein synthesis per cell was actually found to decrease by a factor of 5.2 during early embryogenesis in the mouse, whereas the absolute rate of ribosomal protein synthesis increased 1.4 fold in the same period (LaMarca and Wassarman, 1979). This suggests a special requirement for ribosomal proteins at this time. Total ribosomal protein synthesis increased 11.3 fold per embryo, paralleling the 10 fold increase in rRNA synthesis from the 1- to 8-cell embryo (Clegg and Pikó, 1977). LaMarca and Wassarman (1979) suggested that ribosomal protein may be required for immediate ribosome formation since preribosomal 45s RNA is rapidly cleaved if it does not combine with the appropriate proteins to form an 80s ribonucleoprotein particle (Warner, 1974).

In the same time that the absolute rate of RNA synthesis increases ten fold, total protein synthesis increases less than two times, but picks up again at the blastocyst stage (Schultz *et al.*, 1979). This means that the RNA synthesis surge precedes the increase in protein synthesis, the same (logical) pattern found in rabbit embryo incorporation studies. Most of the RNA in this surge is ribosomal, but tRNA and mRNA are also synthesized.

Temporal control of protein synthesis in embryo development is another question of interest. As mentioned previously, culture of mouse embryos in the presence of actinomycin D, an inhibitor of RNA synthesis, slows development of the embryo but does not arrest it completely (Monesi *et al.*, 1970). In fact, protein synthesis is maintained at 50% of the control levels, indicating the presence of long-lived maternal mRNA. Puromycin, on the other hand, immediately inhibits all protein synthesis, and embryo development subsequently halts.

Synthesis of mRNA can also be inhibited by alpha-amanitin, which acts specifically on nucleoplasmic RNA polymerase II (Stirpe and Fume, 1967; Tata *et al.*, 1972), but also significantly represses formation of 28s and 18s rRNA (Levey and Brinster, 1978). The latter authors suggested that rRNA disruption may occur secondarily due to lack of the enzymes needed for 45s pre-rRNA processing. They concluded that mRNA synthesis was necessary for nucleolar maturation, function, and integrity of the preimplantation embryo. Braude (1979) has shown that alpha-amanitin blocks cavitation, prevents the two to five fold increase in protein synthesis, and changes the sequence of polypeptide synthesis which occurs during early mouse embryo development. He further categorized proteins as belonging to three different classes, depending on their response to alpha-amanitin treatment. Class A polypeptides continued to be synthesized even

though no new mRNA was available. This provided evidence that maternal mRNA remained available during cleavage. Class B proteins were not translated when they normally would have been, and probably represented some of the new proteins required for later development. Class C consisted of polypeptides that ordinarily would have disappeared but continued to be synthesized in the presence of the toxin. This infers the existence of a regulator mRNA coding for a protein that either switches off translation of the class C proteins or is involved in their degradation.

Inhibitors have also been used to demonstrate selective inhibition of specific tissues. Rowinski *et al.*, (1975) used cordycepin and actinomycin D to inhibit RNA synthesis, and cycloheximide to depress protein synthesis in trophoblast and ICM cultures. At appropriate levels, these drugs depressed ICM development but not trophoblast growth. This suggests that the synthetic controls are different in the two tissue types.

There are also examples of naturally occurring inhibitory processes impeding embryonic development. Embryogenesis in  $t^{12}/t^{12}$  lethal mouse mutants does not proceed past the morula stage. It has been shown that radioactive label is incorporated into the nucleoli of morulae, but very little of that label passes into the cytoplasm (Mintz, 1964b). RNA synthesis does not increase to the levels found in normal embryos, although protein synthesis persists until the embryo shows signs of degeneration (Mintz, 1964a). When pregnant mice are ovariectomized, their embryos decrease both RNA and protein synthesis, but continue to exist in a non-implanted condition until their metabolic rate returns to normal under the influence of an external stimulus to the mother (McLaren, 1973). Hamster embryos do not experience experimentally induced delayed implantation when pregnant animals are ovariectomized (Weitlauf, 1971). Ovariectomy of pregnant

hamsters results in embryos that continue to synthesize proteins at a normal rate until they begin a rapid degeneration.

Control of diapausing mouse embryos by uterine factors has been investigated with varying results (Weitlauf, 1976; Van Blerkom *et al.*, 1979). There is one report of a possible uterine control of protein synthesis in pig embryos. Day 16 blastocyst tissue cultures in the presence of endometrium at the same stage of pregnancy had higher rates of protein synthesis than blastocyst tissue cultured alone or with other tissue types (Heap *et al.*, 1977; Heap *et al.*, 1979). Wathes (1980) suggested that steroid hormones may stimulate this increase, but experimental data to support this idea has been inconsistent. Much work remains to be done in elucidating the controls of embryonic protein and RNA synthesis.

## CHAPTER III

INCORPORATION OF  $^3\text{H}$ -URIDINE BY PIG BLASTOCYSTS *IN VITRO*

## Introduction

The majority of embryonic losses in the pig occur early in pregnancy. Ribonucleic acid synthesis, as measured by incorporation of  $^3\text{H}$ -uridine, is a sensitive indicator of gene activity in developing embryos and is therefore potentially useful for determining viability of embryos within and among litters. RNA synthesis in preimplantation embryos has been demonstrated in rats (Austin and Braden, 1953), mice (Mintz, 1964c; Monesi and Salfi, 1967; Woodland and Graham, 1969; Pikó, 1970), Syrian hamsters (Utakoji, 1969) and rabbits (Manes, 1969) and similar approaches are used in the present study of pig embryos. When  $^3\text{H}$ -uridine is used as an RNA precursor the resultant labelled RNA can be identified by autoradiography (Utakoji, 1969), RNA extraction and gel electrophoresis (Warner and Hearn, 1977b) or by DEAE-cellulose filter paper retention (Litman, 1968; Warner and Hearn, 1977b). DEAE filter papers retain both DNA and RNA by electrostatic attraction and matrix effects. Fragments of less than 10 nucleotides are easily removed by extensive washing. This technique has several advantages over conventional acid precipitation of nucleic acids. Blank values can be reduced to less than 0.1% of the added radioactivity and no high molecular weight RNA and DNA is removed by extensive washing therefore recovery of small samples is quantitative and many samples can be processed simultaneously. The main disadvantage is that DEAE-cellulose filter papers are opalescent and decrease the efficiency of scintillation counting by about 15%. However, the reproducibility of this assay far outweighs the loss in counting efficiency (Warner and Hearn 1977b).



