INVESTIGATIONS OF STEROIDOGENIC CAPABILITIES OF THE EARLY EMBRYO

by

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INTRODUCTION

One of the greatest problems facing dairymen today is that of infertility. Many cows will, after having been bred, return to heat with a prolonged estrous cycle. This appears to indicate that fertilization has occurred, with subsequent early embryonic mortality. Early embryonic death may be as high as 15% in reproductively normal cows, but this may approach 65% in cows requiring more than three services (Tanabe and Casida, 1949). If this early embryonic mortality could be prevented or reduced, the economic benefits to the dairyman would be astounding.

In order to be able to prevent this anomaly, it is imperative that we gain considerably more knowledge about the physiological requirements of the embryo during its early development. Beacuse the free-lying blastocyst is a very delicate organism, a small imbalance in its maternal environment or an imperfection in its own genetic make-up may lead to its demise.

The preimplantation embryo may play a role in regulating the use of constituents of its environment or in initiating recognition by the maternal organism and eventual attachment to the uterus. Dickmann and Dey (1973) have postulated that the preimplantation embryo is capable of steroidogenesis and that certain steroids may affect morula-to-blastocyst transformation as well as implantation. Indirect, histochemical evidence indicates the presence of Δ^5-3β-hydroxysteroid dehydrogenase activity in mouse morulae and blastocysts which has been shown to be necessary for steroidogenesis (Dey and Dickmann, 1974c). However, Sherman and Atienza (1977) have disputed the steroidogenic

activity of this enzyme with evidence that the mouse blastocyst is not capable of progesterone or androstenedione biosynthesis when direct precursors are supplied.

The objectives of this investigation were as follows:

(1) to determine if the viable mouse blastocyst could produce estrogen or progesterone in a completely defined culture media void of the maternal environment.

(2) to examine morula, early blastocyst and late blastocyst stages of early embryo development in the mouse and determine if estrogen and progesterone are being produced.

REVIEW OF LITERATURE

Biochemical Aspects of the Developing Embryo

The mammalian preimplantation embryo is a metabolically active organism that goes through many changes from the time it is fertilized by the male gamete. The fusion of pronuclei from sperm and ovum is followed by approximately a 48 hour interval in the mouse during which there are definite structural and metabolic changes (Biggers, 1971). These changes, which have occurred by the 8-cell stage, are probably controlled by the maternal genome. It has been suggested that the restricted patterns of energy metabolism characteristic of the first three cleavage stages are maternally determined and possibly arose during differentiation of the oocyte (Biggers, et. al., 1967).

The eggs of mammals have very little yolk or food reserve and their total lipid content is low (Mintz, 1964). Twelve percent of the total dry mass of 1-cell mouse ova consists of lipids (Loewenstein and Cohen, 1964). Therefore, at this early stage, the embryo is almost completely nutritionally dependent on its environment. Austin and Lovelock (1958) determined that the vitelline membrane and zona pellucida of viable rat, rabbit and hamster embryos were permeable to molecules of molecular weights less than 1200. They attempted to determine metabolic pathways existing in the early embryo as evidenced by the ability of substances of differing molecular weights to pass through the cell membrane.

Biggers, et. al. (1967) reported that pyruvate and oxaloacetate supported the first cleavage division in the mouse ovum in the absence of cumulus cells. However, lactate, phosphoenolpyruvate and glucose

were capable of supporting the first cleavage when cumulus cells were included in culture. This indicated the ability of cumulus cells to metabolize certain substrates into products utilized by the zygote.

When 2-cell mouse embryos were cultured in a medium containing radioactive sodium pyruvate, lactate was found to be the main metabolite accumulated in the medium (Wales and Whittingham, 1970). In addition to pyruvate, energy sources which adequately supported 2-cell mouse embryos were oxaloacetate, phosphoenolpyruvate and lactate (McLaren, 1972). The 2-cell rabbit embryo was sufficiently supported by phosphoenolpyruvate, pyruvate and lactate when used as sole energy sources (Daniel, 1967). These reports correspond well to the observed increase in lactate secretion from the rabbit endosalpinx after mating and ovulation (Mastroianni and Wallach, 1961; Holmdahl and Mastroianni, 1965). The 2-cell mouse embryo can utilize lactate as the sole exogenous energy source, suggesting the importance of lactate dehydrogenase in the oxidation of this substrate (Auerbach and Brinster, 1967). Lactate dehydrogenase activity has, in fact, been shown to be extremely high the first three days of gestation in the mouse (Brinster, 1965a).

Malate did not support 2-cell mouse embryos in vitro (Wales and Biggers, 1966; 1968; Brinster, 1965c). However, malate dehydrogenase was shown to be present in the embryo at this stage (Brinster, 1966b). Thus, it would be expected that, if malate could penetrate 2-cell embryo membranes it would be utilized, provided there is no inhibition of malate dehydrogenase. This suggests that malate is not taken up by the 2-cell embryo (Wales and Biggers, 1968). Indeed, when incubated

with ^{14}C-L-malate, very little accumulation of ^{14}C was detected with 2-cell mouse embryos (Wales and Biggers, 1966).

Glucose did not support growth of 2-cell embryos to the blastocyst stage (Mintz, 1964; Brinster, 1965c; 1965e). This observation suggests that glucose could not be metabolized by the early embryo or that the cells were not permeable to glucose. Insulin did not allow glucose to support 2-cell development to the blastocyst stage either (Brinster, 1965e). Therefore, the failure of glucose to support cleavage was not a result of impermeability, but was probably due to a lack of necessary enzyme systems.

There was a 100-fold increase in the conversion of glucose to CO_2 during the first five days of development in the mouse embryo (Brinster, 1967a). An increase in O_2 consumption by the mouse preimplantation embryo was also evident starting with the 8-cell stage (Mills and Brinster, 1967). Carbon dioxide production doubled between the 2- and 8-cell stages (Brinster, 1967a). However, between these same cleavage stages, permeability of the embryo to glucose only increased slightly (Wales and Brinster, 1968). It therefore seems possible that the increase in utilization of glucose was the result of a change in enzyme activities, specifically the Embden-Meyerhof enzymes (Brinster, 1968). Hexokinase is a regulatory enzyme in the glycolytic sequence and changes in its activity could account for increased utilization of this metabolic pathway (Lehninger, 1975). However, CO_2 production and carbon accumulation from glucose in mouse embryos rose more rapidly than hexokinase activity, indicating that changes in other enzymes,

as well as hexokinase, may be involved in increased glucose oxidation (Brinster, 1968).

Pyruvate and oxaloacetate, which supported development of 2-cell mouse embryos into blastocysts, also support growing 8-cell embryos. Other tricarboxylic acid cycle intermediates which permitted blastocyst formation were malate, citrate and α-ketoglutarate. Succinate, glucose 6-phosphate or fructose 1,6-diphosphate, as sole energy sources, were shown to be able to support 8-cell mouse embryo development (Brinster and Thomson, 1966). Eight-cell embryos incubated with ^{14}C-malate yielded labelled CO_2, which was evidence for the uptake and metabolism of that substrate (Wales and Biggers, 1968).

The activities of lactate dehydrogenase and glucose-6-phosphate dehydrogenase remained constant up to the 8-cell stage but then underwent a 7-fold decrease in activity to the late blastocyst stage (Brinster, 1966b). The decrease in lactate dehydrogenase activity may mean a decrease in dependence on lactate with advancing stages of preimplantation development (Brinster, 1965a). Malate dehydrogenase showed an increased activity in the preimplantation stages. Generally speaking, glucose 6-phosphate dehydrogenase and lactate dehydrogenase play more important roles during the first three days of development and malate dehydrogenase becomes more active at later stages (Brinster, 1966a). These enzyme patterns suggest that glucose oxidation is accomplished via the pentose shunt before blastocyst formation and that, following this period, oxidation appears to be via the Embden-Meyerhof pathway and tricarboxylic acid cycle (Brinster, 1966b).

Concurrent with the metabolic changes that take place in the 8-cell mouse embryo, this stage apparently is the point of interaction between paternal and maternal genomes and their subsequent translation and transcription (Biggers, 1971). The unfertilized egg had abundant RNA and the amount of RNA either slightly increased or decreased during the first few cleavages (McLaren, 1972). When ^{3}H-uridine was incubated with mouse embryos as a precursor of RNA, the rate of RNA synthesis was increased in 2-cell mouse embryos when compared to the rate of synthesis from fertilization to cleavage (Mintz, 1964). Since RNA synthesis was detected during the early cleavage stages and the overall RNA content at this time may decrease, breakdown of RNA must also occur.

Incorporation of ^{3}H-uridine was shown to increase in mouse, rabbit and hamster embryos from the 2-cell stage onward (McLaren, 1972). At the 2-cell stage, the label appeared to be confined to the nucleus and was detected in the cytoplasm for the first time at the 4-cell stage. At the latter stage, labelling of the nucleus was concentrated over the nucleoli, suggesting that synthesis of ribosomal RNA was occurring (McLaren, 1972). Incorporation of ^{3}H-uridine was reported by Monesi and Salfi (1967) to increase sharply at both the morula and blastocyst stages to 90 times its level at ovulation. Data suggest that the largest fraction of cytoplasmic RNA in the morula is of nucleolar origin (Mintz, 1964). Since nucleolar formation is genetically determined, RNA synthesis is an expression of genetic activity during cleavage.

Coincidental with the first major use of the embryonic RNA templates, the rate of protein synthesis increases rather markedly from

the morula to the blastocyst stage (McLaren, 1972). Prior to these stages, the incorporation of ^{3}H-leucine into protein by mouse embryos was present before fertilization but then remained low until the 8-cell stage (Monesi and Salfi, 1967). Greenwald and Everett (1959) have demonstrated similar patterns in uptake of ^{35}S-methionine by the mouse embryo. These results correlate well with the total protein content of the mouse embryo during these developmental stages (Brinster, 1967b).

The first cleavage of rabbit zygotes is known to occur in media free of amino acids (Daniel and Olson, 1968), but the second cleavage requires cysteine, tryptophan, phenylalanine, lysine, arginine and valine. Subsequent cleavage to the morula stage requires methionine, threonine and glutamine. Of the 21 constituent amino acids of bovine serum albumin, only 4 did not support cleavage of 8-cell mouse embryos to the blastocyst stage when employed alone; these being tyrosine, tryptophan, arginine and isoleucine (Brinster and Thomson, 1966). When early cleavage mouse embryos were treated with actinomycin D (10^{-7} M) in vitro, RNA synthesis was reduced by 90 percent, with a concurrent depression in protein synthesis of only 50 percent (McLaren, 1972). This finding was interpreted to mean that, by the 4-cell stage, 50 percent of the protein synthesis still depended upon m-RNA synthesized during oogenesis or just after fertilization, while the other half depended on newly-synthesized m-RNA. Evidently, although RNA and protein are both synthesized by the embryo from an early stage, the embryonic genetic material does not begin to control development until the 8-cell stage.

Effects of Physiological Agents on Preimplantation Development

As the mammalian embryo undergoes cleavage to the blastocyst stage it is either traversing the length of the oviduct or is free-lying within the uterine lumen. Also, during this period of early development, certain physiological substances have favorable and/or necessary effects on the embryo. Krishnan and Daniel (1967) described the secretion of a rabbit uterine protein, "blastokinin" (BKN), which appeared just before the time of blastulation. A maximum secretion rate of BKN was attained at 5 days post coitum and BKN was detected as one of the major protein components of 6-day rabbit blastocoelic fluid. This protein (BKN) was isolated and tested on developing rabbit embryos *in vitro*, showing favorable effects on growth rate.

Using electrophoretic and immunologic techniques, Beier (1968) described a uterine protein, "uteroglobin," found in uterine secretions and blastocoelic fluid of rabbits. It is now held that uteroglobin and blastokinin are identical proteins.

Blastokinin has been characterized as having a molecular weight of 27,000. Peak BKN concentrations were reached in rabbit uterine secretions at five days post coitum and in blastocoelic fluid at about 7 days post coitum (Hamana and Hafez, 1970). Its brief appearance in the blastocyst and uterine fluids suggests that its physiological role is restricted to the phase of embryonic development between hatching of the blastocyst and subsequent implantation.

Beier and Mauer (1975) compared protein patterns of blastocoelic fluids of the rabbit after *in vivo* and *in vitro* development. Large

differences in protein patterns were found between the systems supporting the interpretation that BKN and other proteins originate in the uterine endometrium and were not synthesized by the preimplantation embryo. There were, apparently, diffusion channels present in the thick, dense mucoprotein covering of the rabbit blastocyst, which would permit rapid passage of these proteins into the blastocyst (Kirchner, 1972).

Beier (1968), after administering progesterone and estrogen to intact rabbits, found blastocyst degeneration. He suggested that there may be a direct relationship between the BKN level and blastocyst development. Urzua, et. al. (1970) also demonstrated that BKN is present in the uterine fluids of ovariectomized rabbits receiving progesterone or progesterone and estradiol but not in those receiving only estradiol. By transferring blastocysts to the uteri of castrate rabbits that had previously received progesterone, Arthur and Daniel (1972) demonstrated that the appearance of BKN in the uterine fluids is regulated by this steroid. Bilateral ovariectomy of rabbits at 6.5 days post coitum was shown to completely prevent further growth of the blastocysts in the following 24 hours, as well as to prevent the accumulation of proteins in the blastocoelic fluids (Sugawara and Hafez, 1967). Both of these effects were alleviated by administration of progesterone. This series of studies suggests that progesterone is the stimulus for secretion of BKN from the endometrium of rabbits.

Urzua, et. al. (1970) reported that BKN has the capacity to bind progesterone and only minimal amounts of estradiol. The greatest binding of progesterone occurred on day 5, which is the same day that

peak concentrations of BKN appear in uterine secretions. These findings support those of Beato, et. al. (1975), which demonstrated that a BKN-specific antiserum preparation in guinea pigs completely inhibited the progesterone binding activity of uterine fluid proteins. Arthur, et. al. (1972) reported that BKN binds both progesterone and estradiol. Using equilibrium dialysis and isolated BKN, they showed that progesterone inhibits the binding of estradiol-17β, and vice versa. This inhibition phenomenon may be competitive or non-competitive. If it is a non-competitive type of inhibition, a small amount of estradiol-17β could result in a major change in the amount of bound progesterone. Therefore, small changes in the concentration of estrogen may be an important factor in the control of the interaction between the endometrium, BKN, progesterone and the embryo.

Progesterone and estrogen have other effects on developing blastocysts in vitro in addition to those related to the secretion of BKN. Weitlauf and Greenwald (1968a) detected the incorporation of ^{35}S-methionine by mouse blastocysts using radioautography. They found significant incorporation only after combined administration of progesterone and estrogen to ovariectomized mice. Estradiol did not stimulate the incorporation of ^{14}C-valine into morulae but there was a significant uptake and incorporation of valine by the expanded mouse blastocyst (Smith and Smith, 1971).

Roblero (1973) noted that embryos recovered from ovariectomized, progesterone-treated mice had significantly fewer cells than controls, but more cells when compared to the embryos of ovariectomized mice without treatment. Therefore, the maternal ovarian hormones may control

factors of embryonic development such as cleavage rates in mice. However, in the rabbit, progesterone had no stimulatory or other detectable effect on blastocysts when administered at 1 mg/animal on days 1 to 4 of pregnancy (Adams, et. al., 1961).

Estradiol benzoate, when administered to rabbits on days 4 to 5 post coitum, caused marked uterine hypertrophy and often premature implantation (Adams, et. al., 1961). Although the embryos recovered from these uteri were degenerative, the effect was believed to be due to the grossly altered uterine environment and not a direct effect on the embryos. Daily injection of estrogen, beginning on the day of mating in both mice and rabbits, resulted in oviductal retention of the embryos (Burdick and Pincus, 1935). All ova, whether retained or transported to the uterus, were degenerate by day 4. The major effect of the estrogen administration, secondarily to oviductal retention, was degeneration of the ova before their capability to implant.

