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BIOCHEMICAL ASPECTS OF EMBRYONIC DEVELOPMENT IN PRIMATES FOLLOWING *IN VITRO* FERTILIZATION

By

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ABSTRACT

BIOCHEMICAL ASPECTS OF EMBRYONIC DEVELOPMENT IN PRIMATES FOLLOWING *IN VITRO* FERTILIZATION

By

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These studies were designed to evaluate the viability and biochemical alterations in squirrel monkey ova fertilized and developed *in vitro*.

Exclusion of the vital dye trypan blue and uptake of fluorescein diacetate, by hamster and squirrel monkey (*in vitro* fertilized) ova showed high correlations with *in vitro* development and relative RNA and protein synthesis. ^{3}H-Uridine incorporation in unfertilized squirrel monkey oocytes was diminished with time after HCG administration. There was an increase in ^{3}H-uridine incorporation after fertilization *in vitro*, with another rise following the second cleavage division. ^{3}H-Leucine incorporation decreased with oocyte maturation, and then remained constant to the two-cell stage.

Uptake of estradiol-17β and progesterone by squirrel monkey ova increased with fertilization *in vitro* from 0.59 to 0.87, and 0.21 to 0.49 picomoles/embryo/2 hr, respectively, and to 1.20 and 0.38 at the two-cell stage. Changing the length of FSH treatment prior to HCG-induced ovulation in *Saimiri* did not affect uptake. However, the PMS-HCG superovulatory regimen reduced uptake of both steroids at all

embryonic stages in the hamster. There was no effect on ^{3}H-uridine incorporation.

Unfertilized, immature oocytes from squirrel monkeys consumed 4.85 nanoliters oxygen/oocyte/4 hr. $^{14}CO_2$ production was 41.9 picogram-atoms glucose carbon incorporated/oocyte/4 hr. The molar ratio of $^{14}CO_2$ produced from glucose to total oxygen uptake was estimated at 0.19.

Uptake of 2-deoxyglucose by unfertilized oocytes from squirrel monkeys was not affected by the addition of either 10 nM or 1 μM insulin. There was no change in 2-deoxyglucose uptake at _in vitro_ fertilization. Degenerate ova had significantly reduced levels of 2-deoxyglucose uptake.

Detectable biochemical changes occur in the primate ovum with _in vitro_ fertilization. These include augmented ^{3}H-uridine incorporation and steroid uptake, and diminished incorporation of ^{3}H-leucine. Glucose utilization remains quite low in early primate embryos. These results indicate normal metabolic development of primate embryos fertilized _in vitro_ that is similar to preimplantation development of embryos in other mammalian species. Trypan blue, fluorescein diacetate and 2-deoxyglucose are good viability indicators of early primate embryos.

May a love limitless in its breadth
And knowing no bounds,
From God and man,
'Till eternity resound.

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INTRODUCTION

Biochemical analyses of embryos have been previously used to test viability in nonprimate systems or to investigate and establish normal metabolic pathways. However, little research has focused on the biochemistry of preimplantation development of primate embryos, particularly those embryos derived from _in vitro_ fertilization. The present studies were designed to allow assessment of the embryo's metabolic requirements in an effort to maximize output of _in vitro_ fertilization systems utilizing primates.

The embryos of subprimate species experience little difficulty in developing to the blastocyst stage _in vitro_. Achieving this in nonhuman primates is more difficult. The aims of the present studies, then, were to determine both the viability and metabolism of primate embryos. Several biochemical estimates of such variables were used:

(1) Assessment of viability of squirrel monkey embryos through the use of vital dyes;

(2) Evaluation of ^{3}H-uridine and ^{3}H-leucine incorporation in early preimplantation development;

(3) Determination of uptake of steroid hormones by squirrel monkey embryos through early preimplantation development; and

(4) Ascertainment of the level of utilization of metabolic substrates by monitoring oxygen consumption, carbon dioxide production from glucose and 2-deoxyglucose uptake by primate ova.

LITERATURE REVIEW

Staining with vital dyes as an index of viability

Various vital dyes have been utilized to determine the viability of ova and other cells. These included acridine orange, a fluorescent dye (Austin and Bishop, 1959; Ezzell and Szego, 1979), several cytoplasmic and nuclear stains (Dolan, 1965; Whittingham, 1978), trypan blue (Tennant, 1964; Clines *et al*., 1980) and fluorescein diacetate (Rotman and Papermaster, 1966; McGrath *et al*., 1975; Jarnagin and Luchsinger, 1980).