The objective of the research reported here was to establish the pattern of RNA synthesis in day 8, 9 and 10 pig blastocysts *in vitro*. It was hoped that analysis of this data might elucidate differences in viability of embryos within and among litters.

#### Experimental Procedure

*Preparation of Culture Equipment.* Any materials which came in contact with the embryos or culture medium were thoroughly washed by hand. Culture equipment was soaked in a tissue culture detergent<sup>1</sup> for at least one day, then each piece was brushed and rinsed with at least 15 exchanges of tap water, 3 of distilled water and 1 of deionized distilled water. The equipment was then oven dried, stored in autoclave bags<sup>2</sup> and autoclaved within 10 days of use.

*Preparation of Medium.* Three media were used for embryo recovery and culture. The first was a modified Krebs-Ringer bicarbonate (Davis and Day, 1978) containing 40  $\mu$ M uridine (mKRB; table 1) which was used to flush embryos from the uterus. Embryos were cultured for about 2 hours in mKRB plus 10% fetal calf serum (fcs) and then were transferred to the incubation medium which was mKRB containing 10% fcs and 20  $\mu$ M <sup>3</sup>H-uridine. To make mKRB, dry ingredients were individually weighed and added to a 500 or 1000 ml flask approximately half-filled with deionized distilled water. A magnetic stir bar was used to mix the solution and, after all ingredients had been added, water was added to bring the flask to full volume and the mixture was stirred an additional 15 to 30 minutes. The flask was then cooled to 4°C.

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<sup>1</sup>7X Tissue Culture Detergent, Limbro Chemical Co., Inc., New Haven, Conn.

<sup>2</sup>Will Ross, Inc., Milwaukee, Wis.

TABLE 1. MODIFIED KREBS-RINGER BICARBONATE

Ingredient	Grams/liter	mM
NaCl	7.021	120.13
KCl	.356	4.78
CaCl <sub>2</sub>	.189	1.71
KH <sub>2</sub> PO <sub>4</sub>	.162	1.19
MgSO <sub>4</sub> ·7H <sub>2</sub> O	.294	1.19
NaHCO <sub>3</sub>	2.106	25.00
Glucose	1.000	5.56
Bovine serum albumin <sup>a</sup>	4.000	
Penicillin G	.063	
Streptomycin sulfate	.050	
Uridine - flushing medium	.010	.04
Uridine - incubation medium	.005	.02

<sup>a</sup>Fraction V.

Sterilization was accomplished by filtration ( $0.22\ \mu\text{m}$  pore size)<sup>3</sup> with positive pressure supplied by a bottled gas mixture. The gas was humidified first by bubbling through a 500 ml Erlenmyer flask half-filled with sterile deionized water. The medium was forced directly from the filtration apparatus into sterile 100 ml bottles via a 1 cc syringe and needle which penetrated the serum stopper. A second needle in the stopper vented the bottle. Approximately 80 ml of medium was filtered into each 100 ml bottle and the bottles were gassed with 5%  $\text{CO}_2$  in air for approximately 3 minutes before use.

One day before embryo recovery 10% fcs<sup>4</sup> was added to the medium in which embryos were to be cultured. Fetal calf serum was purchased as a lyophilized powder and stored at  $4^\circ\text{C}$  until it was rehydrated with sterile deionized distilled water. Serum added to medium for culture was first heated at  $56^\circ\text{C}$  for 30 minutes. Any rehydrated serum not used immediately was stored at  $-10^\circ\text{C}$  in 3 ml to 5 ml aliquots.

Preparation of the incubation medium was as follows: sodium bicarbonate ( $\text{NaHCO}_3$ ) was prepared separately at 3.6 times the final concentration (90.0 mM). At high concentrations it precipitated when added to the other ingredients in the dry form. A second solution was prepared containing the remainder of the ingredients at 6 times their final concentration. The concentration of the second solution became 3.6 X when heat-treated fcs was added. The incubation medium consisted of 1 part 3.6X mKRB plus 40% fcs; 1 part 3.6X sodium bicarbonate; 2 parts  $^3\text{H}$ -uridine (1 mCi/ml,  $> 25\ \text{Ci/mmol}$ , New England Nuclear). This resulted in 1X mKRB containing  $20\ \mu\text{M}$   $^3\text{H}$ -uridine and 10% fcs.

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<sup>3</sup>Millipore Corp., Bedford, Mass. 01730

<sup>4</sup>Grand Island Biological Co., Grand Island, N.Y. 14072

The incubation medium was also gassed and all media were stored at 4°C. A few hours before use medium was warmed to 37.5°C.

*Animals.* Embryos were recovered from 26 Yorkshire gilts bred to an unrelated Yorkshire or Duroc boar. The prepuberal gilts were induced to ovulate with one injection of 400 IU of pregnant mare serum gonadotropin (PMSG) and 200 IU of human chorionic gonadotropin (HCG). Gilts were checked twice daily for estrus with a boar and were bred after cycling was established. Eight gilts were injected with 500 IU of HCG at proestrus and were artificially inseminated 36 and 48 hours later. The other 18 gilts were inseminated 12 and 24 hours after estrus was first detected. Onset of estrus (day 0) was defined as the first estrus check that a gilt displayed an immobile reflex (Signoret, 1970).