When morula transfer in rats was proceeded by 2, 3, 4, 5 or 6 days of progesterone treatment, decreasing percentages of morulae developed to term (Dickmann, 1970). The reduced littering rate could be interpreted as being due to over-stimulation by progesterone, lack of estrogen or both. Estrogen and progesterone are known to have many antagonistic and synergistic effects on each other in their roles of monitoring mammalian reproductive functions. An excellent review of many of these interactions has been compiled by Courrier (1950).

Endocrine Effects on Embryonic Development *In Vitro*

The ovarian hormones (estrogen and progesterone) have various effects on embryogenesis in the maternal organism and have been shown

to exhibit definite effects *in vitro*. Progesterone, in doses greater than 2 µg/ml of culture media, was demonstrated to be antagonistic to development of mouse ova (Whitten, 1956b; 1957b). Blastocyst viability was affected to a great extent by progesterone, while estrogen had no antagonistic or protective actions against the effect of progesterone. Progesterone blocked cleavage of rabbit pre-blastocyst embryos at concentrations of 10 µg/ml or more (Daniel and Levy, 1964). Overshadowing this report on the effects of free progesterone was a study by El-Banna and Daniel (1972). They postulated that progesterone may be useful or even essential to the normal development of the rabbit blastocyst when in complex with a carrier protein. The cultured day 5 rabbit blastocysts in media supplemented with either progesterone alone or progesterone in combination with macromolecular components of serum or the fluids of day 5 pregnant rabbits. The embryos grew best, and took up greater amounts of uridine and amino acids in the medium containing progesterone and the uterine components, than either of the other treatments.

Ketchel and Pincus (1964) demonstrated that a 24-hour exposure to estrogens (.1 mg/ml) was lethal to day 1 rabbit fertilized ova *in vitro*. However, an estrogen exposure of five minutes followed by transfer to pseudopregnant recipients showed no apparent effect on recovery of live offspring. Therefore, either five minutes was not sufficient time for estrogen to have its full lethal effect, or the effect was initiated *in vitro* but reversed *in vivo* upon transfer. When incubated with 25 µg/ml estradiol, and several hours remain between the exposure time and expected cleavage, 1-cell rabbit embryos fail

to cleave (McGaughey and Daniel, 1966). If the concentration was reduced to 10 μg/ml, or cleavage was imminent at exposure, the embryos fragmented rather than cleaved. Also, 2- and 4-cell embryos appeared to be more resistant to estrogen and the degree of fragmentation was antagonized by an equimolar inclusion of progesterone. Rabbit embryos, after seven hours of culture in media with any one of the three major estrogens in concentrations of .03 or .06 mM, appeared to have cleaved to at least eight cells, while controls had cleaved only once (Daniel, 1964). These eight cells were different sizes. This may have resulted from fragmentation rather than cleavage. Indeed, the 8-cell embryos contained no nuclei and, at best, only a few chromatin masses were found.

There was a general trend toward resistance to steroids as the embryo progressed in development and the total cell surface became larger (Daniel and Levy, 1964; Kirkpatrick, 1971). After culturing pre-morula rabbit embryos in ^{14}C-progesterone for eight hours, labelling was autoradiographically found on the surface of the embryo and zona pellucida (Daniel and Levy, 1964). It was concluded that progesterone blocks cleavage of rabbit embryos by limiting the supply of protein or amino acids, thereby inhibiting protein synthesis within the embryo. Thus, as development proceeds and the cell surface area increases, the steroid molecules may be spread too sparsely to block protein or amino acid entrance (Kirkpatrick, 1971).

Role of the Embryo in Maternal Recognition of Pregnancy

The developing embryo has been implicated in local and systemic mechanisms of corpus luteum maintenance during the early phase of gestation in several mammalian species. While the embryo is still free-lying within the uterus, there appears to be an embryonic-endometrial-ovarian interaction which "signals" the characteristic maintenance of the corpus luteum longer than its normal life-span in a non-gravid cycle. This embryonic effect is probably anti-luteolytic in nature rather than luteotropic (Rowson and Moor, 1967). The ovine estrous cycle was significantly prolonged after daily intra-uterine infusion of homogenate prepared from frozen tissue of 14 or 15 day sheep embryos. Similar material from 25 day sheep embryos did not lengthen the estrous cycle. The active principle from these embryos was described as being chemical in nature when retained after storage at -20C (Rowson and Moor, 1967).

If sheep embryos were removed from the uterus by day 12 of pregnancy, the life-span of the corpus luteum was not affected (Moor and Rowson, 1966b). A delay in removal to days 13, 14 or 15 caused a marked extension of luteal function. Transfer of embryos to non-pregnant ewes up to 12 days after estrus resulted in pregnancy, but transfer on days 13 or 14 had little or no effect on the life-span of the corpus luteum (Moor and Rowson, 1966a). These combined findings indicated that an anti-luteolytic or luteotropic effect is exerted by the embryo and is essential for maintenance of the corpus luteum of pregnancy after day 12.

Similarly, Dhindsa and Dzuik (1968) investigated the influence on continued pregnancy of killing embryos or fetuses in one uterine horn of the gilt at times from day 4 to day 50 of gestation. They concluded that pig embryos must be present in both uterine horns between days 10 and 12 for continuation of gestation.

Williams, et. al. (1967) interperitoneally injected pseudopregnant rabbits with dried preparations of late-luteal and early-estrual bovine uterus. In 64 percent of the rabbits receiving a single dose of either uterine powder, corpus luteum regression was induced, as well as development of follicles and depression of acetate incorporation into progesterone in in vitro ovarian cultures. These workers proposed that bovine uterine preparations contained a protein uterine luteolytic hormone which affects ovarian function in the rabbit without the direct involvement of LH. This study supported the concept of an embryonic anti-luteolytic effect as opposed to a luteotropic effect.

The mechanism by which the uterus affects lysis of the corpus luteum was shown to depend on the amount of viable endometrium in the gilt (Anderson, 1968). Evidence from auto-transplantation of the uterus in guinea pigs implied that a blood-borne mechanism was involved in controlling corpus luteum life-span (Butcher, et. al., 1962). Anderson (1968), using siblings to demonstrate the successful homo-transplantation of the uterus, implicated a substance transported in the blood as being of primary importance in the control of luteal regression in the rat. However, Barley, et. al. (1966), in prolonging luteal function by bilateral or unilateral hysterectomy of pregnant or pseudopregnant rats, showed that the ability of the uterus to limit

the life-span of the rat corpus luteum was dependent on the proximity of the uterus to the ovary. This interpretation supported a localized luteolytic mechanism in the rat as opposed to a systemic effect. Labhsetwar (1972) demonstrated that prostaglandin $F_{2\alpha}$ did exert definite luteolytic effects and induced ovulation in mice, suggesting it to be the luteolytic substance in this species.

The Mechanism of Implantation

The implantation stimulus is one which involves a direct embryonic effect on the uterus. The embryo is incapable of eliciting this effect until it has been activated. It was suggested that the uterine epithelium kept the trophoblast inactive by secreting only minimal amounts of nutritive medium and the epithelium activated the trophoblast by making more nutrients available (Bergström and Nilsson, 1975). Normal and delayed mouse embryos were incubated in ^{14}C-glucose by Torbit and Weitlauf (1975). Initially, the delayed embryos in short preincubations had low levels of CO_2 production that approached the normal range with longer incubation. These embryos were then said to be activated *in vitro*. The association between CO_2 output and trophoblast outgrowth *in vitro* (and implantation *in vivo*) suggested that metabolic activity had to reach a certain level before implantation could occur (Menke and McLaren, 1970).

Torbit and Weitlauf (1975) postulated that hormones played a role in blastocyst activation by two possible mechanisms. They either stimulated metabolic rate within the embryo, or acted indirectly by altering the uterine environment. Estradiol or progesterone had no effect on CO_2 production by normal or delayed embryos

when they were exposed during a preincubation period to these hormones. This evidence suggested that the mechanism of embryo activation was not direct in nature. Bergström and Nilsson (1975) activated mouse embryos by an injection of estradiol and noted that they became separated from the endometrial surface by a layer of uterine secretion. The trigger for blastocyst activation may be some substance of the uterine secretion, in keeping with an indirect type of activation mechanism.

In order for activation and implantation to occur normally, the embryo and endometrium must have simultaneously reached specific stages in their development. Dickmann and Noyes (1960) have reported that, in the rat, transferred embryos which were one day younger than the gestational stage of the recipient's uterus were damaged before the usual implantation time and did not stimulate a decidual response in the uterus. Those embryos one day older than the uterus did not implant sooner than control embryos, pointing out the need for synchrony between both entities. There were upper and lower limits of endometrial and embryonic development that were compatible with normal development (Noyes and Dickmann, 1960). It is apparent that implantation is dependent on certain factors of both the embryo and the uterus; neither over-riding the other.

In recent years, a series of contractions and dilations have been reported in the rat embryo just prior to implantation (Bitton-Casimiri, et. al., 1971; Psychoyos, 1973). The contraction phase was described as occurring over a one minute interval, after which dilation occurred (Psychoyos, 1973). Expansion of the blastocyst may involve passive water transport, electro-osmosis, pinocytosis

and other membrane effects (Hafez, 1971). After a sequence of contractions in vitro, a large droplet, or several smaller droplets, of a viscous fluid were seen on the outer surface of the contracted blastocysts (Bitton-Casimiri, et. al., 1971). The droplets were pushed against the inner surface of the zona pellucida by the expanding blastocyst and could be seen to emerge into the culture medium, apparently through ruptures in the zona pellucida. These droplets may have contained chemical factors that were involved in the induction of implantation.

There are other conceivable ways by which the blastocyst could initiate the decidual reaction of implantation. For instance, the contractions and dilations of the blastocyst could serve as a mechanical stimulation (Psychoyos, 1973). However, experiments with glass and plastic beads, as imitation blastocysts, eliminated the possibility that the mere distension of the uterus was involved (McLaren, 1968). Beads of 65-120 μ diameter failed to respond with Potamine blue reactivity, a measure of increased vascularization and capillary permeability (Finn and McLaren, 1967).

The other mechanism by which the blastocyst could initiate the decidual reaction is via trophoblast cell invasion of the uterine epithelium (Psychoyos, 1973). Of the 64 cells making up the mouse blastocyst at the time of implantation, 13 of these cells (inner cell mass - ICM) are enclosed and have no surface in contact with the exterior of the embryo (Graham, 1971). The remaining cells (trophoblast) make up the surface of the embryo. Gardner and Johnson (1972) separated mouse blastocysts into their consituent cell types

and demonstrated that trophoblast fragments were as effective in stimulating the decidual reactions as whole blastocysts. The ability to induce decidual changes characteristic of implantation was peculiar to trophoblast cells and not to the inner cell mass. These trophoblast cells gave rise to a few giant cells in vitro which were never found to divide. Giant cells in the mouse have been observed during normal pregnancy, but not in delayed blastocysts (Dickson, 1963). In delayed implantation, the inner cell mass may play a role in inhibiting the giant cell transformation of the overlying trophoblast cells (Gardner and Johnson, 1972).

It was reported that the addition of estradiol benzoate and progesterone was capable of inducing implantation of delayed mouse embryos as late as day 31 post coitum (Smithberg and Runner, 1960). Dickson and Araujo (1966), after treating ovariectomized mice with progesterone and estradiol benzoate, concluded that the trophoblast giant cell transformation could be stimulated by treatment of these ovarian hormones without induction of implantation. However, the mice were killed only 30 to 48 hours after the estrogen injection. This was probably not sufficient time for implantation to have been accomplished, although it points out the fact that giant cell transformation and implantation are two dissociated events.

Wilson (1963) described histologically unique cells within the inner cell mass at the time the blastocyst became situated in the uterine lumen for implantation (84 to 96 hours post coitum). These cells were distinguishable by staining characteristics and nuclear morphology. At 100 hours post coitum, these cells were found between

blastocyst surface and penetrating the uterine epithelium. At 108 to 112 hours post coitum, when the decidual reaction first appeared, these "primary invasive cells" could no longer be distinguished from epithelial cells. The actual function of these cells originating in the inner cell mass is unknown.

Before mammalian embryos implant, they lose their zona pellucida. The structural integrity of the zona pellucida of the mouse embryo was demonstrated to be dependent on disulfide bonds (Inoue, 1973). Restall and Bindon (1971) reported the loss of the mouse zona pellucida to begin between 1400 and 1600 hours on day 4 post coitum, with few zygotes retaining their zona by two hours on day 5 of pregnancy. Zona loss usually precedes the appearance of Potamine blue reactivity in mice (McLaren, 1969). Therefore, zona loss is not a necessary prerequisite for the uterine response.

The zona pellucidae of mouse embryos were lysed by low pH's (Edwards and Gates, 1959; Restall and Bindon, 1971; Inoue, 1973). There was some doubt as to whether or not this was the actual mechanism *in vivo*. Brinster (1965b) determined the optimum pH *in vitro* to be 6.82 for mouse embryos. Bowman and McLaren (1969) demonstrated death of mouse embryos within 24 hours at pH's less than 6.0. Therefore, it was unlikely that zona lysis could be brought about *in vivo* by a shift in pH which would also allow the blastocyst to develop. However, blastocysts may have lower sensitivity to pH *in vivo*, or the zona may be more sensitive than the blastocyst to pH changes.

Dickmann and Noyes (1961) have postulated that when the trophoblast reaches a certain stage of development, it releases a lytic

agent, which facilitates its penetration through the zona. However, Bergström (1972) suggested that gradual thinning of the zona was related to blastocyst expansion and not to a lytic mechanism.

Even if the zona is intact, the passage of certain substances such as CO_2 was not prevented (McLaren, 1969). The injection of air into the mouse uterus simulates the presence of blastocysts by evoking a decidual reaction (Hetherington, 1968a). This decidual stimulation was shown to result largely from its CO_2 content (Hetherington, 1968b). Because the amount of CO_2 production by the mouse embryo is known to increase dramatically between the morula and blastocyst stage (Brinster, 1967a), this could account for the natural stimulation by blastocysts.

For an optimum uterine sensitivity to normal stimulation by the blastocyst, a relatively high level of estrogens is necessary at estrus, followed by a period of several days of progesterone dominance (Finn, 1966; Humphrey, 1967; McLaren, 1972). Evidence suggests that both estrogen and progesterone influences are needed for implantation to occur during normal pregnancy in the mouse (Smithberg and Runner, 1960; Smith and Biggers, 1968), rat (Dickmann, 1967) and Mongolian gerbil (Wu, 1975). In addition, the ratio of progesterone to estrogen was reported to be important for implantation in the rat (Duncan, et. al., 1968). Buchanan, et. al. (1957) suggested that unilateral ovariectomy of armadillos during the implantation delay period altered the circulatory progesterone:estrogen ratio and caused a uterine environment incompatible with blastocyst maintenance.

There is also evidence to suggest that these hormonal influences are unnecessary for implantation. After progesterone and estrogen were removed from serum with Norit A charcoal-dextran, mouse blastocysts were reported to be able to implant in a medium supplemented with this serum (Salomon and Sherman, 1975a). Therefore, since the blastocysts in these cultures attached and trophoblast cells grew out, it was suggested that progesterone and estradiol were not required for deciduoma formation *in vitro*. These results show a discrepancy of action *in vitro* as compared to mechanisms studied *in vivo*. However, trophoblast outgrowth *in vitro* may not be totally analogous to implantation *in utero*.

A decidual response is optimally elicited under progesterone dominance with some estrogen (Finn and Keen, 1963; Yasukawa and Meyer, 1966), although progesterone was not necessary for viability of delayed mouse blastocysts for up to 10 days (Weitlauf and Greenwald, 1968b). Normal implantation in guinea pigs was not interrupted if these animals were ovariectomized on days 3 to 7 post coitum (Deanesly, 1960). However, if ovariectomized on day 2, implantation did not occur unless one injection of progesterone was given at the time of operation. The marked difference in this study between ovariectomy on days 2 and 3 probably is due to the uterus having received an adequate supply of progesterone by day 2.