Trypan blue (TpB) has proven a successful indicator of membrane integrity of cumulus and granulosa cells and embryos (Campbell, 1979; Peluso *et al*., 1982; Thadani *et al*., 1982). Campbell (1979) demonstrated a 1:1 correlation between TpB exclusion and autoradiographic assessments of relative syntheses of DNA, RNA and protein by granulosa cells. Degeneration or atresia of ovarian follicles has also been characterized through the use of a viability index for granulosa cells with TpB (Peluso *et al*., 1982).

The best index of embryo viability currently available delineates the embryo's ability to take up diactyl fluorescein (FDA), a non-fluorescent compound (Church and Raines, 1980; Renard *et al*., 1982; Taylor, 1982). Theoretically, once inside the cell, non-specific esterases cleave the acetate groups from FDA, converting it to the fluorescent compound, fluorescein, which is polar. Bright green fluorescence under high-energy blue or ultraviolet light has been highly correlated with the ability of oocytes to mature in culture and embryos to go to term after transfer to recipients (Mohr and Trounson, 1980; Peluso *et al.*, 1982).

Macromolecular synthesis by the embryo

Measurements of synthesis of macromolecules (e.g., RNA and protein) have provided indications of overall metabolic competency of the ovum and embryo. *In vivo* uptake of ^{3}H-uridine was demonstrable in ovarian oocytes of rats, *Macaca mulatta* and *M. fascicularis* (Baker *et al.*, 1969). RNA was synthesized at a high level by growing mouse oocytes (Bachvarova, 1981). This synthesis was still present, but reduced, in oocytes taken from antral follicles prior to and during germinal vesicle breakdown in mice, monkeys, cattle and swine (existing primarily as HnRNA, rRNA and tRNA) (Oakberg, 1968; Baker *et al.*, 1969; Bloom and Mukherjee, 1972; Bachvarova, 1974; Rodman and Bachvarova, 1976; Wassarman and Letourneau, 1976; Wolgemuth-Jarashow and Jagiello, 1979).

Following fertilization, chromatographic and electrophoretic analyses have detected RNA synthesis by the one-cell stage in the mouse (as poly(A)$^-$ RNA (HnRNA precursor), poly(A)$^+$ RNA (mRNA) and tRNA) and at the two-cell stage (as HnRNA of high molecular weight (mRNA precursor), rRNA, some mRNA and 4sRNA (Woodland and Graham, 1969; Murdoch and Wales, 1971; Knowland and Graham, 1972; Epstein, 1975; Clegg and Pikó, 1982). All classes of RNA were synthesized from the 8-cell stage through blastocyst (including mRNA (Warner and Hearn, 1977b). Prominent rRNA peaks were discovered at the 8-16 cell stage (day 2 of embryonic development, when true nucleoli were labelled with ^{3}H-uridine (Mintz, 1964)). There was a further increase in RNA synthesis from morula to blastocyst stage (day 3) (Ellem and Gwatkin, 1968; Pikó, 1970; Tasca and Hillman, 1970; Epstein and Daentl, 1971; Warner and Hearn, 1977a). ^{3}H-Uridine incorporation which was actinomycin D-sensitive increased 90-fold at mouse morula and blastocyst stages compared to unfertilized controls (Monesi and Salfi, 1967). At low actinomycin D concentrations, RNA polymerase I and therefore rRNA synthesis of mouse embryos were preferentially blocked. There was no effect on development until concentrations of actinomycin D exceeded 0.1 μg/ml (Thomson and Biggers, 1966; Tasca and Hillman, 1970). This led some workers to conclude that little concurrent synthesis of RNA was necessary for protein synthesis in early cleavages of mouse embryos (Schultz, 1975; Brower and Schultz, 1982). However, much maternal message is lost as early as the two-cell stage in the mouse (Pikó and Clegg, 1982) and *de novo* synthesis of RNA may assume an important role thereafter.

RNA synthesis (particularly mRNA and tRNA) in the rabbit embryo has not been demonstrated prior to the 16-cell stage (Schultz, 1973; Manes, 1977). Synthesis of RNA markedly increased by late cleavage stages (64-128 cells) (Manes, 1969, 1971). The RNA was present primarily as nucleolar rRNA (Manes, 1977). Quantitative assessments of total RNA and DNA content of oocytes and embryos have been determined by spectrophotometry (Olds *et al.*, 1973; Henriet *et al.*, 1980).

Innovative studies utilizing gene injection (plasmids produced by fusion of bacterial DNA and the regulator region from embryonic genes of mice) have recently allowed us to further our understanding of regulation of transcription of the embryonic genome (Brinster *et al.*, 1982a; Palmiter *et al.*, 1982a; Stewart *et al.*, 1982) and subsequent gene expression by progeny (Palmiter *et al.*, 1982b; Brinster *et al.*, 1982b).

Protein was found to be synthesized in oocytes from mouse and monkey follicles at the antral stage and undergoing meotic maturation, though synthesis was attenuated considerably (Baker *et al.*, 1969; Schultz *et al.*, 1978, 1979).