*Embryo Recovery.* Embryos were surgically removed from the uterus by flushing with mKRB. For day 8 embryos silastic tubing<sup>5</sup> (20 cm x 0.66 mm I.D.) was inserted into the oviduct through a suture needle point incision just anterior to the tubo-uterine junction and gently pushed approximately 4.5 cm into the lumen near the junction. Twenty ml of mKRB were injected into the uterine lumen near the body of the uterus with a blunt tipped needle and syringe. The medium was gently squeezed through the length of the horn, out of the tubing and into a petri dish. Older embryos were larger and required a glass cannula (I.D. 7 mm) inserted through the uterine wall near the tubouterine junction. The body of the uterus was also flushed for older embryos. The number of corpora lutea on each ovary was recorded. If the number of corpora lutea exceeded the number of embryos, the uterus was flushed a second time. Embryos collected in the flushings were examined

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<sup>5</sup>Dow Corning, Midland, Mich. 48650

under a stereomicroscope, then individually transferred to wells in a culture plate<sup>6</sup> containing fresh mKRB. Finely drawn Pasteur pipets of the appropriate size were used to transfer embryos through several rinses of medium to wash off any cellular debris or other uterine contents. The embryos were then examined for general appearance and measured with an ocular micrometer located in the eye piece of the stereomicroscope. Measurements were taken at the longest axis, and the widest axis perpendicular to it. A few embryos were photographed prior to incubation and representative typical and atypical embryos were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer.

*Incubation and RNA Extraction.* Embryos were transferred to sterile 12 X 75 mm polystyrene tubes with polyethylene caps<sup>7</sup> containing 1.5 ml mKRB plus 10% fcs, or into 16 x 125 mm glass tubes<sup>8</sup> with 2 ml of the same medium. Each tube was gassed with 5% O<sub>2</sub>, 5% CO<sub>2</sub>, 90% N<sub>2</sub> for at least 30 seconds. The small plastic tubes were placed inside a large glass tube (30 x 200 mm) which was gassed for 5 minutes before sealing. The temperature was maintained at 37.5°C in a water-jacketed incubator.

Prewarmed incubation medium was delivered into sterile polystyrene tubes in 50 µl aliquots with a Hamilton syringe<sup>9</sup> and 3 inch needle. Embryos were then transferred to the labelled medium with as little extra fluid as possible. Each tube was gassed with humidified 5% CO<sub>2</sub> in air for 1 minute then placed in a small desiccator which was gassed another 10 minutes inside

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<sup>6</sup>Falcon, Oxnard, C.A. 93030

<sup>7</sup>Falcon, Oxnard, C.A. 93030

<sup>8</sup>Bellco Glass, Inc., Vineland, N.J. 08360

<sup>9</sup>VWR Scientific Inc., Kansas City, MO 64141

the incubator. At the end of either 1 or 2 hours of incubation, the embryos were removed from the tubes and placed in 5 ml of mKRB in 16 x 50 mm tissue culture dishes<sup>10</sup>. They were rinsed in two more changes of 5 ml of mKRB then transferred to polystyrene tubes containing 50  $\mu$ l of 0.01 M Tris buffer at pH 7.4 plus 25  $\mu$ l of yeast RNA<sup>11</sup> (4 mg/ml in Tris).

The tubes were then frozen and thawed three times, followed by sonication for several hours until no embryo fragments were grossly visible. Contents of each tube were spotted on separate DEAE-cellulose filter papers (Whatman DE81, 23 mm diameter)<sup>12</sup> in a clean tissue culture dish. Each filter paper was washed with 15 changes of 5 ml of 0.5 M sodium phosphate (pH 9.2), for at least 10 minutes per wash. The filters were left to soak overnight in the last wash of 7.5 ml. The following day they were rinsed with sodium phosphate, followed by two quick rinses of distilled water, two changes of 95% ethanol and one with ether. The filter was air dried then counted in a Packard Tri-Carb 2425 liquid scintillation counter, using 10 ml of a toluene scintillation fluor<sup>13</sup>. Each vial was counted one minute three times per cycle for five cycles, giving 15 CPM estimates per embryo. Counts per minute (CPM) were converted to femtomoles ( $10^{-15}$  moles) per embryo per hour by the following formula:

$$\begin{array}{l} \text{Incorporated uridine} \\ \text{(femtomoles/hour)} \end{array} = \frac{\text{CPM/hour}}{\text{Quench} \times \text{Efficiency}} \times 1.82 \times 10^{-2}$$

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<sup>10</sup>Falcon, Oxnard, C.A. 93030

<sup>11</sup>Type XI, Sigma, St. Louis, MO 63178

<sup>12</sup>Fisher Scientific Co., St. Louis, MO 63132

<sup>13</sup>Research Products International Corp., Elk Grove Village, Ill. 60007

*RNA Digestion and Hydrolysis.* Embryo homogenates were divided into halves and one half embryo was subjected to digestion by adding 5  $\mu$ l RNase A<sup>14</sup> (1 mg/ml) for 6 hours at 37.5°C, or hydrolysis by adding a volume equal to the homogenate of 0.6 N NaOH and incubating for 18 hrs at 37.5°C. Counts per minute for digested or hydrolysed "halves" was then divided by the CPM for the non-hydrolysed or digested half to determine the proportion of incorporated label which was digestible or hydrolysable. Some homogenate halves were also subjected to 15  $\mu$ l DNase I<sup>15</sup> (10 mg/ml) for 3 hours at 37.5°C followed by an 18 hour alkaline hydrolysis as described above.

*Measurements.* Surface areas of embryos were calculated assuming the blastocyst is a prolate spheroid (Schacht and Foote, 1978). The formula used was:

$$\text{Surface area} = \frac{\sin^{-1} E \cdot a \cdot b \cdot 2\pi}{E} + 2\pi b^2$$

$$\text{where: } E = \frac{\sqrt{a^2 - b^2}}{a}$$

and: a = largest radius  
b = smallest radius

and the sine function is calculated in radians

For data analysis all embryos were classified as either typical or atypical. Mean and median values were calculated for typical and atypical blastocysts within each age group and reported on the basis of femtomoles <sup>3</sup>H-uridine incorporated per embryo per hour or per mm<sup>2</sup> surface area of embryo per hour. Inspection of the data indicated a non-normal distribution, and

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<sup>14</sup>Sigma, St. Louis, MO 63178

<sup>15</sup>Sigma, St. Louis, MO 63178

median values were lower than mean values since the distribution was skewed markedly to the left. Therefore data were analyzed using the Wilcoxon signed ranks test (Snedecor and Cochran, 1967) since it does not require the assumption of normality.