Although both progesterone and estrogen were necessary for unimpaired implantation in most rodents, the immediate stimulation for sensitization was given by estrogen which, in the mouse, acted on the uterus on the morning of the fourth day of pregnancy (McLaren,

1972). A peak of plasma estradiol concentration was noted in the mouse between 1000 to 1200 hours of day 4 (McCormack and Greenwald, 1974). Wu (1975) reported that estrogen sufficient for implantation in the Mongolian gerbil was secreted before 2100 hours of day 5. This surge of estrogen, after progesterone sensitization, was reported to result in successful implantation in mice (Humphrey, 1967; Smith and Biggers, 1968). Doses of estradiol in excess of those supporting a maximal decidual reaction resulted in inhibition of implantation in mice (Duncan, et. al., 1968; Smith and Biggers, 1968).

Shelesnyak, et. al. (1963) demonstrated that a single injection of estradiol could be a substitute for the estrogen surge in rats. Finn (1966) indicated that the period of sensitivity induced by the surge on day 4 in the mouse was followed by a period of refractoriness, making it increasingly more difficult for embryos to implant after the optimal interval. Humphrey (1969) transferred blastocysts to progesterone-treated ovariectomized mice which had been injected with estradiol at different times after the operation. His conclusion was that estrogen was not released as a surge before implantation but, rather, was released continuously after ovulation.

Although estrogen was shown to enhance the implantation process, the injection of progesterone into spayed, unprimed animals was capable of inducing the attachment reaction (Martin, et. al., 1970). The attachment reaction is characterized by the opposing uterine luminal surfaces coming into close contact. Hedlund and Nilsson (1971) demonstrated the presence of the attachment reaction after a four day treatment of ovariectomized mice with progesterone. Implantation was

blocked in this study by insufficient contact between the trophoblast and uterine endometrium. Estrogen may have a function in bringing these two tissues into closer opposition. Implantation was noted to occur in ovariectomized hamsters without any exogenous estrogen, suggesting that this species can implant with progesterone alone (Orsini and Meyer, 1959).

The exact mechanism of steroid stimulation of implantation is unknown. Yoshinaga (1961) suggested two possible means by which estrogen could affect implantation: systemic and local. Estrogen could act systemically by stimulating the secretion of progesterone or by acting on the ovary or pituitary. None of the anterior pituitary hormones in themselves could initiate implantation, although combinations of prolactin and FSH or LH and FSH appear to be capable of inducing this phenomenon in the mouse (Gidley-Baird and Emmens, 1975). During delayed implantation in lactating mice, gonadotropins were capable of inducing implantation (Whitten, 1955). The local mechanism may be elicited as a permissive factor directly on the blastocyst or uterus (Yoshinaga, 1961).

Administration of estrogen resulted in the mobilization of glycogen in the myometrium and accumulation of water in the endometrium (Telfer and Hisaw, 1957). These workers found estrogen to increase the rate of oxidative metabolism in uterine tissues. Prasad, et. al. (1968) reported that estrogen enhanced the synthesis of DNA, RNA and protein in the blastocyst as well as RNA and protein in the uterus. These effects may be elicited via the second messenger of estrogen (cyclic-AMP), since this substance induced implantation when deposited

into the uterine lumen of delayed implantation mice (Holmes and Bergström, 1975). Smith and Smith (1971) demonstrated a significant stimulation of ^{14}C-valine uptake by estradiol-17β, adding credence to a localized effect of estrogen on the blastocyst. Delayed implanting blastocysts from animals treated with progesterone showed a significant increase in CO_2 production after the injection of estradiol (Torbit and Weitlauf, 1975). In turn, CO_2 production by the blastocyst was reported to initiate the decidual response in mice (Hetherington, 1968b). Estradiol was shown to bind to rabbit blastocysts in vitro (Bullock and Bhatt, 1973; Bhatt and Bullock, 1974). Since the minimum effective dose of estrogen to induce implantation in lactating rats by local application was only 1/20 the effective systemic dose (Yoshinaga, 1961), the estrogen surge in rodents probably has a preponderance of local actions.

Possible Role for Embryonic Steroidogenesis

Dickmann (1969) transferred rat morulae to the uteri of long-term ovariectomized recipients. These eggs were recovered three days later as blastocysts and transferred again to pseudopregnant rats, after which 39% developed to term. These results seem to indicate that rat morulae can be transformed into blastocysts in the absence of ovarian hormones. The presence of Δ^5-3β-hydroxysteroid dehydrogenase (HSD) was determined histochemically, as a deposition of formazan granules, in the blastocysts of rats after incubation with dehydroepiandrosterone (Dickmann and Dey, 1974a). The 3β-HSD enzyme is characteristic of tissues that form hormones with the α and β unsaturated ketone structures; the enzyme oxidizing the Δ^5-3-ol structure

(Samuels, et. al., 1951). In this study, 3β-HSD activity was first observed 30 to 26 hours earlier than the normal implantation time in the rat, suggesting a role of embryonic steroidogenesis in the implantation process. Dickmann and Dey (1973) therefore postulated that the preimplantation embryo was a source of steroid hormones which controlled the morula-to-blastocyst transformation as well as implantation.

With this same histochemical technique, 3β-HSD was found in embryonic tissues on the day of implantation in the rat (Dey and Dickmann, 1974b), rabbit (Dickmann, et. al., 1975a; b), mouse (Dey and Dickmann, 1974c) and hamster (Dickmann and Sen Gupta, 1974). In addition, 17β-HSD was identified in preimplantation embryo cultures of the rat (Dey and Dickmann, 1974a) and hamster (Dickmann and Sen Gupta, 1974) when estradiol-17β was employed as the substrate. This enzyme affects the conversion of estrone and estradiol.

Dickmann and Dey (1974a) explored three factors that conceivably could regulate HSD activity in rat blastocysts. To determine if the uterine environment influenced HSD activity in rat blastocysts, embryos were prevented from entering the uterus by ligating the utero-tubal junction. On day 5 of pregnancy, all blastocysts recovered from the oviducts were positive for HSD. In addition, rats were hypophysectomized to determine whether pituitary gonadotropins had an influence on HSD activity in blastocysts. On day 5, again, all blastocysts were positive for HSD activity. The third factor studied was ovarian hormone influence on HSD activity. Using delayed implantation embryos, the results indicated that neither the endogenous ovarian hormones nor

exogenous progesterone had an influence on HSD activity in rat blastocysts. The overall conclusion was that HSD activity was autonomous in blastocysts.

Ovarian hamster eggs, as well as unfertilized and fertilized ova of the same species were studied histochemically in reference to their content of Δ^5-3β, 20α, or 20β-HSD's and possible progestagen production (Niimura and Ishida, 1976). Substrates and cofactors appropriate for each of the three enzymes were utilized *in vitro*. No enzyme activity was found in ovarian eggs, but all three enzymes were present in tubal eggs. Enzyme activity had not risen by the time of blastocyst formation. This might suggest that progestagens in hamster eggs may participate in other physiological activities during development, as well as in blastocyst formation.

Activity of 3β-HSD in rabbit embryos was found to begin at the morula stage and was sustained through the late blastocyst stage (Dickmann, *et. al.*, 1975a). Seamark and Lutwak-Mann (1972) showed that free-lying rabbit blastocysts contain progesterone in higher amounts than fluid from attached blastocysts. They found evidence for 20α-hydroxyprogesterone and 17α-hydroxyprogesterone in the fluid of attached blastocysts. However, the presence of these progestins in uterine fluid suggested they may be conveyed to the blastocysts by the endometrial secretions. Huff and Eik-Ness (1966) incubated rabbit blastocysts with radioactive progesterone, pregnenolone, 17α-hydroxypregnenolone, androstenedione and sodium acetate. The results indicated that rabbit blastocysts could biosynthesize cholesterol and pregnenolone

from acetate and could promote various biotransformations of various steroids.

If, indeed, the mammalian preimplantation embryo is producing steroids, the stimulus to do so is unknown. A gonadotropin (LH or HCG) has been detected in rabbit blastocysts prior to implantation with a radioreceptor assay for HCG (Haour and Saxena, 1974). The concentration of the LH or HCG in blastocyst fluid was 10 times higher than in the blood of pregnant rabbits at the time of implantation. Trophoblast cells of 10 day old human embryos were estimated to secrete 1.4 x 10^{-2} IU HCG/cell/day (Braunstein, et. al., 1973).

Pig blastocysts, endometrial tissue and uterine washings were incubated with radioactive androstenedione, dehydroepiandrosterone, progesterone, pregnenolone, estrone, estrone sulphate and cortisol (Perry, et. al., 1973). From these incubations, evidence was obtained for the presence within the blastocyst of aromatase, 17-20 desmolase and 3-sulphatase enzyme systems. These enzymes are concerned with the production of estrogens from neutral steroids, progesterone and conjugated steroids, respectively. Also, Perry and Heap (1973) determined, through other labelled substrate studies, that the 16-day-old pig blastocyst can convert androstenedione and dehydroepiandrosterone to estrogens, but there was a low conversion of pregnenolone to progesterone. Flood (1974) observed no histochemical activity of steroidogenic enzymes in 10-day-old pig blastocysts, but by 12 days there was vigorous utilization of 3β-hydroxy-5β-androstan-17-one in the trophoblast cells and a less rapid metabolism of the Δ^5-3β and 17β-hydroxy substrates. This coincides well with the time of maternal recognition of pregnancy in the

pig (Dhindsa and Dziuk, 1968). Therefore, the embryo may signal its presence by some active process, such as synthesis of estrogen in the pig embryo between days 10 to 12 (Jones, et. al., 1976). There may be other physiological functions of this estrogen produced by the pig conceptus around the time of implantation. Wildt, et. al. (1976) demonstrated that a systemic administration of progesterone and estrogen together for 10, 5 and 2 consecutive days during the implantation interval led to increased litter size at term. One possible effect could be a stimulation of amino acid uptake as shown in preimplantation mouse embryos (Smith and Smith, 1971), although there was no observed effect of estrogen on the quantitative or qualitative pattern of protein synthesis in vitro by the pig embryo (Jones, et. al., 1976).

Another possible function of embryonic steroidogenesis was suggested for human embryos. Hulka (1971) hypothesized that the trophoblast is capable of secreting a substance that abolishes immune responses with a local, rather than systemic, effect. He suggested that, since a steroid-producing graft may have a better chance of surviving than a non-hormone-producing graft, the endocrine activity of the trophoblast may be a factor in local survival at the implantation site. However, no data was forthcoming that demonstrated an immunosuppressive activity of the hormones of pregnancy in prolonging trophoblast survival.

Sherman and Atienza (1977) cultured mouse preimplantation and post-implantation embryos with pregnenolone or progesterone. Possible production of progesterone or androstenedione by the cultures was then quantified by chromatography and radioimmunoassay. These workers were

unable to detect conversion of pregnenolone to progesterone by preimplantation embryos. After trophoblast outgrowth, however, progesterone was produced. These results suggest that enzyme activities such as Δ^5-3β-HSD, which are involved in metabolism of progestins and androgens, are not acquired until after implantation. The peak progesterone production was observed on days 8 to 9. Hydroxysteroid dehydrogenase activity was observed in post-implantation mouse trophoblasts by other workers (Deane, et. al., 1962; Chew and Sherman, 1973; 1975; Salomon and Sherman, 1975b).

Steroid Quantification Methods

Today, the quantitative measurement of progesterone, estrogen and many other steroids is a daily routine in many laboratories. Progesterone is measured by competitive protein binding (CPB) assays and also by radioimmunoassays (RIA), while estrogens are most commonly quantified by RIA. Radioimmunoassays are based on the competitive binding of labelled and unlabelled hormone to an antibody produced against that hormone and a sensitive method for measuring the degree to which the effective binding sites are filled. The antisera used is commonly produced by the immunization of rabbits or sheep with a steroid containing a bovine serum albumin derivative at a certain position remote from distinguishing functional groups (Midgley and Niswender, 1970).

Johansson (1969; 1976) described a CPB assay for human peripheral plasma progesterone. This technique employs naturally-occurring steroid-binding proteins. However, with this technique, one is restricted to working with relatively unstable proteins that have a limited

range of affinities and are obtained from different donor animals that are in various physiological states (Midgley and Niswender, 1970).

In the past, several chemical methods were used to measure progesterone. Reynolds and Ginsburg (1942) developed a quantitative method based on uv absorption at 240 mμ of Δ^4-3-ketosteroids such as progesterone. Haskins (1950) considered that the relatively high extinction coefficient at 240 mμ made progesterone readily measurable at optimum concentrations of 1 to 20 μg/ml of ethanol. A method based on organic extraction and partition between organic solvents, with final separation of the individual steroids by partition chromatography and subsequent polarographic estimation was developed by Butt, et. al. (1951). Edgar (1953) described a method of progesterone assay based on the extraction and purification methods of Butt, et. al. (1951), with final separation by chromatographic partition on filter paper and uv absorption spectroscopy. By this procedure, .1 μg of progesterone per ml of blood could be measured. Short (1958) described a system using solvent partition, paper chromatography and uv absorption spectroscopy of the chromatogram eluate. This procedure was capable of distinguishing between progesterone and two closely related compounds: 20α and 20β pregnenolone.

Riondel, et. al. (1965) formed the 3,20-bisthiosemicarbazone derivative of progesterone, which is more polar than the parent compound. This derivative was then hydrolyzed to 3-thiosemicarbazone, which could be chromatographed as a highly specific purification step. A modification of this procedure, which involves five chromatograms, was employed by Sommerville, et. al., (1963) to measure progesterone in amounts as low as .1 μg.

Fluorescence methods sensitive enough to measure .05 µg of progesterone were described by Touchstone and Murawec (1960) and Heap (1964). Fluorometric determination of progesterone has been applied to human plasma during pregnancy and the menstrual cycle (Short and Levett, 1962). Along with these chemical methods, several workers attempted to qualify and quantify progesterone by bioassay. Hooker and Forbes (1947) developed an assay using a change in mouse endometrial stromal nuclei as an end point after progesterone injection. This method was sensitive to a concentration of .3 µg progesterone per ml of fluid.

Futterweit, et. al. (1963) used gas-liquid chromatography for estimating progesterone in human pregnancy plasma. Gas chromatographic methods were developed for the assay of plasma progesterone with sensitivities in the order of .01 to .02 µg of progesterone (Collins and Sommerville, 1964; Yannone, et. al., 1964). Van der Molen and Groen (1965) quantified ng levels of progesterone using gas-liquid chromatography of the monochloroacetate derivative of this steroid, followed by electron capture detection. This technique has been used to follow progesterone patterns in peripheral plasma of gilts during the estrous cycle (Stabenfeldt, 1969). A detailed description of the procedure used in gas-liquid chromatography of progesterone has been given by Van der Molen and de Jong (1976).

The CPB assay procedure has been applied to the determination of plasma progesterone with a specificity of .1 ng (Neill, et. al., 1967; Surve, et. al., 1968; Yoshimi and Lipsett, 1968; Johansson, 1969; 1970; Edquist and Lamm, 1971; Shemesh, et. al., 1971). The RIA procedure was used by Abraham, et. al. (1971) to measure progesterone content

of plasma after celite-column separation of cross-reacting steroids in the ether extract. This assay was sensitive to 25 pg of progesterone.

The assay of estrogens had an early beginning. Astwood (1938) developed a bioassay for estrogens by the changes which occur *in utero* after a single injection. The uterus of an immature rat underwent an increase in weight due almost entirely to an accumulation of water. After an injection of .1 g of estrogen, the uterine weight reached a maximum by six hours. Brown (1955) and Bauld (1956) developed similar chemical methods for the separate estimation of estrone, estradiol and estriol in human urine using a colorimetric measurement technique. The sensitivity of these methods was 2.5 to 10 µg/24 hours of urine.