Much of the protein synthesis detectable at the two-cell stage in mouse embryos represents a control at the post-transcriptional level, utilizing mRNA's synthesized prior to fertilization (Braude *et al.*, 1979; Cascio and Wassarman, 1982). There is little indication of an enhanced rate of protein synthesis at fertilization (Monesi and Salfi, 1967; Brinster, 1971a; Brinster *et al.*, 1976; Abreu and Brinster, 1978). Only methionine incorporation (Schultz *et al.*, 1979) and synthesis of the FP1-6 proteins were augmented (Cascio and Wassarman, 1982). In fact, there is some evidence for decreased protein synthesis with fertilization (Chen *et al.*, 1980). Qualitative patterns remain essentially unchanged in early embryo development (Van Blerkom and Brockway, 1975; Schultz *et al.*, 1979). However, utilizing a double-isotope labelling technique (^{3}H- and ^{35}S-methionine) and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), Chen *et al.* (1980) have compared 95 individual proteins prior to and subsequent to fertilization. These workers demonstrated significant increases in synthesis of only 6 specific proteins and a decline in the rates of 11 proteins. Amino acid incorporation increased as embryonic development proceeds further in the mouse (Mintz, 1964; Monesi and Salfi, 1967; Tasca and Hillman, 1970; Brinster, 1971a) and pig (Motlik *et al.*, 1980). Amino acid incorporation (Epstein and Smith, 1973; Brinster *et al.*, 1976) and uptake at ^{35}S-methionine (which was Na^+-dependent and competitive) (Kaye *et al.*, 1982) increased several-fold between day 2 (two-cell) and day 4 (blastocyst). Autoradiographic techniques have been used to demonstrate ^{3}H-lysine incorporation at syngamy in porcine embryos with nuclear label disappearing by the four-cell stage (Motlik *et al.*, 1980). Nuclear methionine and tryptophan were still present at 4- and 8-cell stages, respectively. Tryptophan was presumably incorporated into non-histone proteins assuming a role in genomic regulation.

Studies of amino acid incorporation must consider the pool size of the endogenous precursor, which increases concomitantly with embryonic development (Sellens *et al.*, 1981; Schultz *et al.*, 1981). Therefore, uptake values are of major importance. With long culture periods (>13 hr), protein degradation also significantly increased by the blastocyst stage (Merz *et al.*, 1981). Several specific proteins present in embryos have been analyzed. Tubulin synthesis in mouse embryos rose slightly after fertilization, increased to 14-fold by blastocyst stage and accounted for 2% of the total protein synthesis (Abreu and Brinster, 1978; Schultz *et al.*, 1979). Actin, synthesized by the unfertilized mouse oocyte (Osborn and Moor, 1982), increased 10-fold by the 8-cell stage and 90-fold by the blastocyst stage. This represented 5.7% of the total protein synthesis. High levels of alpha-fetoprotein, transferrin, fetuin and glycoprotein synthesis have been measured by radioimmunoassay, ^{35}S-methionine and ^{3}H-leucine incorporation and 2-D PAGE before and after implantation in mouse, bovine, sheep, and pig embryos (Janzen et al., 1982; Godkin et al., 1982; Masters et al., 1982; Adamson, 1982).

Steroid metabolism by the embryo

Embryos of several species lack the capacity for steroid synthesis and metabolite interconversions until the peri-implantation period (Dickmann and Dey, 1974; Dickmann, 1979; Gadsby et al., 1981). Acetate incorporation into cholesterol by mouse embryos was enhanced in vitro from the blastocyst to the early somite stage (Carson et al., 1982). These embryos were capable of converting ^{3}H-pregnenolone to progesterone and acylpregnenolone. Rabbit blastocysts showed high aromatase activity by day 6 of development (Hoversland et al., 1981; Wu and Lin, 1982b). Other lipids were also synthesized. Enhanced fatty acid levels were present by days 11-13 in bovine blastocysts (Menezo et al., 1982). PGE_2 and $PGF_{2\alpha}$ were produced from ^{3}H-arachidonate by days 14-19 (Lewis et al., 1982).

Although many workers have demonstrated steroid synthesis by the embryo, only a few have attempted to assess radiosteroid uptake by the embryo (Smith, 1968; Bhatt and Bullock, 1974; Wu and Lin, 1982a). These studies were not validated autoradiographically. It is known that an appropriate hormonal milieu is required for proper development of embryos (Stone et al., 1977; Dickmann et al., 1977; Warner and Tollefson, 1978), and early embryos initially respond to endogenous levels of maternal steroids (Smith, 1968; Weitlauf and Greenwald, 1968; Smith and Smith, 1971). Responses to these steroids may be measured in terms of RNA synthesis, since steroid hormones classically cause derepression of genes (O'Malley *et al*., 1973). However, workers have failed to demonstrate increases in incorporation of ^{3}H-uridine by mouse embryos treated with estrogen or progesterone, *in vitro* (Warner and Tollefson, 1977, 1978). Estrogen has only been shown to increase uptake of amino acids by implanting blastocysts (Smith and Smith, 1971). Therefore, these studies suggest changes of a mitotic or membrane nature being effected.