## Results and Discussion

*Products of  $^3\text{H}$ -Uridine Incorporation.* Calculations of incorporation of  $^3\text{H}$ -uridine into high molecular weight RNA are based on the assumption that all the label is in the form of RNA. It has previously been shown that  $^3\text{H}$ -uridine can be converted to cytidine and thymidine via ribonucleotide reductase, and then incorporated into DNA (Comings, 1966; Alexandre, 1977). In the present study treatment of RNA extracts with RNase and alkaline hydrolysis did not remove all of the radioactivity over background levels (table 2). Treatment with DNase, however, essentially removed this residual radioactivity. Warner and Hearn (1977b) found a similar proportion of  $^3\text{H}$ -uridine was incorporated into the DNA of mouse embryos of various ages. They ignored this small amount in calculations of incorporation into RNA. We also have not corrected our observations and therefore a small amount of the  $^3\text{H}$ -uridine incorporated must be assumed incorporated into DNA.

*Typical versus Atypical Blastocysts.* Typical embryos, the predominant morphological type, possessed a relatively smooth trophoblast, were moderately translucent to light, and in larger, older blastocysts, the trophoblast was frequently wrinkled. In contrast, atypical blastocysts displayed one or more of the following characteristics: dark (not translucent) when examined with transmitted light, a rough, bumpy contour, complete collapse of the blastocoel, or exceptionally small relative to the other blastocysts in the litter. These small blastocysts were often nearly transparent to transmitted light. Figure 2 shows both typical and atypical pig blastocysts.



TABLE 2. DIGESTION AND HYDROLYSIS  
OF BLASTOCYSTS LABELLED WITH  $^3\text{H}$ -URIDINE

Alkaline hydrolysis CPM <sup>a</sup>			Alkaline hydrolysis plus DNase digestion CPM		
Untreated half embryo	Treated half embryo	% CPM remaining <sup>b</sup>	Untreated half embryo	Treated half embryo	% CPM remaining <sup>c</sup>
525	40	7.6	972	0	0
955	45	4.7	3077	39	1.3
335	19	5.7	4045	0	0
322	34	10.6	624	0	0
639	153	23.9	272	0	0
			432	24	5.6
Mean = 10.5			Mean = 1.1		

<sup>a</sup>Counts per minute.

<sup>b</sup>After hydrolysis.

<sup>c</sup>After hydrolysis and DNA digestion.