Procedures for the determination of estrogens in human urine have been described using gas-liquid chromatography (Thijssen, 1976) and fluorimetry (Brown, 1976). Values as low as .1 and 2 µg, respectively, could be measured using these methods.

Competitive protein binding assays have been developed for quantification of estradiol using the sex hormone-binding globulin of blood plasma (Murphy, 1968; Dufau, 1976) and a specific estrogen binding molecule in the high-speed supernatant of a uterine homogenate of rabbits (Korenman, 1968; 1976; Korenman, *et. al.*, 1969; Corker and Exley, 1970; Exley, 1976) and of sheep (Shutt, 1969). This assay was capable of measuring 100 pg of estrogen.

The preparation of specific antibodies for the RIA of estradiol-17β by injecting rabbits with the hapten, 17β-estradiol-6-(O-carboxymethyl) oxime-BSA has been described (Exley, *et. al.*, 1971). The solid-phase RIA of estradiol with a sensitivity of 6 to 10 pg (Abraham, 1969; Abraham,

et. al., 1970) has been reported. An RIA for estradiol with a sensitivity of 5 pg was developed by Korenman, et. al. (1974) and this procedure has been discussed in detail by Abraham (1976). Estrone, estradiol-17α and estradiol-17β were estimated via RIA with sensitivities ranging from 11 to 50 pg (Mikhail, et. al., 1970; Robertson, et. al., 1972; Dobson and Dean, 1974).

In Vitro Culture of Preimplantation Mouse Embryos

As early as 1949, Hammond reported the development of 8-cell mouse embryos to the blastocyst stage in a balanced salt solution with glucose and egg white. This medium was improved upon, and was more defined, by Whitten (1956a) who added bovine serum albumin in place of egg white. The successful culture of mouse 2-cell embryos to the blastocyst stage was accomplished by Whitten (1957a) when he showed that the addition of 1 mg Ca lactate/ml of the medium was beneficial. Soon after, Mastroianni and Wallach (1961) demonstrated that the lactate content of rabbit oviductal secretions increased on the first three days of pregnancy. Brinster (1963) reported that lactate alone was a sufficient energy source for these stages. Pyruvate, as well as pyruvate and lactate, are better than lactate alone for the development of the 2-cell stage to blastocyst in the mouse (Brinster, 1965c). Whittingham and Biggers (1967) were the first to achieve cleavage of mouse zygotes to 2-cell embryos in vitro and Biggers, et. al., (1967) showed that only pyruvate and oxaloacetate could serve as energy sources for this first cleavage division. Most defined media today contain glucose. The addition of glucose to a medium that contains lactate and pyruvate does not significantly increase the number of early cleavage stages developing

to blastocysts *in vitro*. Its inclusion, however, provides a safeguard in cases where other energy sources may become depleted before the termination of the culture period (Whittingham, 1971).

Brinster (1965d) reported the optimum bovine serum albumin concentration for *in vitro* culture of mouse embryos to be from 1 mg/ml to 10 mg/ml. This optimum concentration was probably a function of its nitrogen supply and its ability to act as a polymer at the cell-medium interface. Bovine serum albumin also has chelating effects, is a regulator of oxidation-reduction potential and is a protector of cell surfaces.

Two-cell mouse embryos developed to blastocysts when bovine serum albumin was replaced with an equivalent mixture of amino acids and when a nonprotein polymer, polyvinylpyrolidone (PVP), was included (Brinster, 1965d). This polymer maintained the viscosity of the medium and prevented the embryos from sticking to glass (Whittingham, 1971). It was apparent that bovine serum albumin was required in the medium as a nitrogen source and also to control cell-surface phenomena. Haidri, *et. al.* (1971) reported that .1 mg PVP/ml of media, when substituting for protein, allowed development of mouse embryos as measured by the formation of polar bodies, but led to floating embryos which stuck to glass surfaces. In the same study, .5 mg PVP/ml of medium overcame the handling problems but was inhibitory to development.

Streptomycin and penicillin are included in culture media as protectors against microorganisms that may contaminate the culture system (Biggers, *et. al.*, 1971).

An atmosphere of 5% CO_2 with a 100% humidity was essential to inhibit a change in medium pH (Biggers, et. al., 1971). The optimum pH for mouse ova in vitro was reported to be 6.82, with an optimum osmolarity of .276 osmols (Brinster, 1965b). The bicarbonate content of uterine and tubal fluids of rabbit were higher than the corresponding plasma, possibly from the hydration of CO_2 (Vishwakarma, 1962). Therefore, bicarbonate is used in most embryo culture media as the pH buffer.

It is therefore evident that in vitro culture conditions for the mouse embryo have been thoroughly described. This opened up a new area of opportunity for the reproductive physiologist. Through the culture of embryos, considerable knowledge has been gained in attempts to regulate the reproductive status of humans and animals.

MATERIALS AND METHODS

Experimental Design

Two experiments, using a total of 4180 embryos flushed from ICR albino mice, were designed to test the hypothesis that the mouse preimplantation embryo produces progesterone and estradiol. In Experiment I, 20, 40 or 60 early blastocysts were incubated in 10, 10 and 6 cultures, respectively (Figure 1). These embryos were all flushed at 1300 to 1400 hours on day 4 of pregnancy. Each culture had two controls, a 24-hour incubated viability control and an immediate freeze control. The purpose of Experiment I was to demonstrate a dose response relationship in hormone production with increasing numbers of embryos/culture and to determine the number of embryos needed to be cultured at one time in order to detect steroid hormone production.

Experiment II was designed to investigate possible hormone production at the morula, early blastocyst and late blastocyst stages (Figure 2). The morula were flushed on day 4 of pregnancy at 0400 to 0700 hours, early blastocysts at 1300 to 1400 hours on day 4 and late blastocysts at 2200 to 2400 hours on day 4.

At the end of the incubation period, all cultures except viability controls were maintained at -90C. Prior to hormone isolation, each tube of embryos and .25 ml of BMOC-2 was thawed at room temperature, extracted with diethyl ether, chromatogramed and assayed for progesterone and estradiol.

Figure 1: Experiment I design (all embryos in early blastocyst stage).

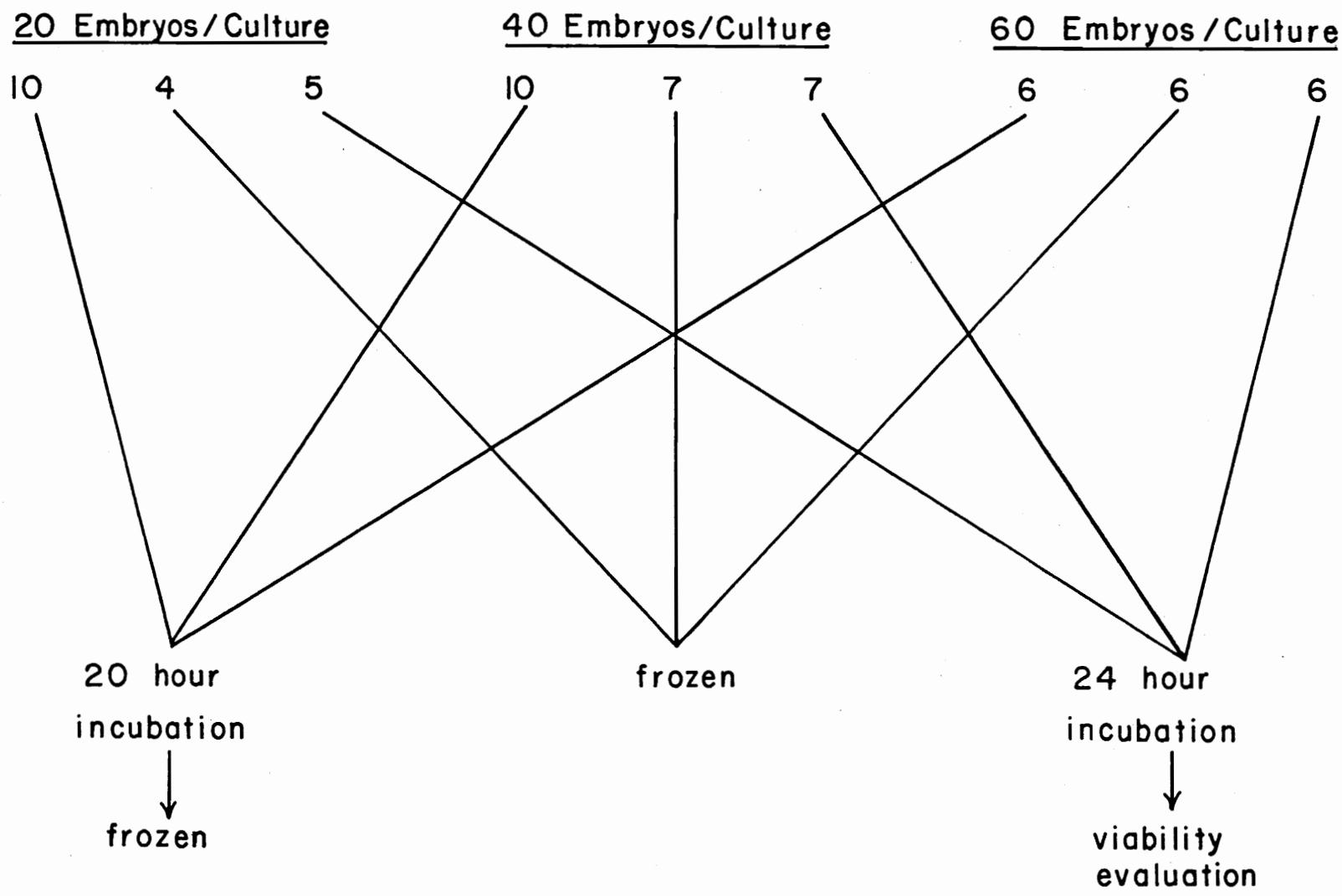
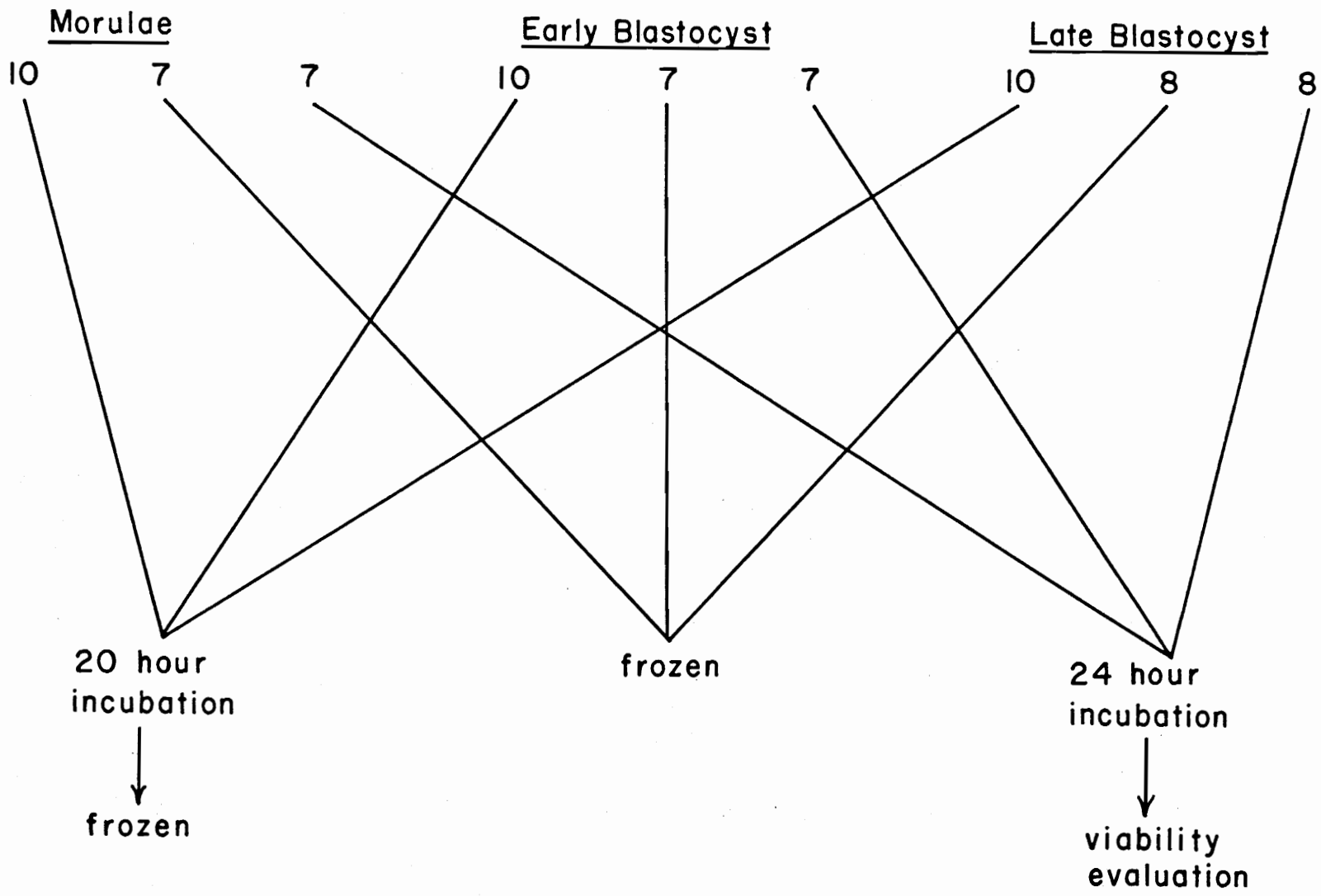

20 Embryos/Culture
40 Embryos/Culture
60 Embryos/Culture
10
4
5
10
7
7
6
6
6
20 hour
incubation
frozen
frozen
24 hour
incubation
viability
evaluation

Figure 2: Experiment II design (all cultures contained 40 embryos).


Morulae
Early Blastocyst
Late Blastocyst
10
7
7
10
7
7
10
8
8
20 hour incubation
frozen
frozen
24 hour incubation
viability evaluation

Flushing Techniques

Random-bred ICR albino mice were allowed to cycle normally and mated at a stocking rate of two females/male while housed in a 14:10 (light:dark) light cycle. Observation of vaginal plugs was conducted between 0800 and 1000 hours daily. The day on which a vaginal plug was detected was considered day 1 of pregnancy. At varying times on day 4, the uteri were excised and flushed retrograde with 1 ml of culture media (BMOC-2, Appendix 1) into a .90 mm sterile watch glass enclosed within a petri dish.

Disposable pasteur pipettes were heated over an alcohol flame and drawn to an internal diameter slightly larger than a mouse blastocyst (.10 mm). With these pipettes, fitted with a rubber mouthpiece, embryos from each flushing were immediately transferred to a fresh, sterile watch glass containing 1 ml of BMOC-2. Embryo manipulation subsequent to flushing was carried out under a stereomicroscope at 40X. Embryos within each stage that were flushed on a particular day were washed three times and pooled prior to culture.

Culture Methods

From the daily embryo pool, washed embryos were placed into one of three treatments (Figures 1 and 2). Treatment 1 involved incubation for 20 hours, after which media with embryos were frozen at -90C until analysis for steroids. The control (treatment 2) was immediately frozen at -90C and treatment 3 (viability control) was cultured for 24 hours, at which time the contents were poured into a watch glass and embryos were microscopically evaluated for viability. Embryos

were cultured in 13 X 100 mm glass tubes. All glassware was sterilized with dry heat at 190C for two hours and warmed to 37C prior to use. Embryos were cultured at 37C with 5% CO_2 in air, at 100% humidity, flowing through the incubating chamber (Biggers, et. al., 1971; Rafferty, 1970). All manipulations of embryos were performed under a plexiglass hood to eliminate or minimize air-borne impurities.