Energy metabolism of the embryo

Oxidative pathways involved in substrate metabolism (primarily glucose) have been elucidated for ova from various species. Oxygen consumption increased during gonadotropin-induced maturation of unfertilized rabbit and rat oocytes (Lindner *et al*., 1974; Dekel *et al*., 1976; Magnusson *et al*., 1977; Magnusson and Hillensjö, 1981; Magnusson *et al*., 1981). Fertilized ova from rabbits showed increased oxygen utilization during preimplantation development (Fridhandler, 1961). Consumption of oxygen by rabbit embryos reached highest levels at the blastocyst stage, primarily supporting the Na^+-K^+-ATPase used in active transport of ions (Benos and Balaban, 1980). Mills and Brinster (1967) and Sugawara and Umezu (1961) demonstrated a 3.5-fold increase in oxidation by the mouse blastocyst over that of the unfertilized egg. Further significant increases occurred at the 8-cell and subsequent stages.

Oxygen consumption has been assessed simultaneously with CO_2 production (see below; Brinster, 1968; Hammerstedt, 1975). The resulting respiratory quotient was an important indicator of overall metabolic competency and preference for energy substrate.

Measurements of $^{14}CO_2$ production from universally- or specifically-labelled glucose have been used to determine the metabolic pathways utilized by the preimplantation embryo (Brinster, 1967a). Carbon dioxide production increased 100-fold over the first five days of development in the mouse, with a five-fold increase occurring at the time of fertilization. Incubation with specifically-labelled glucose (^{14}C in the C-1 or C-6 position) resulted in a C-1/C-6 ratio of 1.6, indicating an active TCA cycle early in mouse development (Brinster, 1967a). The Embden-Meyerhof pathway does not assume a prominent role until the last two days before implantation (Thomson, 1967). Conversely, in the rabbit, inhibitors of oxidative metabolism have demonstrated both an active pentose shunt (C_1/C_6 = 10 one day after fertilization, then declines) (Fridhandler, 1961; Wales and Whittingham, 1970; Wales, 1973) and an active TCA cycle in preimplantation embryos (Quinn and Wales, 1973a; Kane and Buckley, 1977). Glycolysis was not quantitatively observed until the blastocyst stage (Wales and Whittingham, 1970). Experiments comparing oxidation of various substrates proved pyruvate to be the primary energy substrate utilized by two-cell mouse embryos (Biggers _et al_., 1967) and primate oocytes (Brinster, 1971b). Brinster (1971b) demonstrated a greater capability for pyruvate oxidation for the monkey oocyte than for the mouse ovum. LDH activity was, however, 80-fold greater in mouse oocytes than either rabbit or primate (rhesus monkey, squirrel monkey and human) oocytes (Brinster, 1967b). The values for pyruvate and glucose oxidation by primate oocytes were equivalent to those of the unfertilized rabbit ovum (Brinster, 1968, 1969), but two- to five-fold greater than for the unfertilized mouse ovum (Brinster, 1967a). This may have been attributable merely to oocyte size, as the volume of rabbit and primate ova is roughly 3.5 times that of the mouse (Brinster, 1971b). Metabolic pathways have also been elucidated through the use of inhibitors of carbohydrate, protein and nucleic acid metabolism (Thomson and Biggers, 1966; Thomson, 1967).

Comparisons of viability between mouse blastocysts developing _in vitro_ and _in vivo_ have been made by monitoring CO_2 production (Menke and McLaren, 1970). The mean CO_2 output and trophoblast outgrowth of blastocysts (highly correlated) cultured _in vitro_ from the 8-cell stage were significantly reduced when compared to _in vivo_ controls. The addition of fetal calf serum to the medium augmented both viability indices. Other metabolic determinations of embryo viability have included ATP content, and pyruvate and lactate uptake (Quinn and Wales, 1973b). All indices were diminished with retarded embryonic development. _In vitro_ uptake by bovine blastocysts has proven to be a good index of subsequent development _in vivo_ (Renard _et al_., 1980; Renard _et al_., 1982). Of 13 transferred embryos which took up more than 2.5 μg glucose/embryo/hr, 69% maintained pregnancy at day 50. Only 14% of ova considered non-viable by the assay did so. ^{3}H-Glucose uptake increased over days 5-7 post-coitus in the rabbit blastocyst (Benos and Biggers, 1981). This uptake was not dependent on external sodium.