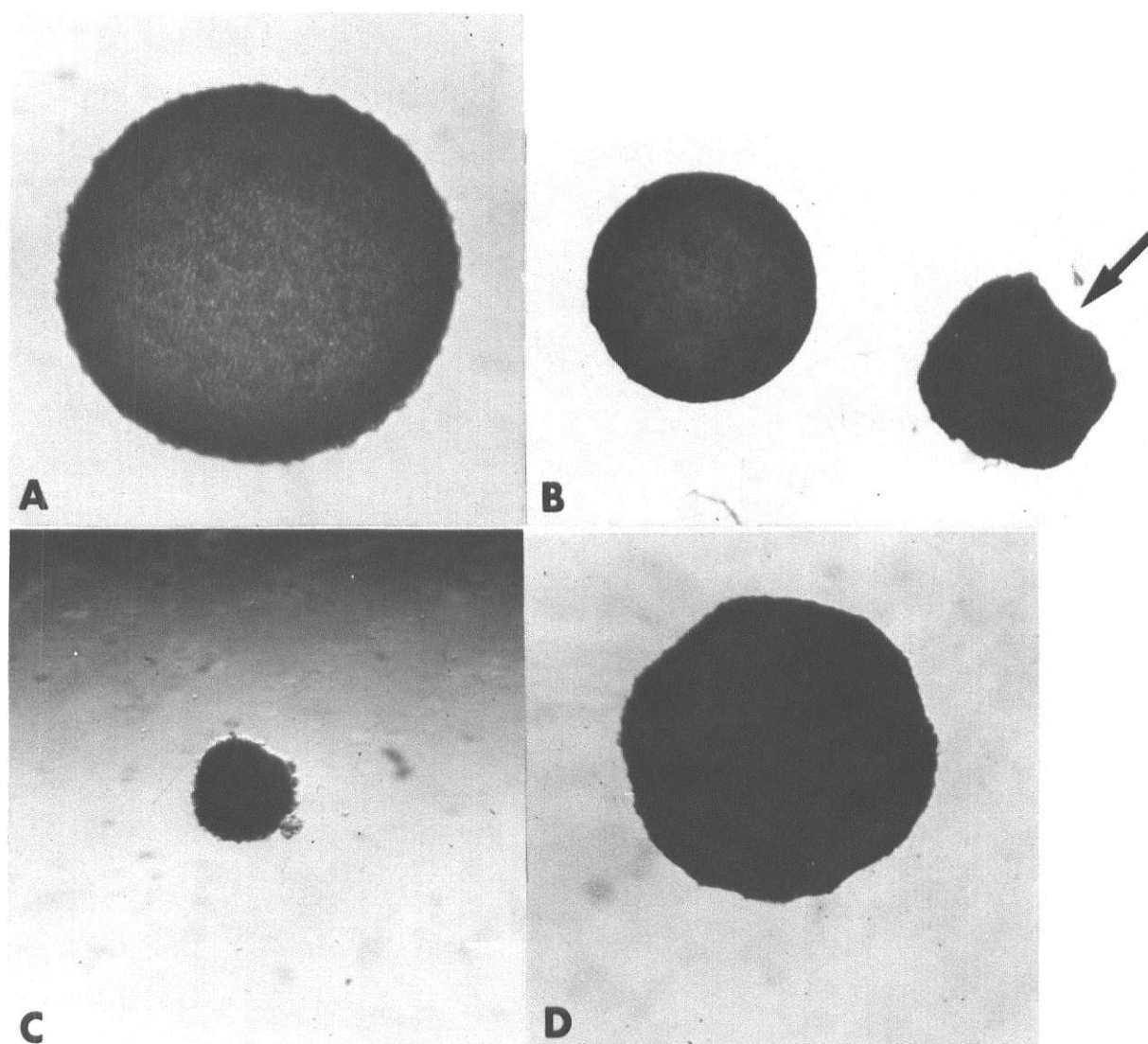


Figure 2. (A) Typical 8 day pig blastocyst (190X approx.)  
(B) Typical 8 day embryo plus atypical (arrow) rough and opaque embryo (45X approx.).  
(C) Atypical 9 day embryo, incorporated 45 fmol/hr. (35X approx.).  
(D) Atypical 10 day embryo, incorporated 81 fmol/hr. (40X approx.).

A few embryos had torn trophoblasts but were otherwise morphologically normal. Since the tears possibly occurred during recovery, and torn blastocysts incorporated as much or more  $^3\text{H}$ -uridine as typical embryos in their potential litter (table 3), they were considered typical. Atypical embryos consistently incorporated less  $^3\text{H}$ -uridine than typical embryos (tables 4 and 5) regardless of whether they were considered on a per embryo or per  $\text{mm}^2$  of surface area basis ( $P < .005$ ).

*Experiments.* Experiment I was undertaken to establish the pattern of  $^3\text{H}$ -uridine incorporation in day 8 embryos and was conducted in the spring and summer. Experiment II included day 9 and 10 blastocysts and was conducted in the summer and early fall. Table 6 summarizes all data on the basis of embryo age and season. Most of the day 9 and 10 embryos were incubated in the fall at which time levels of incorporation were considerably lower than during the spring or summer. Incorporation was higher for day 8 embryos than for day 9 or 10 embryos. However, since they were not incubated in the same season or with the same batch of  $^3\text{H}$ -uridine it is not possible to determine if these differences are due to embryo age or to other experimental variables.

Data for days 9 and 10 (experiment II) where all embryos had been incubated, was used to compare the frequency distribution of incorporation per embryo and per unit area of embryo (figures 3 and 4). When expressed as  $\text{fmol } ^3\text{H}\text{-uridine incorporated/embryo/hour}$ , differences were detected between day 9 and 10 blastocysts with the distribution for day 10 blastocysts shifted to the right for both typical ( $P < .005$ ) and atypical ( $P < .02$ ) embryos. However differences diminished and were no longer statistically significant when the data was expressed on a per unit surface area basis. Therefore differences between days 9 and 10 were likely due to embryonic growth characterized by a relatively constant incorporation per unit surface area.

TABLE 3. INCORPORATION OF  $^3\text{H}$ -URIDINE BY TORN EMBRYOS

Embryo description	No. embryos	Fmol/embryo/hr <sup>a</sup>	Fmol/mm <sup>2</sup> embryo/hr <sup>a</sup>
Typical, day 10	12	421 (200-607)	62.7 (18.3-97.9)
Torn <sup>b</sup> , day 10	3	534 (511-720)	56.7 (54.3-65.9)

<sup>a</sup>Median (range).

<sup>b</sup>Otherwise typical.

TABLE 4.  $^3\text{H}$ -URIDINE INCORPORATION  
BY TYPICAL AND ATYPICAL BLASTOCYSTS<sup>a</sup>

Day	Typical embryos		Atypical embryos	
	No. of embryos	Fmol/embryo/hr (Fmol/mm <sup>2</sup> embryo/hr)	No. of embryos	Fmol/embryo/hr (Fmol/mm <sup>2</sup> embryo/hr)
Experiment I				
8	32	581.9 (566.3)	9	76.6 (231.0)
Experiment II				
9	67	290.0 (115.8)	23	130.5 (57.2)
10	68	489.3 (149.7)	13	158.4 (44.1)

<sup>a</sup>All values are means.

TABLE 5.  $^3\text{H}$ -URIDINE INCORPORATION  
BY TYPICAL AND ATYPICAL BLASTOCYSTS<sup>a</sup>

Day	Typical embryos		Atypical embryos	
	No. of embryos	Fmol/embryo/hr (Fmol/mm <sup>2</sup> embryo/hr)	No. of embryos	Fmol/embryo/hr (Fmol/mm <sup>2</sup> embryo/hr)
Experiment I				
8	32	341.5 (390.2)	9	34.0 (86.2)
Experiment II				
9	67	80.0 (38.6)	23	14.0 (19.1)
10	68	278.5 (46.5)	13	91.0 (25.1)

<sup>2</sup>All values are medians.

TABLE 6. SEASONAL VARIATION IN  $^3\text{H}$ -URIDINE  
INCORPORATION BY TYPICAL PIG BLASTOCYSTS

	Early spring	Spring	Summer	Fall
Day 8				
No. embryos	11	13	8	
No. donor gilts	2	2	1	
Fmol/embryo/hr <sup>a</sup>	1238.0	281.8	167.3	
Fmol/mm <sup>2</sup> embryo/hr <sup>a</sup>	959.0	413.8	275.6	
Day 9				
No. embryos		2	12	53
No. donor gilts		1	1	6
Fmol/embryo/hr		232.5	1209.7	84.0
Fmol/mm <sup>2</sup> embryo/hr		184.2	439.3	36.3
Day 10				
No. embryos		12	6	50
No. donor gilts		1	1	5
Fmol/embryo/hr		598.6	2323.7	242.9
Fmol/mm <sup>2</sup> embryo/hr		380.9	639.3	35.9

<sup>a</sup>All values are means.