The culture media (BMOC-2, appendix 1) was prepared weekly. Sodium hydroxide (1N) was used to adjust the pH of the media to 6.82 (Brinster, 1965b). The medium was then filtered with a 0.45 μ Millipore filter prior to storage at 4C until use. Media required for each day's cultures and manipulations was taken from this refrigerated source and the required amount of albumin was added. Prior to use, the media was warmed to 37C.

Cleaning of Glassware for Hormone Analysis

All glassware, except pipettes, was brushed with detergent, rinsed 10 times with tap water, soaked in hydrochloric acid for two hours, rinsed 10 times with distilled water and dried at 100C. Glassware contaminated with tritium was rinsed with acetone twice before being cleansed as previously described. Pipettes were rinsed with acetone twice if contaminated with tritium, rinsed with tap water, soaked in chromic acid overnight, rinsed 10 times in tap water, rinsed 10 times in distilled water and dried at 100C.

Hormone Standards and Isotopes

Progesterone (preg-4-ene-3, 20 dione) and estradiol-17β (estra-1, 3, 5 (10)-triene-3, 17β-diol) were obtained from Sigma Chemical Com-

pany. A working standard solution (1 pg/µl) of each was prepared by diluting in absolute ethanol.

Labelled [1, 2, 6, 7-^{3}H] progesterone, [1, 2-^{3}H] progesterone, [2, 4, 6, 7-^{3}H] estradiol-17β and [6, 7-^{3}H] estradiol-17β were obtained from New England Nuclear. Dual-label isotopes were used as tracer isotopes in extraction procedures, while the four-label isotopes were used in RIA procedures.

Extraction and Isolation Procedures

Approximately 4800 dpm (15 pg) of 1,2-^{3}H progesterone and 4800 dpm (14 pg) of 6,7-^{3}H estradiol were added to 13 X 100 mm screwcap tubes and dried prior to embryo incubation. The tritiated steroids added prior to culture were used as internal standards for correction of extraction losses. Samples were then extracted twice with 3 ml of diethyl ether. Each sample was shaken for one minute, frozen at -90C and the ether fraction poured off. The pooled ether extracts were dried in 15 X 125 mm glass tubes and stored at 4C until further isolation of steroids.

Sephadex LH-20 columns were utilized to separate progesterone and estradiol. A filter paper disk (Whatman 2) was placed at the bottom of a 2.5-ml glass syringe topped with a slurry of pre-soaked Sephadex LH-20. Benzene/methanol (9:1) (Chenault, 1973) was used to soak the LH-20, as well as prepare columns and elute samples. The column was packed to 1.5 ml and capped with a filter paper disk. The columns were then washed with at least 5 ml of solvent before adding a sample.

In order to characterize progesterone and estradiol elution patterns with this system, 140,000 dpm of each were separately added to

Figure 3: Elution patterns of ^{3}H-progesterone and ^{3}H-estradiol separately applied to Sephadex LH-20 columns.

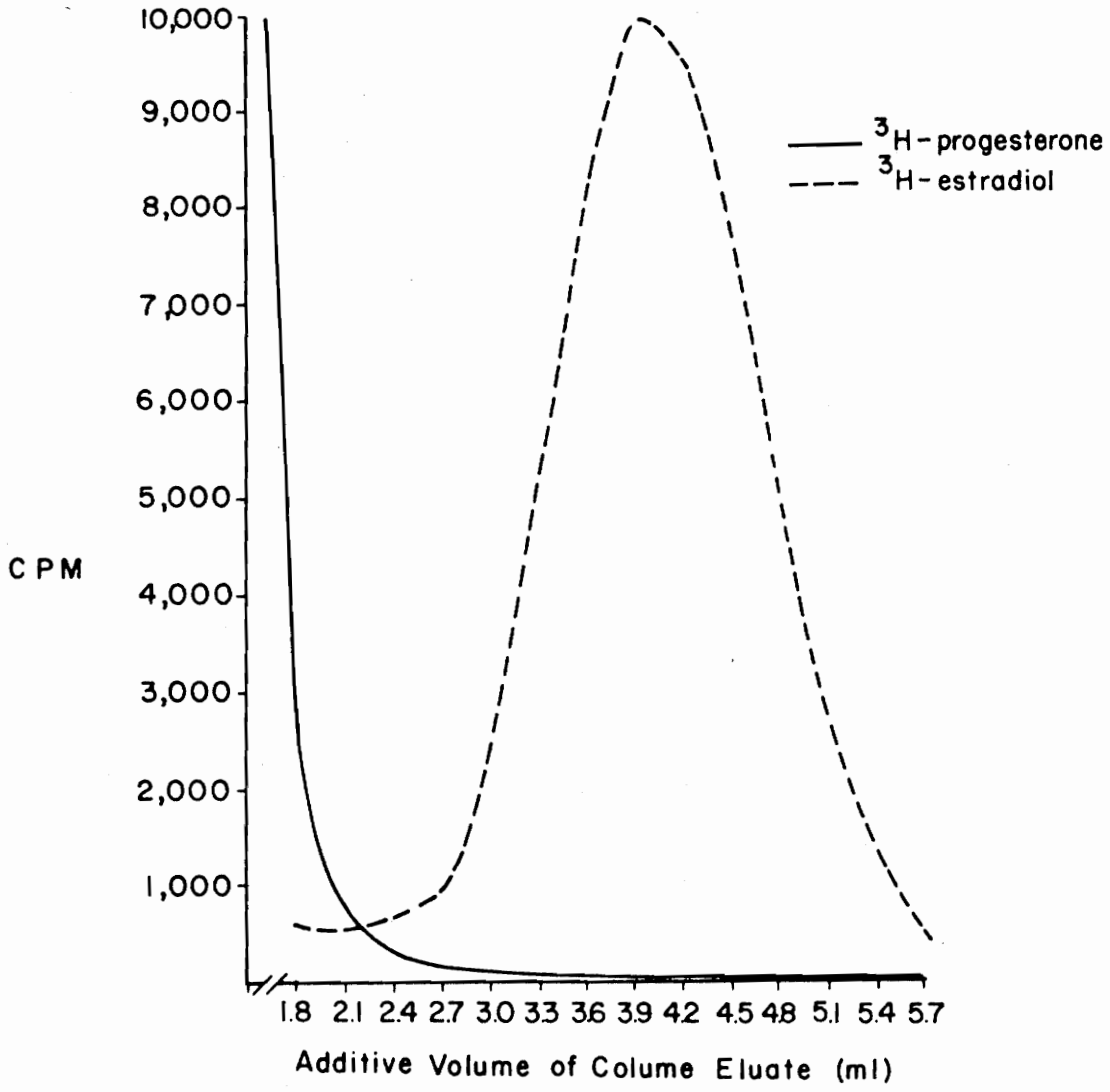
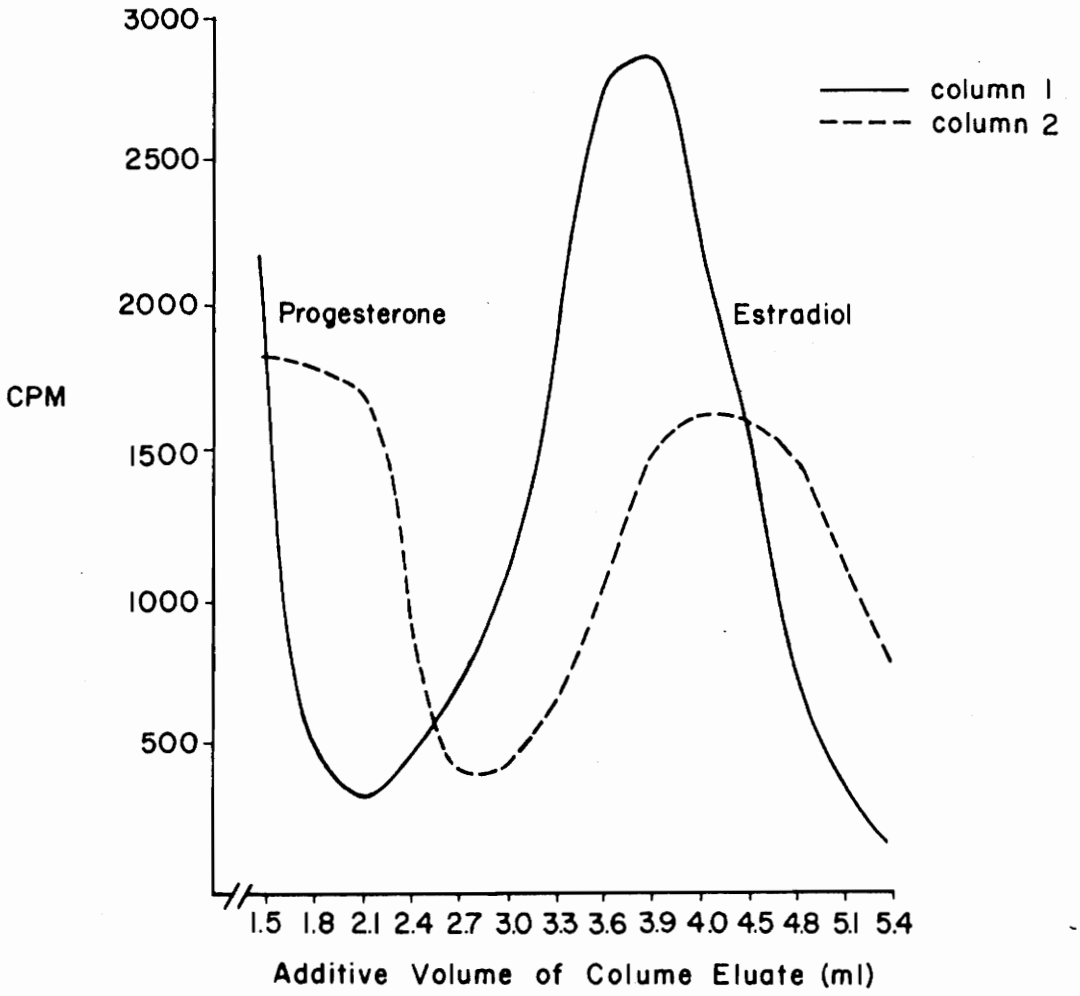

10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
CPM
^{3}H - progesterone
^{3}H - estradiol
1.8 2.1 2.4 2.7 3.0 3.3 3.6 3.9 4.2 4.5 4.8 5.1 5.4 5.7
Additive Volume of Colume Eluate (ml)

Figure 4: Elution patterns of ^{3}H-progesterone and ^{3}H-estradiol to Sephadex LH-20 columns as a mixture.


3000
2500
2000
1500
1000
500
CPM
column 1
column 2
Progesterone
Estradiol
1.5 1.8 2.1 2.4 2.7 3.0 3.3 3.6 3.9 4.0 4.5 4.8 5.1 5.4
Additive Volume of Colume Eluate (ml)

LH-20 columns in .2 ml of solvent. Aliquots of .3 ml were then collected and counted. The separate patterns of elution of progesterone and estradiol in this system are shown in Figure 3. Figure 4 shows the elution patterns of labelled progesterone and estradiol when added to a column as a mixture. It was determined that progesterone and estradiol could be separated with Sephadex column chromatography employing the described procedure. As shown in Figure 2, the normal elution pattern was as follows: progesterone in the first 2.7 ml and estradiol in the next 3 ml. Between samples, the columns were washed with at least 5 ml of solvent.

Dried ether extracts of embryo cultures were redissolved in .2 ml of benzene:methanol (9:1) and transferred to the columns with pasteur pipettes. This was allowed to enter the column before .2 ml was again added from the sample tube. Known fractions were collected in 10 X 75 mm culture tubes by adding known volumes of solvent to the column. Tubes containing the progesterone and estradiol were dried.

Radioimmunoassays

Radioimmunoassays are based on the competitive binding of labelled and unlabelled hormone on an antibody produced against that hormone. When increasing amounts of unlabelled hormone are present, less radioactivity remains attached to the antibody. The amount of antibody and labelled hormone added to each sample is the same. Therefore, if a standard curve of known amounts of unlabelled hormone is set up, the amount of hormone in each unknown sample can be determined. The free hormone is separated from the bound fraction by absorption to dextran-coated charcoal and centrifugation.

Prior to RIA of the samples, the dried column eluent was redissolved in 2 ml of methylene chloride:methanol (9:1). A .2 ml aliquot was taken and allowed to dry in a scintillation vial. Five ml scintillation fluid (3 ℓ toluene and 12 g omnifluor) was then added to the vial. It was counted to determine procedural losses through extraction and column chromatography. The remaining 1.8 ml was split into .5 and 1 ml fractions for duplicate assay estimates. These fractions were dried in 10 X 75 mm culture tubes in preparation for the RIA procedure.

Progesterone Radioimmunoassay

The progesterone antiserum used in this study was prepared in sheep against progesterone-11α BSA and purchased from Dr. G. D. Niswender of Colorado State University. It was stored at -90C in 2-ml aliquots after being diluted 1:500 with phosphate buffered saline (PBS-ga, Appendix 3).

Progesterone standards were prepared containing amounts of 0, 0, 20, 50, 100, 500, 1000, 1500 and 2000 pg. These were pipetted from a solution containing 1 pg/µl using Eppendorf pipettes. Each progesterone assay involved duplicate standard curves to which 40 to 50 unknowns were compared. When all sample and standard tubes were dried, 100 µl of progesterone antibody (1:500) was added. The tubes were then permitted to incubate for 30 minutes at room temperature before adding 200 µl of ^{3}H-progesterone solution (∿23,000 dpm) and vortexing. After addition of the tritiated progesterone, the tubes were incubated in a 37C water bath for five minutes. At the end of this period, they were vortexed and incubated in an ice bath for 40 minutes. At this point, .8 ml dextran-coated charcoal (Appendix 3) was added to each tube

except the initial 0 tube. To this 0 tube was added .8 ml of PBS-ga. The charcoal suspension was incubated in an ice bath for 10 minutes prior to centrifuging at 4000 g for 10 minutes in a refrigerated centrifuge. Following centrifugation, .5 ml of supernatant was removed from each assay tube and added to 5 ml of scintillation fluid (previous recovery scintillation fluid plus 20% v/v triton* C-100).

A standard curve was determined by plotting the logit of percent ^{3}H-progesterone bound ($\ell n \frac{\% \text{ bound}}{1-\text{bound}}$) vs the $\log_{10}$ of quantity of progesterone in the standards. The quantity of progesterone in the unknown samples was determined by comparing the logit of percent binding to the standard curve and its corresponding progesterone content.

The precision of any RIA depends on the specificity and sensitivity of the antibody used. The antibodies produced against one steroid will bind varying amounts of other steroids as well. This is termed crossreactivity. The crossreactivity of our progesterone antibody, and thus its specificity, was tested by running standard curves with other steroids. The crossreactivity of these selected steroids is shown in Table 1.

To test the accuracy of the progesterone quantification technique, known amounts of progesterone (1 and 2 ng), as well as estradiol (1 and 2 ng), were added to freshly prepared BMOC-2. The extraction, column chromatography and progesterone RIA procedures were completed as described. The amounts of progesterone measured are shown in Tables 2 and 3.

Table 1. Crossreactivity* of selected steroids in the radioimmunoassay for progesterone.

Hormone	% Crossreactivity
Progesterone	100.0
Testosterone	38.9
Androstenedione	29.9
Corticosterone	16.7
Cortisol	0
Estradiol	0
Estrone	0

*Crossreactivity = pg of progesterone at 65% binding/pg of steroid at 65% binding.

Table 2. Accuracy of measuring progesterone and estradiol in BMOC-2 (1 ml) using radioimmunoassay.