Glucose utilization, and hence, the cellular metabolic rate of various mammalian tissues have been assessed by the cells' ability to take up radiolabelled 2-deoxy-D-glucose (2-DG) (Sokoloff et al., 1977; Van den Broeck and Van Steveninck, 1981; Astic and Saucier, 1982). 2-DG is a nonmetabolizable analogue of glucose that, depending on circumstances, is transferred across cell membranes by facilitated transport (Kotyk and Michaljanicova, 1974) (like fructose, mannose and glucose outside kidney and intestinal epithelium (White, Handler and Smith, 1968)) or, as is more often the case, by active transport (Jasper and Van Steveninck, 1975) (utilizing the same carrier as galactose (Parra et al., 1980) and 3-O-methylglucose (Segal and Ingbar, 1980)). 2-DG is phosphorylated by glucokinase (like mannose and glucose) to 2-deoxyglucose-6-phosphate (2-DG-6P). 2-DG-6P inhibits glucose phosphate isomerase and cannot be further metabolized. Analyses of 2-DG uptake therefore provide valid information about the utilization of glucose (Brooks, 1982).

METHODS AND MATERIALS

Animals

Forty mature female hamsters (Mesocricetus auratus) (8-10 weeks of age) were monitored for postovulatory vaginal discharge for at least three cycles before being placed on experiment. Hamsters normally received a superovulatory regimen of 30 I.U. pregnant mare's serum (PMS) (Serotropin, Teizo, Tokyo, Japan) i.p. on the morning of Day 1 (day of ovulatory plug). This was followed by 30 I.U. HCG (A.P.L., Ayerst Laboratories, Inc., NY) i.p. 76 hr later (Mizoguchi and Dukelow, 1980). In the experiments on steroid uptake, animals of group A received no exogenous gonadotropins, and ovulated naturally. All animals, regardless of treatment, were then mated on the evening of Day 4. Following mating, animals were sacrificed at varying times to obtain embryos at different stages of development.

Squirrel monkeys (Saimiri sciureus) of Bolivian and Guyanan origin (Primate Imports, Charles River, Inc., Port Washington, NY) were housed and fed as previously described (Kuehl and Dukelow, 1979). Adult females of the same subspecies were used for related studies (Bolivian type for macromolecular and steroid studies, Guyanan type for metabolic studies). Animals were exposed to fluorescent lighting on a 14L:10D cycle with ambient temperature controlled at 21±2°C. In the present experiments, animals were used during the breeding season (October through May). They normally received an ovulatory regimen of

4 days of 1 mg of FSH i.m. daily (Burns-Biotec Laboratories, Inc., Omaha, NE). However, in group B in the experiments on steroid uptake, animals received 5 days of FSH. This reflected the hormonal regimen designed for induction of single and double ovulations in *Saimiri* (Dukelow, 1970), which requires increased FSH during the anovoulatory season (Kuehl and Dukelow, 1975). On the final day of FSH treatment, 250 I.U. HCG was administered i.m., 16 hr prior to laparoscopy for follicular aspiration (Dukelow, 1979).

Production and collection of embryos

Hamster embryos were recovered at 40 hr after mating for two-cell embryos and 60 hr for four-cell embryos from the oviduct and at 72 hr for eight-cell embryos, 78 hr for morulae and 86 hr for blastocysts from the uterus (Ghosh *et al*., 1982).

Squirrel monkey oocytes were recovered at laparoscopy (Kuehl and Dukelow, 1975, 1979). If processed immediately, oocytes were mechanically denuded of cumulus cells with glass pipettes (unless stated otherwise). Some oocytes were allowed to mature for 21 hr in tissue culture medium (TC-199, GIBCO, Grand Island, NY) which was modified to contain 1 mM pyruvate, 100 μg/ml gentamycin and 1 U/ml heparin (Asakawa *et al*., 1982). Twenty percent fetal calf serum (FCS) (heat inactivated for 30 min at 56°C) was added prior to insemination *in vitro*. Semen was collected by electroejaculation (Kuehl and Dukelow, 1974). Embryos were collected 24 hr after insemination for one-cell, 48 hr for two-cell and 52-60 hr for three- to four-cell embryos.

Prior to experimental processing, ova and embryos from one animal or group of animals were divided among the various treatments.

Techniques were usually validated with hamster ova (where larger numbers of embryos were available), and further modified for squirrel monkey ova. Some ova were also subjected to suboptimal (>0.5°C/min freezing and 500°C/min thawing rates, Leibo, 1977) and optimal (0.25°C/min freezing and 3°C/min thawing rates, DeMayo *et al*., 1983) freezing procedures to artificially yield groups of ova in varying states of degeneration.