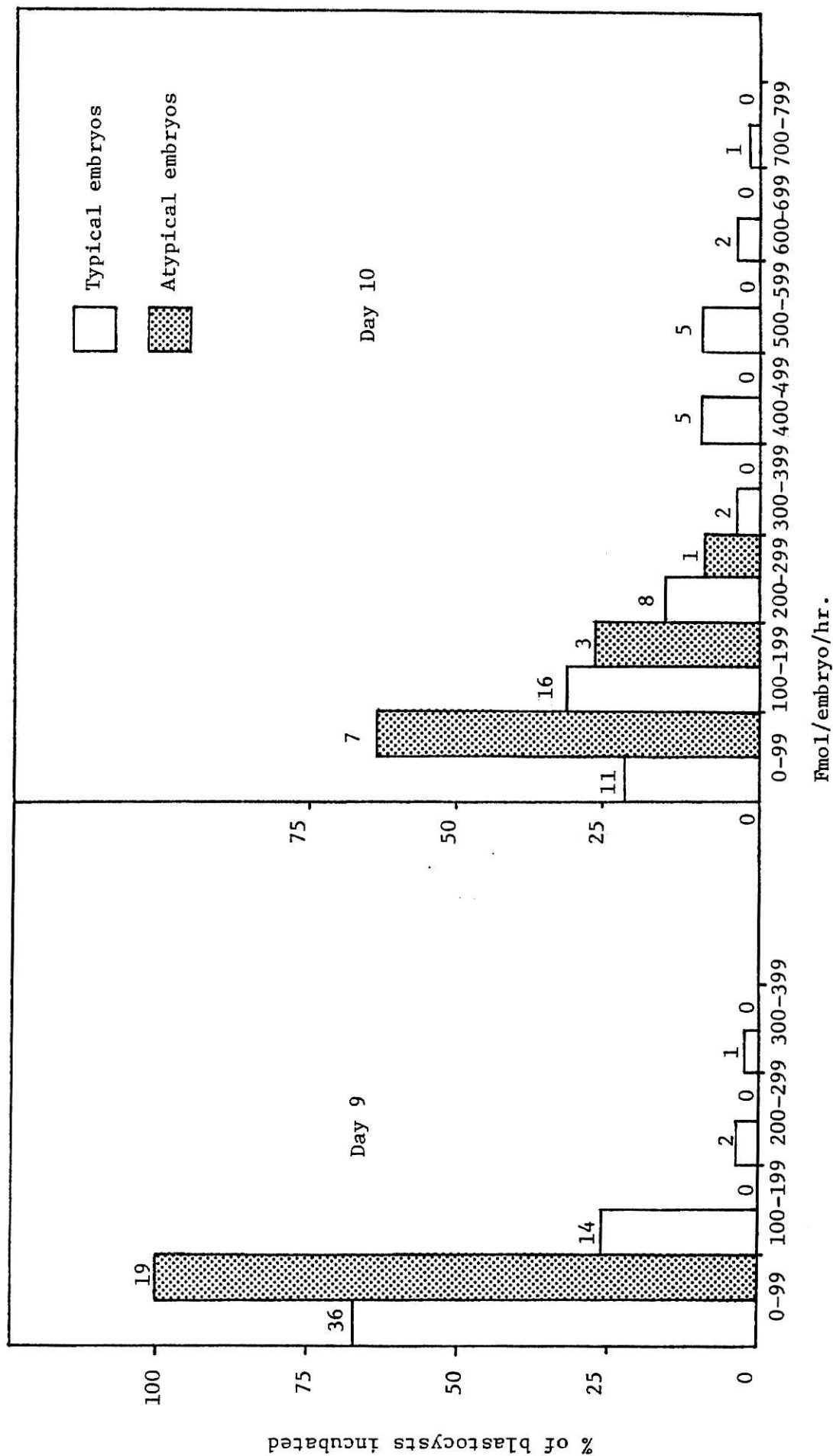


Figure 3. Incorporation of  $^3\text{H}$ -uridine by typical and atypical blastocysts recovered 9 and 10 days after estrus. Value over each column refers to number of embryos in that class.