Amount Added (ng)		Amount Measured[a] (ng)	
Estradiol	Progesterone	Estradiol	Progesterone
1	0	1.05 ± .07[b] (6)[c]	.14 ± .07 (4)
2	0	2.15 ± .09 (4)	.20 ± .10 (4)
0	1	.05 ± .01 (6)	1.10 ± .19 (5)
0	2	.05 ± .01 (4)	2.12 ± .27 (4)
1	1	.96 ± .03 (6)	.87 ± .22 (4)
2	2	1.94 ± .07 (4)	2.44 ± .18 (3)
1	2	.86 ± .05 (4)	2.21 ± .12 (4)
2	1	1.88 ± .10 (4)	1.24 ± .19 (4)
1 ml BMOC-2		.03 ± .01 (6)	.07 ± .04 (3)
1 ml H_2O		.03 ± .10 (4)	.17 ± .08 (4)

[a]Estimates have been adjusted for procedural losses.

[b]$\bar{x}$ ± SE

[c](n) number of cultures

Table 3. Overall accuracy of measuring hormones added to BMOC-2

Progesterone Added (ng)	Progesterone Measured (ng)	n[b]	CV[c]
0	.17 ± .09[a]	8	66
1	1.07 ± .03	13	10
2	2.24 ± .03	11	5
Estradiol Added (ng)	**Estradiol Measured (ng)**	**n**	**CV**
0	.05 ± .01	10	20
1	.96 ± .01	16	3
2	1.99 ± .02	12	3

[a] ($\bar{x}$ ± SE)

[b] (n) number of samples

[c] coefficient of variation (%)

Estradiol Radioimmunoassay

Estradiol antibody was obtained from Dr. G. D. Niswender of Colorado State University. It was produced by immunizing sheep with estradiol-6-BSA. The antibody was diluted to 1:1000 and stored at -90C until use.

Estradiol standards of 0, 0, 1, 5, 10, 25, 50, 100, 200 and 500 pg were measured from a standard solution of 1 pg/µl. Approximately 20 unknowns were run with each standard curve. Sephadex column eluent was dried, redissolved and aliquotted for assay as previously described for progesterone. To begin the assay, .5 ml of PBS-ga was added to each tube followed by a 15-minute incubation at 45C. At the end of this incubation period, the tubes were vortexed. To each tube 100 µl of estradiol antibody (1:19,000) was added followed by a room-temperature incubation (∿25C) for 30 minutes. Approximately 24,000 dpm of ^{3}H-estradiol in 100 µl PBS-ga was then added to each tube, which was followed by vortexing and a 1 hour room temperature incubation. At the end of incubation, the tubes were placed on ice and .5 ml of dextran-coated charcoal (Appendix 3) was added to each tube except the initial 0 tube. To this tube, .5 ml PBS-ga was added. Following a 10 minute incubation in an ice bath, the assay tubes were centrifuged at 4000 g for 10 minutes. One-half ml of supernatant was removed from each tube and placed in a counting vial with 5 ml scintillation cocktail containing triton. The vials were counted for five minutes. Comparison of unknown tubes to the standard curve was similar to that described for the progesterone assay.

The antibody used in this assay was not entirely specific for estradiol and was found to crossreact with certain steroids as shown in Table 4. However, in the chromatographic step, estradiol was isolated from the crossreactivity steroids.

In order to validate this estradiol assay, varying combinations of estradiol and progesterone were added to BMOC-2. Following extraction, column chromatography and RIA of the BMOC-2, the amount of hormone added prior to extraction was successfully measured (Tables 2 and 3).

Statistical Analysis

Data in both experiments were subjected to a simple analysis of variance with a one-way classification (Snedecor and Cochran, 1967).

Table 4. Crossreactivity* of selected steroids in the radioimmunoassay for estradiol.

Hormone	% Crossreactivity
Estradiol	100.0
Estrone	16.9
Androstenedione	15.1
Testosterone	13.0
Corticosterone	4.5
Progesterone	0.6
Cortisol	< 0.1

*Crossreactivity = pg of estradiol at 40% binding/pg of steroid at 40% binding.

RESULTS AND DISCUSSION

Experiment I

The individual treatment means and standard deviations for progesterone and estradiol content in Experiment I are presented in Table 5. Diagramatic illustration of progesterone concentrations in control and incubated cultures is depicted in Figure 5. Although progesterone concentration was not different ($P>.05$) between the varying number of embryos cultured, there was a trend for the lower numbers of control embryos to have a higher progesterone content than corresponding incubated cultures. At 60 embryos/culture, the trend is reversed. Progesterone was not significantly higher ($P>.05$) in the incubated treatment than in the control treatment (Table 6).

There were no significant differences ($P>.05$) in the amount of estradiol between incubated or control embryos nor between the varying numbers of embryos/culture (Table 7). These data are graphically represented in Figure 5.

In Experiment I, with 20, 40 and 60 embryos/culture, the 24-hour viabilities were $98.0 \pm 2.6\%$, $94.5 \pm 4.7\%$ and $93.3 \pm 7.8\%$ ($\bar{x} \pm SD$), respectively. When hormone concentrations were adjusted for viability neither treatment nor embryo numbers significantly influenced progesterone or estradiol concentrations ($P>.05$).

The results do not show a dose-response relationship in hormone production by the early mouse blastocyst. This strongly suggests that the embryos are not, in fact, generating the two steroids in our defined media. However, there appears to be a considerable amount of

Table 5. Progesterone and estradiol content (ng) for incubated mouse blastocysts and control embryos.

Number of Embryos	Incubated	Recovery	Control	Recovery
	Progesterone			
20	.309 ± .159[a] (10)[b]	100	.362 ± .117 (5)	100
40	.289 ± .186 (10)	100	.404 ± .166 (7)	100
60	.343 ± .189 (6)	100	.222 ± .115 (6)	100
	Estradiol			
20	.120 ± .108 (10)	70.9	.123 ± .017 (5)	73.3
40	.147 ± .147 (10)	75.0	.125 ± .117 (7)	76.2
60	.123 ± .113 (6)	70.9	.107 ± .109 (6)	80.8

[a] mean ± SD

[b] (n) number of embryo cultures

[c] mean percent recovery following extraction and isolation prior to RIA

Figure 5. Progesterone and estradiol content for varying numbers of cultured early blastocysts (Experiment I).

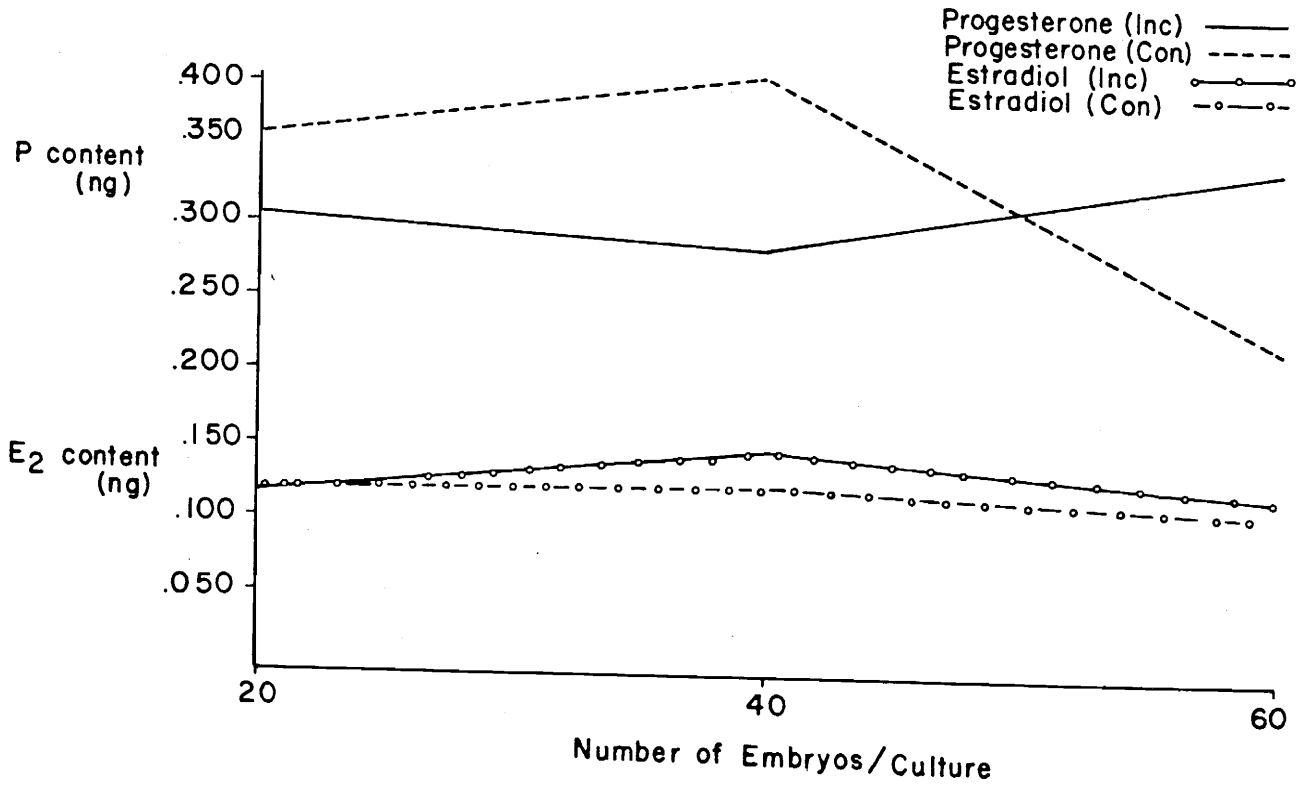

Progesterone (Inc)
Progesterone (Con)
Estradiol (Inc)
Estradiol (Con)
P content (ng)
E_2 content (ng)
.400
.350
.300
.250
.200
.150
.100
.050
20
40
60
Number of Embryos/Culture

Table 6. Analysis of variance for progesterone content for cultures in Experiment I.

Source	df	Mean Square
Number of embryos	2	.004
Treatment	1	.013
Error	39	.035

Table 7. Analysis of variance for estradiol content for cultures in Experiment I.

Source	df	Mean Square
Number of embryos	2	.004
Treatment	1	.002
Error	39	.014

progesterone contained even in the controls (Figure 5). This can be explained in part by blank values of .18 ng obtained in the validation assay for progesterone (Table 3) and tracer isotope which could have been assayed.

Another considerable contributor to the progesterone content of both incubated and control samples may have been endogenous hormone within the embryo itself. Six-day rabbit blastocysts were found to contain .5 ng/blastocyst (Seamark and Lutwak-Mann, 1972). This stage of development in the rabbit corresponds to the developmental stage of the mouse blastocyst used in the present study. The progestins in rabbit blastocysts were suggested to be transferred inside the embryo from endometrial secretion, as opposed to being synthesized by the blastocyst cells. Also, rabbit uterine washings were found to contain progestins.

Two consecutive blastocyst washings were sufficient to remove all the free estradiol associated with the exterior of the blastocyst (Table 8). Since the structure of progestins is very similar to estradiol, we may also conclude that the progesterone was removed as well.

Estradiol, also, was characterized as being contained in the embryo cultures of Experiment I (Figure 5). The same sources of blank levels of progesterone may also be true for the presence of estradiol, although at lower levels. Blank values of .06 ng/ml were obtained in the estradiol validation assay (Table 3) and maximum amount of tracer isotope which could have been assayed. Mouse blastocysts have been shown to take up and bind ^{3}H-estradiol <u>in vitro</u> (Smith, 1968). Therefore, estradiol originating in the uterine secretions may have been

Table 8. Estradiol content of uterine flushings and media from two consecutive washings.

Fluid	Estradiol pg/.5 ml[a]
Uterine flushing	31.0
First wash	12.0
Second wash	1.2

[a]mean content of 5 flushings

contained within the embryos in the present study. There was a considerable amount of estradiol in the uterine washings (Table 8), which was removed by two consecutive blastocyst washings, eliminating this as a background source.

The possibility exists that the blastocyst may have contained estradiol at the time of flushing, in addition to progesterone. An estrogen surge in the mouse on the morning of day 4 of pregnancy has been characterized (McLaren, 1972; McCormack and Greenwald, 1974). In the present experiment, early mouse blastocysts were flushed a few hours after this surge. Estrogen may have been transferred to the blastocysts by this time and possibly contributed to estradiol concentrations in the cultures.

The progesterone results of Experiment I, illustrated graphically in Figure 5, can be interpreted in two ways. First of all, since there was a trend for the mean progesterone content of the incubated cultures to be higher than the corresponding control level, one might conclude that 60 was the minimum number of embryos needed per culture in order to detect steroid production. The difference between incubated and control progesterone at 60 embryos/culture was .121 ng. With a dose response, we would then expect to detect a difference of .08 ng at 40 embryos/culture. Our progesterone RIA was sensitive to .02 ng. Therefore, a difference of .08 ng would be within our sensitivity range. Because a dose response relationship between embryo numbers and progesterone content was not demonstrated, a second interpretation, that of no differences between incubated and control cultues in progesterone content, is taken. Indeed, incubated culture progesterone content was

not significantly higher (P>.05) than progesterone content of the corresponding controls.

Figure 5 shows a trend for progesterone to be higher in the control samples compared to the incubated tubes at both 20 and 40 embryos/culture. This difference was not significant (P>.05). A possible explanation may be a type of feedback mechanism within the embryo. One could expect an in vivo secretion by the embryo to be utilized and, subsequently, removed. However, in vitro, any secretion by the embryo would tend to accumulate. The embryo may have an inherent mechanism to stop production of a steroid when present above certain levels in its localized environment.

Steroid hormones could be involved in an endproduct feedback to inhibit further production. Estradiol was shown not to be degraded at incubation temperatures (McGaughey and Daniel, 1966). We would expect the same of progesterone. Indeed, there were no differences in recovery of tracer progesterone and estradiol between incubated and control samples (Table 5).

There were no differences between incubated and control estradiol concentrations at either 20, 40 or 60 embryos/culture. This suggests that, either no estradiol is produced by early blastocysts under our culture conditions, or levels of production are so low that our quantification techniques could not detect them. Our estradiol assay was sensitive to 5 pg.

In Experiment I, we have shown that certain basal levels of progesterone and estradiol were present in both control and incubated embryos. These steroids have varying effects on developing embryos in

vivo. Progesterone has been implicated in stimulating the secretion of blastokinin in the rabbit uterus (Sugawara and Hafez, 1967; Beier, 1968; Uruzua, et. al., 1970; Arthur and Daniel, 1972). Weitlauf and Greenwald (1968a) found significant incorporation of ^{35}S-methionine by mouse blastocysts after the combined administration of progesterone and estrogen to ovariectomized mice. Roblero (1973) noted that progesterone-treated mice had more blastomeres/embryo than embryos of ovariectomized mice without treatment. He concluded that maternal hormones may control factors of embryonic development such as cleavage rates in mice.

Progesterone and estrogen also were shown to have inhibitory effects on in vitro embryonic development. When morula transfer in rats was preceded by 2, 3, 4, 5 or 6 days of maternal progesterone treament, decreasing percentages of morulae developed to term (Dickmann, 1970). These deleterious effects could be interpreted as being over-stimulation by progesterone, lack of estrogen or both.

In vitro, progesterone in doses greater than 2 µg/ml of culture media was reported to be antagonistic to development of mouse ova (Whitten, 1956b; 1957b). Estrogen had no antagonistic or protective actions against the effect of progesterone. Cleavage of pre-blastocyst rabbit embryos was inhibited by progesterone at concentrations of 10 µg/ml or more (Daniel and Levy, 1964). El-Banna and Daniel (1972) cultured day 5 rabbit blastocysts in media supplemented with either progesterone alone or progesterone in combination with macromolecular components of serum or fluids of day 5 pregnant rabbits. Embryos grew best and took up greater amounts of uridine and amino acids in the

medium containing progesterone and the uterine components than either of the other treatments. They postulated that progesterone may be useful or even essential to the normal development of the rabbit blastocyst when in complex with a carrier protein.

A 24-hour exposure to estrogens (.1 mg/ml) was lethal to day 1 rabbit embryos in vitro (Ketchel and Pincus, 1964). When incubated with 25 µg/ml estradiol, and several hours remained between the exposure time and expected cleavage, 1-cell rabbit embryos failed to cleave (McGaughey and Daniel, 1966).