Staining with vital dyes

Ova were transferred to depression slides containing 10 µl of 15 µM FDA in Dulbecco's phosphate-buffered saline (PBS) and held at room temperature for one min. The ova were then examined for 10 sec under a fluorescence microscope (Leitz BG12 and BG38 exciter filters and a K510 long pass barrier filter, The Microscope Co., New Castle, PA). Ova were classified as either positive (bright fluorescence) or negative (faint or no fluorescence). Examinations were also made for exclusion or uptake by ova of 0.2% TpB. Ova were washed and further processed to correlate with morphologic studies and macromolecular synthesis.

Evaluations of macromolecular synthesis

Ova and embryos were incubated in 0.25 ml of 2.8 (Daentl and Epstein, 1971) and 5.6 µM 5-^{3}H-uridine (for hamster and squirrel monkey ova, respectively; S.A. 18 Ci/mmole) which supplemented the modified TC-199. Incubations took place for 3 hr in an atmosphere of 5% CO_2 in air. Ova and embryos were washed 10X in medium containing unlabelled uridine in 1000-fold excess and solubilized in 100 µl of

0.14 M 2-mercaptoethanol and 0.1% sodium dodecylsulphate in phosphate buffer (pH 7.4) (Fishel and Surani, 1978). Samples were heated to 65°C for one hr in a water bath, duplicate aliquots removed and added to Whatman GF/C glass fiber filters (Fisher Scientific, Pittsburgh, PA), and air-dried. RNA was precipitated by addition of 30 ml cold 10% TCA and 30 ml cold ethanol (EtOH) to the discs under light suction filtration (Sartorius-Membranfilter, Göttingen, W. Germany). Treatments of selected filters with either ribonuclease (RNase) A from bovine pancreas (0.4%, Sigma, St. Louis, MO) and 0.5 N NaOH reduced ^{3}H-uridine incorporation into TCA-precipitable material to background levels, thereby serving as controls. Total uptake was determined by assaying discs without prior TCA/EtOH treatment. Discs were assayed for radioactivity in 10 ml ACS (Amersham, Arlington Hts., IL) in a Searle Model 6891 liquid scintillation counter. Machine efficiency was 64.5% for ^{3}H and quenching was 5.7% with an external standard. Aliquots of the final wash were processed as procedural blanks (usually 30-60 CPM). The moles of precursor incorporated/embryo/unit time were calculated using the known specific activity of the precursor and the incorporated radioactivity (Epstein, 1975).

Autoradiography

Squirrel monkey ova were incubated 3 hr in modified TC-199 supplemented with 5.6 μM 5-^{3}H-uridine (S.A. 18 Ci/mmole) or 0.4 μM L-4,5-^{3}H-leucine (S.A. 50.4 Ci/mmole) and treated as described above. Following washing, ova were fixed 24 hr in Bouin's fluid and processed for autoradiography (AR) (Weitlauf and Greenwald, 1971). Ova were embedded in paraffin and serially sectioned at 5 μm. For uridine, alternate paraffin sections were mounted on two sets of slides and the paraffin removed. One set was treated with RNase A (0.1 mg%, activity 85 Kunitz units/mg, Sigma, St. Louis, MO) in sodium phosphate buffer (0.1%, pH 7.4). The other set of slides received only buffer treatment. All slices were incubated at 37°C for 1 hr and were subsequently treated with 5% TCA for 10 min at 4°C. For leucine, since Bouin's fixative removes unincorporated amino acids (Weitlauf and Greenwald, 1971), no further treatment was required. All slides were washed 15 min in tap water, air-dried and dipped in Kodak NTB-3 emulsion at 42°C in the dark. The slides were exposed two weeks, developed, fixed and stained with hematoxylin and eosin (Peluso and Hutz, 1980). Two blank slides in each set were exposed to light and two processed in the dark to control for negative and positive chemography, respectively. ^{3}H-Uridine and -leucine incorporation into RNA and protein, respectively, were determined by counting reduced silver grains over three different areas of 300 μm^2 each of nucleoplasm or cytoplasm with a micrometer reticle. These counts were averaged and background counts from similar averaging of equivalent areas 200 μm from the ovum were subtracted. (For the case of uridine, the background counts were the same as RNase-treatment.) The area over which grains were counted was converted to 1000 μm^2 for ease in calculations and graphic representation.

Steroid autoradiography was according to Uriel _et_ _al_. (1973). In brief, slides of sectioned ova were incubated 2 hr in PBS containing

0.005 μg/ml of either 2,4,6,7-^{3}H-estradiol-17β (E_2) or 1,2,6,7-^{3}H-progesterone (P) (S.A. 94 Ci/mmole each), washed 1.5 hr in running tap water and autoradiographed as described above. Positive controls were nonradioactive E_2 and P (each in 1000-fold excess) to compete for radioactive E_2 and P, respectively.