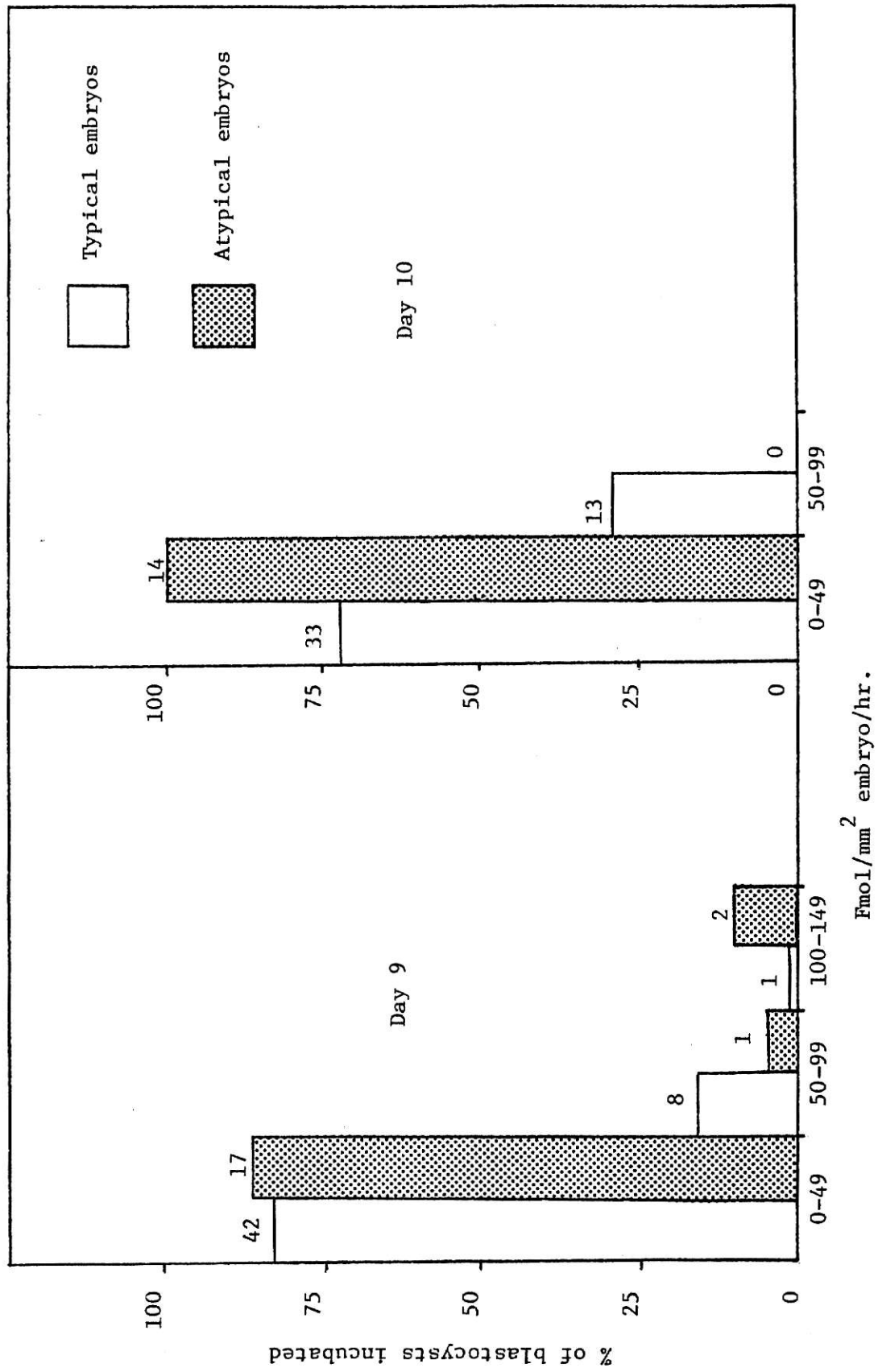


Figure 4. Incorporation of <sup>3</sup>H-uridine by typical and atypical blastocysts per unit surface area. Embryos were recovered 9 and 10 days after estrus. Value over each column refers to number of embryos in that class.

This relationship was further analyzed by investigating the correlation between surface area and  $^3\text{H}$ -uridine incorporation for days 9 and 10. Correlation coefficients for typical blastocysts at these two ages ( $r = .735$  and  $.667$ , respectively) were not different ( $P > .2$ ), and when pooled gave a correlation coefficient of  $.7035$ . The values for atypical embryos at days 9 and 10 ( $r = .499$  and  $.426$ ) also were not different ( $P > .45$ ), and when pooled gave a correlation coefficient of  $.476$ . Therefore approximately half of the variation in  $^3\text{H}$ -uridine incorporation by typical embryos can be explained by embryo size, but only 23% of the variation in incorporation by atypical blastocysts can be attributed to their size. The weaker correlation for atypical embryos suggests that their surface area is not as closely related to  $^3\text{H}$ -uridine incorporation as it is for typical embryos. Sources of variation other than embryo size may include differences in the ability to take up  $^3\text{H}$ -uridine and phosphorylate nucleosides which would affect the size of UTP pools, and the capacity to synthesize RNA. Any of these functions may be related to blastocyst viability.

The distribution of typical, atypical and missing ova (based on the number of CL observed) is presented in table 7. Eggs or blastocysts recovered as a percent of CL usually averages 80 to 90% during the first week of pregnancy (Perry and Rowlands, 1962; Hunter, 1974). The majority of this discrepancy before blastocyst hatching is probably due to procedural error (i.e. failure to recover all of the eggs). After 7 days few unfertilized eggs retain the zona pellucida and therefore would either disintegrate or become unidentifiable. There is no reason to expect differences in the percent blastocysts present which would be recovered for the interval studied here since they are all preattachment stages. However, if atypical embryos die and disintegrate between days 8 and 10, one would expect a greater

TABLE 7. DISTRIBUTION OF TYPICAL,  
ATYPICAL AND UNACCOUNTED FOR OVA

Embryo age (days)	No. of corpora lutea	% of corpora lutea accounted for by:		% ova not accounted for
		Typical embryos	Atypical embryos	
Experiment I				
8	66	27.3	60.6	12.1
Experiment II				
9	99	32.3	63.7	4.0
10	106	13.2	69.8	17.0

percent of the CL represented by atypical blastocysts at the earlier stages. The data in table 7 suggest such a trend. Since day 8 recoveries were not conducted during the same period as day 9 and 10 recoveries, the most meaningful comparison is between the latter two embryo ages. The percent atypical blastocysts decreased dramatically from day 9 to day 10 ( $P < .005$ ). This would be expected if atypical embryos were able to develop, although perhaps aberrantly, for a period of time. This possibility is supported by the observation of some, although significantly less ( $P < .005$ ),  $^3\text{H}$ -uridine incorporation by a typical blastocysts. Also, some atypical embryos are able to undergo trophoblast expansion *in vitro* (O. I. Dawydiak and D. L. Davis, unpublished observations). Furthermore, the percent normal embryos at all 3 days remains approximately constant, with the atypical and lost ova combined representing 30.2 to 39.4% of the CL counted. Based on these observations we suggest that atypical embryos are indeed abnormal and likely contribute substantially to the embryonic wastage observed by day 25.

A related consideration is the unknown effect of atypical embryos on pregnancy maintenance. In three cases there were less than four typical embryos, and in one of these all the blastocysts recovered were atypical. Polge *et al.* (1966) have shown that when fewer than four embryos are present, the cycle length is extended although the pregnancy is not maintained, and this may have been the fate of these three pregnancies had they been allowed to continue. In addition, it may be that atypical embryos can extend the cycle length. Possibly both of these situations contribute to the incidence of bred females returning to estrus after a period of 25 or more days.