Therefore, steroid hormones found within the embryo (Experiment I) may have either stimulatory or inhibitory effects on development. If, in fact, their effect was inhibitory in nature, the blastocyst may not be affected because there was a general trend toward resistance to steroids as embryos progressed in development and the total cell surface became larger (Daniel and Levy, 1964; Kirkpatrick, 1971).

Experiment II

Experiment II was designed to test the steroidogenic capabilities of two other developmental stages, thereby exploring proposed hypotheses of other workers (Dickmann and Dey, 1973) that hormones may be produced at these stages and may be prerequisite to early embryogenesis. Dickmann (1969) demonstrated that rat morulae were transformed to viable blastocysts after transfer to long-term ovariectomized recipients. He proposed that the rat morulae may be producing steroids at this stage to aid in this transformation. Also, Dickmann and Dey (1974a) demonstrated the presence of Δ^5-3β-hydroxysteroid dehydrogenase (HSD) activity in blastocysts of rats after their incubation with

dehydroepiandrosterone. Experiment II was therefore designed to test the steroidogenic capabilities of mouse morulae, early blastocysts and late blastocysts. Late blastocysts represented the developmental period corresponding to the process of implantation *in vivo*.

Progesterone and estradiol concentrations in relation to embryonic stage are presented in Table 9. Overall viabilities for morulae, early blastocysts and late blastocysts were 95.8 $\pm$ 5.4%, 94.5 $\pm$ 4.7% and 97.3 $\pm$ 4.7% ($\bar{x}$ $\pm$ SD), respectively. There were significant differences due to stage and treatment ($P<.01$) in progesterone content of cultures in Experiment II (Table 10). Progesterone was higher ($P<.05$) in early blastocysts (Figure 6) than either morula or late blastocyst stages. As in Experiment I, control samples contained more progesterone than incubated samples. Because incubated levels of progesterone were not greater than controls, the stage effect is not due to a greater production of progesterone by the early blastocysts as compared to the other stages. However, it may be explained by a higher endogenous progesterone content of the blastocyst at the time they were flushed from the uterus. This may be interpreted to mean that maternal progesterone is transferred to the early blastocyst and may be facilitory in the implantation process. A period of several days of progesterone dominance is needed in the mouse, following estrus, for optimal uterine sensitivity to implantation stimulation (Finn, 1966; Humphrey, 1967; McLaren, 1972).

Estradiol contents of cultures in Experiment II are shown in Table 9. Differences due to stage or treatment (Table 11) were not

Table 9. Progesterone and estradiol content (ng) for incubated mouse blastocysts and control embryos.

Stage of Development[a]	Incubated	Recovery (%)	Control	Recovery (%)
		Progesterone		
Morula	.192 ± .084[b] (10)[c]	98.3	.306 ± .152 (5)	100
Early blastocyst	.289 ± .186 (10)	100	.404 ± .166 (7)	100
Late blastocyst	.157 ± .089 (10)	100	.241 ± .142 (7)	100
		Estradiol		
Morula	.082 ± .068 (10)	85.6	.089 ± .090 (5)	92.4
Early blastocyst	.147 ± .147 (10)	75.0	.125 ± .117 (7)	76.2
Late blastocyst	.088 ± .072 (10)	72.5	.086 ± .102 (7)	75.5

[a] morula - 76 to 79 hours post coitum (P.C.)
early blastocyst - 85 to 86 hours P.C.
late blastocyst - 94 to 96 hours P.C.

[b] mean ± SD

[c] number of embryo cultures

Table 10. Analysis of variance for progesterone content for cultures in Experiment II.

Source	df	Mean Square
Stage	2	.096*
Treatment	1	.174**
Error	45	.021

*(P<.05)

**(P<.01)

Figrue 6. Progesterone and estradiol content for different stages of cultured embryos (Experiment II).

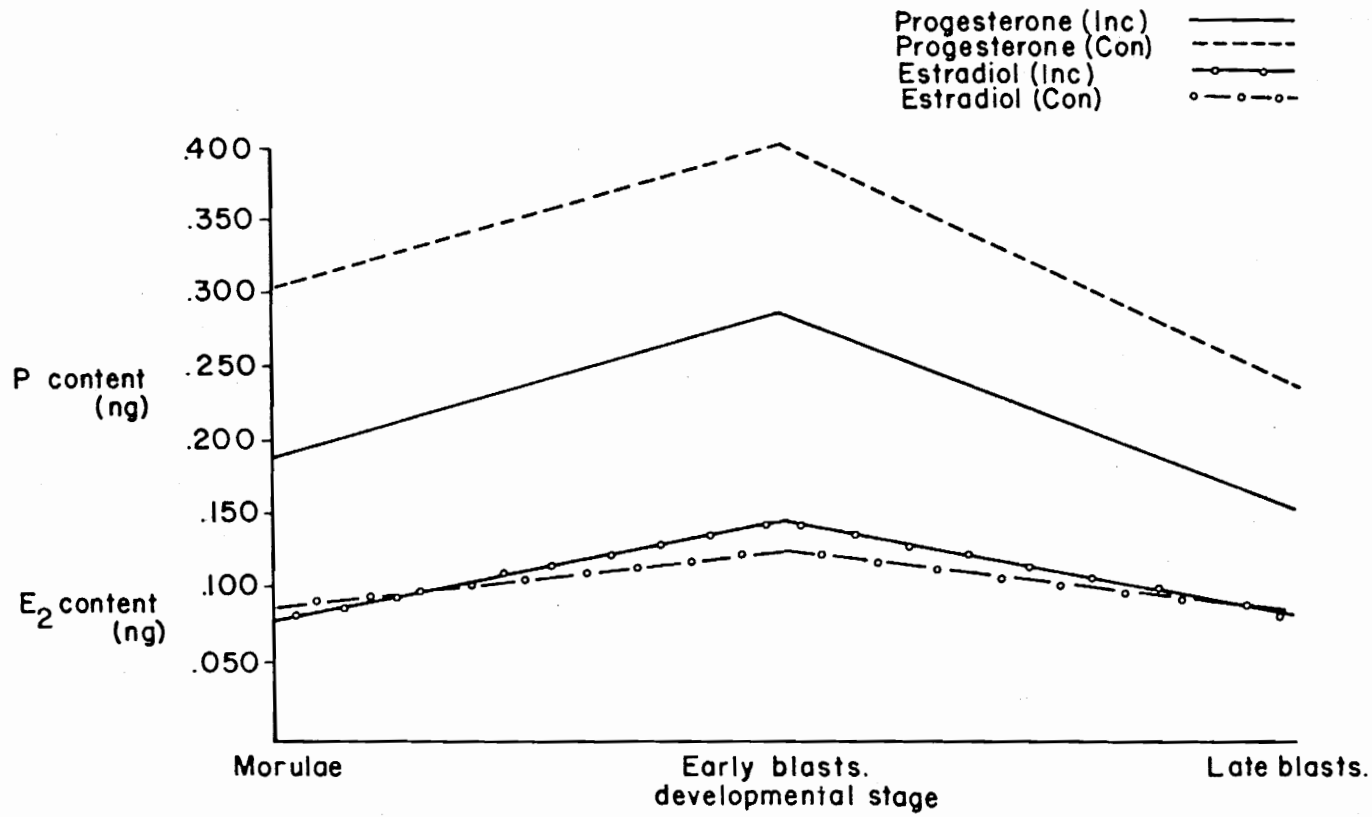

Progesterone (Inc)
Progesterone (Con)
Estradiol (Inc)
Estradiol (Con)
.400
.350
.300
.250
.200
.150
.100
.050
P content (ng)
E_2 content (ng)
Morulae
Early blasts.
developmental stage
Late blasts.

Table 11. Analysis of variance for estradiol content for cultures in Experiment II.

Source	df	Mean Square
Stage	2	.019
Treatment	1	.001
Error	45	.011

shown to be statistically significant (P>.05). Estradiol content of Experiment II cultures is shown graphically in Figure 6.

The presence of a basal quantity of estradiol, thought to be transferred from the maternal secretions prior to flushing, was discussed in Experiment I.

Much evidence has been put forth in the past few years for possible steroidogenic capabilities of mammalian preimplantation embryos. Seamark and Lutwak-Mann (1972) demonstrated the presence of progestins in free-lying blastocysts and showed these levels to be higher than those in the corresponding uterine fluids. These workers concluded that the progestins found in the blastocysts were probably transported from the uterine fluids. However, Huff and Eik-Ness (1966), after incubating rabbit blastocysts with radioactive steroid precursors, indicated that these embryos could biosynthesize cholesterol and pregnenolone from acetate and could promote various steroid biotransformations. The activity of 3β-hydroxysteroid dehydrogenase (HSD) in rabbit embryos was found to begin in morulae and was sustained through the late blastocyst stage (Dickmann, et. al., 1975a).

Dickmann and his co-workers have given indirect histochemical evidence for the activity of certain steroidogenic enzymes in the preimplantation embryos of several rodent species. In the rat, slight HSD activity was noted in morulae and by day 5, all cells of the rat blastocyst showed formazan granule deposition (Dickmann and Dey, 1974a). The 3β-HSD enzyme was also demonstrated in blastocysts of corresponding developmental stages in the rabbit (Dickmann, et. al., 1975a; 1975b),

mouse (Dey and Dickmann, 1974c) and hamster (Dickmann and Sen Gupta, 1974). When using estradiol-17β as substrate, the 17β-HSD enzyme was characterized in blastocysts of the rat (Dey and Dickmann, 1974a) and hamster (Dickmann and Sen Gupta, 1974) at the same stage of development.

In their work with this histochemical technique, Dickmann and Dey (1973) postulated that the preimplantation embryo was a source of steroid hormones which controlled the morula-to-blastocyst transformation as well as implantation. In working with these specific stages in the mouse (Experiment II, Table 9, Figure 6), no steroidogenic capabilities were demonstrated by morulae, early blastocysts or late blastocysts. In their histochemical studies, Dickmann and Dey (1974b) used precursors of specific enzymes in incubations with blastocysts, whereas in the present experiment, a defined media was employed with no added direct steroid precursors. However, glucose, pyruvate and lactate were all present in the culture media (Appendix 1).

There was a 100-fold increase in the conversion of glucose to CO_2 during the first five days of development in the mouse embryo (Brinster, 1967a), demonstrating an increase in glycolytic capabilities up to the time of implantation. An increase in utilization of the Embden-Meyerhof enzymes is also evident by the 8-cell stage in the mouse (Wales and Brinster, 1968). Even in the absence of the glycolytic enzymes, pyruvate and lactate may have possibly entered the tricarboxylic acid cycle as acetyl CoA.

These evidences for the operating glycolytic sequence and TCA cylce in preimplantation mouse embryos also demonstrates the presence of acetyl CoA in the embryo at this point in development. All the carbon atoms of cholesterol were found to be derived from acetate (Lehninger, 1975). Huff and Eik-Ness (1966) indicated that rabbit blastocysts could synthesize cholesterol from labelled acetate. Since cholesterol is the precursor of steroid molecules, and its synthesis by the embryo has been shown, true steroidogenesis by the embryo should not require a direct steroid precursor in vitro. The presence of glucose, pyruvate or acetate would be sufficient.

The results of Experiment I show that with 20, 40 or 60 early mouse blastocysts, incubated in a defined medium with glucose, pyruvate and lactate, no progesterone or estrogen biosynthetic capabilities could be detected. When expanded to include morulae and late blastocysts, no progesterone or estradiol were found. It was indeed possible that more than 40 or 60 embryos may be needed to produce sufficient steroid to enable their quantification by RIA. Using the t-test (Snedecor and Cochran, 1967), .12 ng would have been a significant treatment difference (α=.05) using the present experimental design. At 60 embryos/culture this means a production of 2.0 pg/embryo of hormone would have been significant. Furthermore, Sherman and Atienza (1977) were unable to demonstrate progesterone synthesis by up to 300 mouse blastocysts per culture.

The possibility of biosynthetic utilization and metabolism of progesterone and estradiol in the present study is remote because tracer

isotope added before the initiation of culture was not metabolized (Tables 5 and 9).

Progesterone utilization as a precursor in the steroidogenic pathway is not evident because of the high recovery of the label in both incubated and control samples. Any metabolite produced from ^{3}H-progesterone would have been removed from the sample by the chromatographic step. Sherman and Atienza (1977) indicated that 4- to 5-day mouse blastocyst cultures were unable to metabolize progesterone, while 10- to 11-day post-blastocyst cultures did metabolize progesterone into at least three products determined by chromatographic analysis.

These same workers have recently concluded that mouse embryos were not capable of progesterone and androstenedione synthesis *in vitro* until day 6.5 of equivalent gestation age. This age corresponds to post-implantational development. At this time, mouse embryos transformed pregnenolone into progesterone and dehydroepiandrosterone into androstenedione, indicating 3β-HSD activity.

It appears that steroidogenic capabilities of preimplantation embryos could have been detected by culturing 40 embryos. Our results tend to eliminate the possibility that progesterone and estradiol were being metabolized. Direct evidence has been given that viable mouse embryos prior to the time of implantation, are not capable of progesterone or estradiol biosynthesis in a defined medium *in vitro* with simple precursors supplied.

Before it is concluded that early embryos do not produce steroids *in vivo*, several other areas must be searched. The uterine secretions in mice may contain direct precursors which can be transformed into

steroids by the embryo. Culturing embryos in uterine secretions and/or various steroid precursors in different combinations would answer this question.

The luteotropic or anti-luteolytic effect which the embryo exerts on the uterus is left unresolved. The possibility that embryo-secreted steroids aid in morula-to-blastocyst transformation and/or stimulate the implantation response has been seriously questioned by this study. It does appear, however, that a continuous secretion by the ovine embryo, probably beginning on day 12 of pregnancy, has either a luteotrophic or anti-luteolytic effect in the recognition of pregnancy (Moor and Rowson, 1966a; 1966b; Rowson and Moor, 1967).

Sheep embryonic homogenate, when repeatedly infused into the uterus, maintained the corpora lutea beyond the regular estrous cycle (Rowson and Moor, 1967). Since this material was active after freezing and thawing at -20C, the possibility remains that the active compound could be protein in nature. In this same study, heat-inactivated embryos had no effect on the cycle length, implicating a protein molecule which would be denatured by this treatment. Also, homogenized pig embryos of the same developmental stage were not effective, indicating a species specificity. Steroid molecules are similar between species. However, one may expect proteins to exhibit species specificities.

Krishnan and Daniel (1967) and Beier (1968) described the presence of a protein (blastokinin) in rabbit uterine secretions and blastocoelic fluids which ahad a favorable effect of developing rabbit

embryos *in vitro*. Beier and Mauer (1975) compared protein patterns of blastocoelic fluids of the rabbit after *in vivo* and *in vitro* development. Large differences in protein patterns were found between the systems. This was interpreted to mean that blastokinin originated in the uterine endometrium and was not synthesized by the preimplantation embryo. However, this does not preclude the synthesis of other proteins in the blastocyst.

The protein content of mouse embryos decreased from 27.77 ng at the 1-cell stage to 20.58 ng in morulae (Brinster, 1967b). However, after the morula stage, there is an increase in protein content with blastocyst formation. Therefore, the anti-luteolytic agent in the embryo may be synthesized in the embryo.

SUMMARY

ICR albino mouse embryos were used to determine their possible production of progesterone or estradiol. A total of 4180 embryos were used in two experiments. In Experiment I, 20, 40 and 60 early blastocysts were flushed at 1300 to 1400 hours on day 4 of pregnancy and incubated in 10, 10 and 6 cultures, respectively. These cultures were maintained in 13 X 100 mm tubes with .25 ml of BMOC-2 for 20 hours under an atmosphere of 5% CO_2 in air at 37C. Also, 20, 40 and 60 early blastocysts in 4, 7 and 6 tubes, respectively, were frozen at -90C after flushing. Viability was determined by incubating 20, 40 and 60 early blastocysts in 5, 7 and 6 cultures for 24 hours, at which time percent normal development was microscopically evaluated. The overall viability in Experiment I was 95.4%.