Measurements of steroid uptake

^{3}H-Estradiol or ^{3}H-progesterone (S.A. 94 Ci/mmole each) were dried and dissolved in 0.1 ml EtOH. Ova were then incubated for 2 hr in 0.2 ml of modified TC-199 supplemented with 0.06 μM of either radioactively-labelled steroid. (This amount provided for maximum uptake in preliminary trials). Ova were washed 10X in phosphate buffer, dissolved in 0.1 ml of tissue solubilizer (Soluene, Packard, Downers Grove, IL), and the solution assayed for radioactivity as described above in 10 ml of methanolic ACS to reduce chemiluminescence.

Metabolic assessments

Oxygen consumption

Oxygen consumption of ova was measured by the method of Benos and Balaban (1980). This utilized a polarographic oxygen electrode of the Clark type with micromodifications (Model 5331, Yellow Springs Instruments, Yellow Springs, OH). Hamster ova or squirrel monkey oocytes with cumulus were incubated for 4 hr in a sealed glass chamber (0.5-0.8 ml volume) containing modified TC-199 medium (Seamark *et al.*, 1976). The medium, previously equilibrated with 5% CO_2 in air, was surrounded by a water jacket controlled at 37±0.01°C by a Haake Model

FE2 circulating water bath (Haake, Karlsruhe, W. Germany). The chambers' contents were stirred continuously with magnetic stirrers and additions made with a microsyringe through the access ports. Oxygen consumption of ova was calculated from the observed decrease in O_2 tension per unit time (YSI O_2 Electrode References, 1974) as compared to a control chamber containing no ova. A positive control was the addition of 1×10^{-4}M KCN, which reduced the oxygen consumption of ova to baseline levels. Consumption of oxygen by squirrel monkey oocytes was estimated by taking 10% of the total uptake of the oocyte-cumulus complex as the approximate value for oocytes alone (Dekel *et al.*, 1976).

Carbon dioxide production

CO_2 production by oocytes was monitored by modifications of published techniques (Brinster, 1967; Menke and McLaren, 1970) to those used by Dey *et al.* (1979). Oocytes from squirrel monkeys were incubated for 4 hr at 37°C in 5% CO_2 in air in Dulbecco's PBS (titrated to pH 7.3 with 0.5 N NaOH), supplemented with 100 μg/ml gentamycin. (PBS has been shown to lack embryotoxic or metabolic effects in a 4 hr culture period (Quinn and Wales, 1973b,c).) The only energy substrates added were 1.80×10^{-4} or 5.56×10^{-4}M D-U-^{14}C-glucose (S.A. 296 mCi/mmole). Incubations were in equilibrated test tubes sealed with rubber stoppers, and containing plastic center wells (Kontes Glassware, Vineland, NJ). The incubations were terminated and $^{14}CO_2$ liberated from the medium at the end of the culture period with an injection of 0.25 ml 0.05 M potassium hydrogen phthalate buffer (pH 4.0). NCS (0.25 ml, Amersham, Arlington Hts., IL) was then injected into the center wells to absorb the $^{14}CO_2$. The tubes were agitated one hr in a metabolic shaker (100 cycles per min). The tubes were then discarded and the center wells containing the $^{14}CO_2$-labelled NCS were dropped into liquid scintillation vials with 10 ml standard PPO-POPOP (6 g/L and 75 mg/L, respectively, in toluene; Spectrafluor, Fisher Scientific, Pittsburgh, PA). Efficiences and quenching were assessed using a $Na_2{}^{14}CO_3$ standard. Picogram-atoms of carbon incorporated were calculated utilizing the method of Menke and McLaren (1970). Blanks contained all components except oocytes. Background levels produced 100-200 cpm, significantly less than experimental tubes.

2-Deoxyglucose uptake

Ova from squirrel monkeys were preincubated one hr in 0.2 ml TC-199 containing 5.56 mM D-glucose (non-radioactive) and washed in saline 5 times. They were then incubated in 0.2 ml of modified PBS containing 1 μM 2-deoxy-D-1-^{3}H-glucose (S.A. 25 Ci/mmole) (Dunn and Mallucci, 1980) as the only energy substrate. (Incubations with 2-deoxyglucose had no detrimental effect on *in vitro* development of early embryos (Kane and Buckley, 1977) or on viability of other cell types as assessed by trypan blue (Segal and Ingbar, 1980).) Ova were cultured for 3 hr with and without the addition of 10 nM or 1 μM insulin (Segal and Ingbar, 1980) from bovine pancreas (24.3 U/mg; ICN Pharmaceuticals, Cleveland, OH). Following incubations, ova were washed 10X in nonradioactive medium and solubilized in 100 μl of 0.14 M 2-mercaptoethanol and 0.1% sodium dodecylsulphate in sodium phosphate buffer (0.1%, pH 7.4) for 1 hr at 65°C. Aliquots of the final wash served as procedural blanks (Fishel and Surani, 1978). Machine efficiency for ^{3}H was 57.6% and quenching was 5% using an external standard. Although 2-DG depresses ATP levels in some cell types, the technique allows accurate measurements of initial substrate utilization (Parra et al., 1980).