Since abnormalities are apparent in day 8 embryos, morphologically identifiable as blastocysts, it is likely that aberrations occur during the peri-hatching period. It is at this time that the critical changes in RNA

and protein metabolism become apparent in mammalian embryos. During this period the morula undergoes cavitation to form a blastocyst, and trophoblast and ICM differentiate. Any mistakes in the normal sequence of developmental events would become evident at this time. How long abnormal embryos may continue to develop is unknown, but abnormal trophoblasts or fragments have been detected at 12 to 14 days of pregnancy (Corner 1923). It would be interesting at this point to increase the number of observations and to score even earlier embryonic stages.

*Conclusions.* Morphologically atypical pig blastocysts incorporated less  $^3\text{H}$ -uridine into high molecular weight RNA than did typical blastocysts at 8, 9 and 10 days of age. These studies have shown that aberrant embryonic development is apparent as early as day 8 and may be the first indication of dying embryos contributing to the high losses seen by day 25. In addition, we believe that measurement of  $^3\text{H}$ -uridine incorporation may prove to be a useful tool for evaluating embryonic development, and our results demonstrate the importance of considering the morphology of embryos when studying their biosynthetic capabilities.

## CHAPTER IV

## SUMMARY

Embryos were surgically recovered from artificially inseminated Yorkshire gilts 8, 9 or 10 days after estrus. The blastocysts were measured and morphologically scored as typical (smooth trophoblast, moderately translucent to light) or atypical (opaque, rough, collapsed or exceptionally small). Embryos were then incubated in a medium containing  $^3\text{H}$ -uridine, the high molecular weight RNA extracted and radioactivity counted.

Atypical embryos gave consistently lower values than typical embryos at all ages ( $P < .005$ ). Incorporation of  $^3\text{H}$ -uridine on a per embryo and per unit surface area basis was higher for day 8 blastocysts than for days 9 or 10. However, all day 8 embryos were incubated in the spring or summer and most day 9 and 10 embryos were incubated in the fall. The lowest levels of incorporation occurred in the fall, making it impossible to separate the effects of season and age on incorporation of  $^3\text{H}$ -uridine.

The pooled correlation coefficient for the relationship between surface area and  $^3\text{H}$ -uridine incorporation in typical day 9 and 10 embryos was .7035. Therefore, approximately 50% of the variation in incorporation by typical embryos could be explained by differences in blastocyst size. The pooled correlation coefficient for atypical 9 and 10 day embryos was only .476, indicating that 23% of the variation in incorporation was due to size. Other sources of variation in these embryos may include the ability to take up uridine, phosphorylate nucleosides and synthesize RNA. These functions are likely related to blastocyst viability.

The proportion of corpora lutea represented by typical, atypical and unaccounted for ova changes between days 8, 9 and 10. Percent atypical

blastocysts are similar for days 8 and 9 but decrease dramatically on day 10 ( $P < .005$ ). It is possible that aberrant embryos, still recognizable as blastocysts at days 8 and 9, disintegrate and disappear by day 10. The proportion of typical embryos does not vary as much (.606 to .698) and is representative of the embryos surviving to day 25 of pregnancy (.6-.7; Hanly, 1961; Wrathall, 1971). Therefore embryos as early as day 8, which have an atypical morphology and tend to incorporate less  $^3\text{H}$ -uridine into RNA may account for much of the early losses in pregnancy.

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INCORPORATION OF  $^3\text{H}$ -URIDINE BY PIG BLASTOCYSTS IN VITRO

by

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AN ABSTRACT OF A MASTER'S THESIS

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The majority of embryonic losses in the pig occur early in pregnancy. Ribonucleic acid synthesis, as measured by incorporation of  $^3\text{H}$ -uridine, is a sensitive indicator of gene activity in developing embryos and is therefore potentially useful for determining viability of embryos within and among litters.

Twenty-six Yorkshire gilts were checked for estrus twice daily and were artificially inseminated 12 and 24 hours after onset of estrus (day 0). Embryos were recovered surgically on days 8, 9 or 10, and corpora lutea counted. Blastocysts were measured and their morphology recorded. Blastocysts that were opaque, rough, collapsed or very small and translucent were classified as atypical. All embryos were incubated individually in a modified Krebs-Ringer bicarbonate medium containing 10% fetal calf serum and  $20\text{ }\mu\text{M}$   $^3\text{H}$ -uridine for 2 hours at  $37.5^\circ\text{C}$ . Blastocysts were then washed and radioactivity counted.

Typical embryos incorporated more uridine per embryo and per  $\text{mm}^2$  surface area than atypical embryos ( $P < .005$ ) at all ages. More uridine was incorporated at day 8 than at days 9 or 10. There may also have been a seasonal effect since day 8 blastocysts incorporated more uridine earlier in the year, and day 9 and 10 blastocysts incorporated more in the summer than in the spring or fall. Low values were obtained in the fall, and since most 9 and 10 day blastocysts were incubated in the fall, season and embryo age are confounded.

The correlation coefficient for the relationship between surface area and uridine incorporation in typical day 9 and 10 embryos was .7035. This suggests that approximately 50% of the variation in incorporation by typical embryos is explained by differences in blastocyst size. The correlation



coefficient for atypical day 9 and 10 embryos was .476, indicating that only 23% of the variation in incorporation was due to size. The lower incorporation and poorer relationship of blastocyst size to incorporation by atypical embryos may have resulted from defects in their ability to take up uridine and/or synthesize RNA, both of which are likely related to embryo viability.

The typical blastocysts recovered per corpus luteum remained approximately the same at all ages, varying from .588 to .676. However, the proportion of atypical blastocysts decreased dramatically between days 9 and 10 (.284 to .163;  $P < .005$ ). It is possible that some atypical embryos identifiable as blastocysts on days 8 and 9 disintegrate by day 10. These results indicate embryos with atypical appearance are metabolically inferior and that such embryo defects appear as early as 8 days after the onset of estrus.