Incubated and control cultures were extracted with diethyl ether and progesterone and estradiol were separated on Sephadex LH-20 columns prior to quantification by radioimmunoassay.

In Experiment I, culture progesterone and estradiol were .31 $\pm$ .17 ng ($\bar{x} \pm$ SD) and .13 $\pm$ .12 ng compared to .33 $\pm$ .16 ng and .12 $\pm$ .10 ng/sample for the controls. There were no significant effects ($P>.05$) of embryo numbers or treatment on the hormone content of these cultures. Therefore, it was determined that early blastocysts, in a defined medium *in vitro*, were incapable of progesterone or estradiol synthesis.

Experiment II was designed to test the steroidogenic capabilities of three different developmental stages of embryos. Morulae flushed

at 0400 to 0700 hours on day 4, early blastocysts flushed at 1300 to 1400 hours on day 4 and late blastocysts obtained at 2200 to 2400 hours on day 4 were incubated in 10 cultures each. Each culture contained 40 embryos. Forty embryos of these three stages were frozen in 5, 7 and 7 tubes, respectively. Viabilities, averaging 95.8% in this experiment, were determined on 5, 7 and 7 cultures containing the identical stages as above.

In Experiment II, incubated progesterone and estradiol were .21 $\pm$ .13 and .11 $\pm$ .10 ng compared to .32 $\pm$ .16 and .10 $\pm$ .10 ng/culture for the corresponding controls. In this experiment, there were significant effects ($P<.01$) of stage and treatment on progesterone content, with controls containing higher concentrations than the incubated treatments. There were no differences due to stage or treatment in estradiol concentration ($P>.05$).

These results do not show production of steroids by the early mouse embryo. The data suggest that the mouse embryo does not produce quantifiable levels of progesterone or estradiol in a defined medium at either the morula, early blastocyst or late blastocyst stages, although viability is maintained.

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Appendix 1. COMPOSITION OF BRINSTER'S MEDIUM FOR OVUM CULTURE (BMOC-2)[a]

REAGENT		
Glucose		1.0 g
Sodium chloride	(NaCl)	5.546 g
Sodium pyruvate		0.056 g
Potassium chloride	(KCl)	0.356 g
Calcium chloride	($CaCl_2$)	0.189 g
Monopotassium phosphate	(KH_2PO_4)	0.162 g
Magnesium sulfate	($MgSO_4 7H_2O$)	0.294 g
Sodium bicarbonate	($NaHCO_3$)	2.106 g
Sodium lactate	(85% liquid)	2 ml in 100 ml of 3X distilled H_2O & adjusted to pH 7.4 with 1N NaOH
Potassium penicillin G		100 units/ml
Dihydrostreptomycin sulfate		50 µg/ml
Bovine serum albumin		5 mg/ml
Deionized water		to make 1 liter

[a]Brinster, 1965e

Appendix 2. List of materials

Culture Reagents and Instrumentation

500 random-bred ICR albino mice
.90 mm watch glasses (Arthur H. Thomas)
100 X 15 mm petri dishes (Kimble Products)
disposable pasteur pipettes (Arthur H. Thomas)
sterilizing oven (Precision Scientific Company)
isotemp incubator (Fisher Scientific Company)
5% CO_2 in air (Airco)
Brinster's Medium for Ovum Culture (Appendix 1)
stereomicroscope (American Optical Company)
surgical scissors, hemostat, forceps (Arista Surgical Supply)
metofane (Pitman-Moore, Inc.)
Millipore filter (Millipore Corporation)
1 cc tuberculin syringes (Pharmaseal Laboratories)
Luer-lok needles - 23 gauge (Becton, Dickinson and Company)

Assay Reagents and Instrumentation

acetone (P. B. & S. Chemical Company)
benzene (Fisher Scientific Company)
methylent chloride (J. T. Baker Chemical Company)
methanol (Fisher Scientific Company)
ether (Mallinckrodt)
absolute ethanol (Mallinckrodt)
omnifluor* (New England Nuclear)
triton* X-100 (Amersham/Searle Corporation)
toluene (Mallinckrodt)
Sephadex LH-20 (Sigma Chemical Company)
clinical grade dextran - m.w. 73,200 (Sigma Chemical Company)
activated charcoal Norit A (Sigma Chemical Company)
gelatin - unflavored (Knox Gelatine, Inc.)
Deionizer (Cole-Parmer Instrumental Company)
B-D Yale, 2.5 ml glass syringes (Becton, Dickinson and Company)
Delta 300 liquid scintillation counting system - Model 6890
(Searle Analytic, Inc.)
automatic diluter (Repipet-Labindustries)
magnetic stirrer PC-353 (Corning Glass Works)
super-mixer (Arthur H. Thomas)
Mettler balance (Mettler Instruments Company)
ultra low freezer - Model ULT-657 (Revco, Inc.)
Sorvall RC-5 superspeed refrigerated centrifuge (DuPont Instruments)

Appendix 2. List of materials (continued)

selectapette pipetter (Clay Adams)
micropipetter (Arthur H. Thomas)
10 X 75 mm Pyrex borosilicate culture tubes (Corning Glass Works)
Eppendorf pipettes (Arthur H. Thomas)
1 ml glass pipettes (Corning Glass Works)
Silent 700 electronic data terminal (Texas Instruments, Inc.)
5.5 cm Whatman filter paper (Arthur H. Thomas)

Appendix 3. Assay reagent preparation.

REAGENTS

1. Solution A. (0.2 M)

$NaH_2PO_4 \cdot H_2O$	27.6 g
Distilled H_2O	1000 ml

2. Solution B. (0.2 M)

$Na_2HPO_4 \cdot 12\ H_2O$	71.64 g
or Na_2HPO_4 (anhydrous)	28.4 g
Distilled H_2O	1000 ml

3. Phosphate Buffer (PBS-a), pH 7.0

Sodium Azide	1 g
Sodium Chloride	9 g
Solution A.	195 ml
Solution B.	305 ml
Distilled H_2O	500 ml

4. 0.1% Gelatin in phosphate buffer (PBS-ga)

Gelatin (Knox unflavored)	100 mg
PBS-a.	100 ml

5. Dextran-Charcoal Solution

Ingredient	Estradiol Assay	Progesterone Assay
Charcoal (Norit A)	625 mg	250 mg
Dextran (T70 of T80)	400 mg	25 mg
PBS-ga	100 ml	100 ml

Appendix 4. Experiment I results

Tube #	# Embryos	% Viability	Progesterone (ng/culture) Incubated	Control	Extraction Recovery (%)	Estradiol (ng/culture) Incubated	Control	Extraction Recovery (%)
145a	20	95.0	.348	----	100.0	.052	----	52.7
145b	20	95.0	.321	----	100.0	.095	----	67.2
145c	20	----	----	.447	100.0	----	.084	67.6
156a	20	95.0	.126	----	100.0	.257	----	54.3
156b	20	95.0	.170	----	100.0	.205	----	84.5
156c	20	----	----	.440	100.0	----	.171	62.2
157a	20	100.0	.190	----	100.0	.204	----	60.3
157b	20	100.0	.186	----	100.0	.291	----	48.9
157c	20	-----	----	.367	100.0	----	.187	71.7
158a	20	100.0	.598	----	100.0	.030	----	90.7
158b	20	100.0	.241	----	100.0	.035	----	64.9
162a	20	100.0	.539	----	100.0	.015	----	89.1
162b	20	100.0	.368	----	100.0	.016	----	78.9
162c	20	-----	----	.195	100.0	----	.048	100.0
146a	40	92.5	.288	----	100.0	.056	----	85.0
146b	40	92.5	.295	----	100.0	.081	----	62.8
146c	40	----	----	.244	100.0	----	.232	48.7
147a	40	100.0	.067	----	100.0	.374	----	50.4
147c	40	-----	----	.127	100.0	----	.265	63.6
149a	40	87.5	.369	----	100.0	.239	----	75.3
149b	40	87.5	.343	----	100.0	.202	----	77.2
149c	40	----	----	.446	100.0	----	.250	81.2
148a	40	100.0	.210	----	100.0	.032	----	85.5
148c	40	-----	----	.512	100.0	----	.015	70.1

Appendix 4. Experiment I results (continued)

Tube #	# Embryos	% Viability	Progesterone (ng/culture) Incubated	Progesterone (ng/culture) Control	Progesterone (ng/culture) Extraction Recovery (%)	Estradiol (ng/culture) Incubated	Estradiol (ng/culture) Control	Estradiol (ng/culture) Extraction Recovery (%)
151a	40	100.0	.631	----	100.0	.022	----	62.8
151c	40	-----	----	.376	100.0	----	.042	88.6
154a	40	95.0	----	----	100.0	.399	----	84.9
154c	40	----	----	.552	100.0	----	.025	90.3
155a	40	95.0	0	----	100.0	.026	----	79.9
155b	40	95.0	.397	----	100.0	.040	----	85.8
155c	40	----	----	.568	100.0	----	.048	91.1
150a	60	100.0	.472	----	100.0	.230	----	83.8
150c	60	-----	----	.120	100.0	----	.266	66.6
152a	60	100.0	.229	----	100.0	.228	----	70.1
152c	60	-----	----	.230	100.0	----	.185	74.6
153a	60	98.3	.131	----	100.0	.218	----	54.8
153c	60	----	----	.102	100.0	----	.147	59.7
163a	60	90.0	.361	----	100.0	.014	----	91.7
163c	60	----	----	.386	100.0	----	.019	100.0
165a	60	80.0	.642	----	100.0	.006	----	83.0
165c	60	----	----	.166	100.0	----	.003	99.4
166a	60	91.7	.223	----	100.0	.041	----	41.8
166c	60	----	----	.327	100.0	----	.019	84.3

Appendix 5. Experiment II results

Tube #	Stage	% Viability	Progesterone (ng/culture) Incubated	Control	Extraction Recovery (%)	Estradiol (ng/culture) Incubated	Control	Extraction Recovery (%)
169a	morula	85.0	.172	----	85.3	.028	----	90.4
169c	morula	----	----	.175	100.0	----	.017	100.0
170a	morula	97.5	.234	----	100.0	.039	----	100.0
170b	morula	97.5	.185	----	98.1	.026	----	97.5
$170c_1$	morula	----	----	.183	100.0	----	.023	93.2
170d	morula	97.5	.154	----	100.0	.021	----	100.0
$170c_2$	morula	----	----	.174	100.0	----	.015	100.0
175	morula	100.0	.160	----	100.0	.024	----	96.0
175c	morula	-----	----	.545	100.0	----	.036	94.0
176a	morula	100.0	.161	----	100.0	.113	----	80.1
176b	morula	100.0	.195	----	100.0	.124	----	89.1
176c	morula	-----	----	.345	100.0	----	.199	88.2
177a	morula	90.0	.335	----	100.0	.195	----	67.6
177b	morula	90.0	.294	----	100.0	.185	----	66.9
177c	morula	----	----	.285	100.0	----	.174	79.0
181	morula	100.0	.027	----	100.0	.067	----	68.1
181c	morula	-----	----	.285	100.0	----	----	64.5
146a	early blastocyst	92.5	.288	----	100.0	.056	----	85.0
146b	early blastocyst	92.5	.295	----	100.0	.081	----	62.8
146c	early blastocyst	----	----	.244	100.0	----	.232	48.7
147a	early blastocyst	100.0	.067	----	100.0	.374	----	50.4
147c	early blastocyst	-----	----	.127	100.0	----	.265	63.6
149a	early blastocyst	87.5	.369	----	100.0	.239	----	75.3
149b	early blastocyst	87.5	.343	----	100.0	.202	----	77.2
149c	early blastocyst	----	----	.446	100.0	----	.250	81.2

Appendix 5. Experiment II results (continued)

Tube #	Stage	% Viability	Progesterone (ng/culture) Incubated	Control	Extraction Recovery (%)	Estradiol (ng/culture) Incubated	Control	Extraction Recovery (%)
148a	early blastocyst	100.0	.210	----	100.0	.032	----	85.5
148c	early blastocyst	-----	----	.512	100.0	----	.015	70.1
151a	early blastocyst	100.0	.631	----	100.0	.022	----	62.8
151c	early blastocyst	-----	----	.376	100.0	----	.042	88.6
154a	early blastocyst	95.0	----	----	100.0	.399	----	84.9
154c	early blastocyst	----	----	.552	100.0	----	.025	90.3
155a	early blastocyst	95.0	0	----	100.0	.026	----	79.9
155b	early blastocyst	95.0	.397	----	100.0	.040	----	85.8
155c	early blastocyst	----	----	.568	100.0	----	.048	91.1
171a	late blastocyst	85.0	.154	----	100.0	.034	----	81.4
171c	late blastocyst	----	----	.305	100.0	----	.068	89.8
172a	late blastocyst	100.0	.159	----	100.0	.016	----	98.4
172c	late blastocyst	-----	----	.445	100.0	----	.041	86.5
173a	late blastocyst	100.0	----	----	100.0	----	----	----
173c	late blastocyst	-----	----	.170	100.0	----	.027	90.3
174a	late blastocyst	100.0	.309	----	100.0	.051	----	78.5
174b	late blastocyst	100.0	.216	----	100.0	.014	----	88.0
174c	late blastocyst	-----	----	.172	100.0	----	.015	96.5
178a	late blastocyst	100.0	.128	----	100.0	.123	----	76.3
178c	late blastocyst	-----	----	.267	100.0	----	.098	81.4
179a	late blastocyst	100.0	.103	----	100.0	.129	----	85.3
179b	late blastocyst	100.0	.251	----	100.0	.249	----	59.6
179c	late blastocyst	-----	----	.326	100.0	----	.308	83.8
180a	late blastocyst	95.0	.027	----	100.0	.106	----	76.8
180b	late blastocyst	95.0	----	----	100.0	.116	----	80.2
180c	late blastocyst	----	----	0	100.0	----	.042	68.5
180d	late blastocyst	95.0	.068	----	100.0	.043	----	74.6

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INVESTIGATIONS OF STEROIDOGENIC CAPABILITIES OF THE EARLY EMBRYO

by

Kenneth E. Grube

(ABSTRACT)

ICR albino mouse embryos (n=4180) were used to determine production of progesterone and estradiol. In Experiment I, cultures containing 20 (n=10), 40 (n=10) or 60 (n=6) early blastocysts were incubated in 13 X 100 mm tubes with .25 ml BMOC-2 for 20 h under 5% CO_2 and air at 37C. Also, 20 (n=4), 40 (n=7) and 60 (n=6) control embryos were frozen at -90C after flushing. Viability was determined by culturing 20 (n=5), 40 (n=7) and 60 (n=6) for 24 h at which time percent normal development was microscopically evaluated.

In Experiment II, 40 embryos at either morula, early blastocyst or late blastocyst (n=10) stage were cultured similarly. Viability and control steroid levels were determined on n=5, n=7 and n=7 cultures. Incubated and control cultures were extracted with diethyl ether and progesterone and estradiol isolated on Sephadex LH-20 columns prior to quantification by radioimmunoassay.

Viability for all cultures was 95.6 $\pm$ .05% ($\bar{x}\pm$SD). In Experiment I, incubated progesterone and estradiol were .31 $\pm$ .17 and .13 $\pm$ .12 ng/culture compared to .33 $\pm$ .16 and .12 $\pm$ .10 ng/culture for controls. In Experiment II, incubated progesterone and estradiol were .21 $\pm$ .13 and .11 $\pm$ .10 ng compared to .32 $\pm$ .16 and .10 $\pm$ .10 ng/culture for controls. There were no effects of embryo numbers, stage of development or treatment on hormone content. These data suggest that the

early mouse embryo does not produce progesterone or estradiol when cultured in a defined medium, though viability is maintained.