Statistical analyses

Since scintillation counts normally follow a Poisson distribution (Steel and Torrie, 1980), such data were usually transformed ($\sqrt{X}$) prior to further analysis. Steroid data were transformed to log (X+1). Comparisons were analyzed by Student's t-test, or randomized one-way ANOV or factorial designs. Student-Newman-Keuls test was used to compare multiple groups. The rank sum (Mann-Whitney U) and Kruskal-Wallis tests were performed if data was still nonparametric following transformation. Vital dye data were compared by χ^2 (contingency tables) or Fisher exact test, or by simple linear correlation. Percentage data were evaluated following angular transformation ($\sin^{-1}\sqrt{X}$). Simple linear regression analyses were done to validate oxygen consumption data. $P<0.05$ was considered to be significant.

RESULTS

Control ova obtained from hamsters took up FDA and fluoresced brightly, excluded TpB, and appeared morphologically normal after a 3 hr culture period (Table 1A). These ova also incorporated ^{3}H-uridine to a significant extent (Table 1B). A suboptimal freezing procedure reduced all indices of viability to zero. Although an optimal freezing procedure for hamster ova reduced viability, ova judged normal by vital stain criteria incorporated and took up ^{3}H-uridine to the same extent as controls. Although TpB exclusion and FDA uptake were normally highly correlated with each other in ova of both species over various treatments ($r = 0.99$, Figure 1), there were discrepancies in groups with intermediate viability after culture (Table 1A). In this third group, there was a drop of viability after culture (as assessed morphologically) to TpB levels before and after culture. The percentage of ova assessed viable by FDA was lower than the other indices both prior and subsequent to culture. There was no effect of incubation with radioprecursor on the viability of control ova over the 3 hr. The use of vital dyes had no effect on radiouridine uptake by hamster two-cell embryos, as estimated by radioautography. Control embryos (no stains, n=4) had grain counts of 108 ± 5 versus 95 ± 9 grains/ 1000 μm^2 for embryos incubated 1 min in each of TpB and FDA and

TABLE 1A

Validation of Trypan Blue (TpB) and Fluorescein Diacetate (FDA) in Hamster Ova

Treatment	TpB Exclusion		FDA Uptake		Morphologically Normal	
	B.C.[1] (%)	A.C.[1] (%)	B.C. (%)	A.C. (%)	B.C. (%)	A.C. (%)
Control	$\frac{150}{150}$ (100)	$\frac{149}{150}$ (99.3)	$\frac{149}{150}$ (99.3)	$\frac{149}{150}$ (99.3)	$\frac{150}{150}$ (100)	$\frac{150}{150}$ (100)
Suboptimal Freezing	$\frac{3}{148}$ $(2.0)^{*}$	$\frac{0}{148}$ $(0)^{*}$	$\frac{0}{148}$ $(0)^{*}$	$\frac{0}{148}$ $(0)^{*}$	$\frac{3}{148}$ $(2.0)^{*}$	$\frac{0}{148}$ $(0)^{*}$
Optimal Freezing	$\frac{482}{586}$ $(82.3)^{*ac}$	$\frac{461}{574}$ $(80.3)^{*ac}$	$\frac{440}{586}$ $(75.1)^{*b}$	$\frac{421}{575}$ $(73.2)^{*b}$	$\frac{490}{576}$ $(85.1)^{*a}$	$\frac{455}{576}$ $(79.0)^{*c}$

[1] B.C. = before culture, A.C. = after 3 hr of culture.

*Significantly different from respective control group ($p<0.05$).

^{abc}Numbers within a row lacking a common superscript differ significantly ($p<0.05$).

TABLE 1B

Validation of Trypan Blue (TpB) and Fluorescein Diacetate (FDA) in Hamster Ova

Treatment	^{3}H-Uridine[1]	
	Incorporation	Uptake
Control	0.69±0.15	6.50±1.50
Suboptimal Freezing	---[2*]	---[2*]
Optimal Freezing	0.55±0.15[3]	8.36±2.15[3]

[1] Femtomoles ^{3}H-uridine/ovum/3 hr; number of ova per RNA group was 73-110.

[2] Not detectable from background levels.

[3] Only ova judged viable by both TpB and FDA assays were used.

[*] Significantly different from respective control group ($p<0.05$).

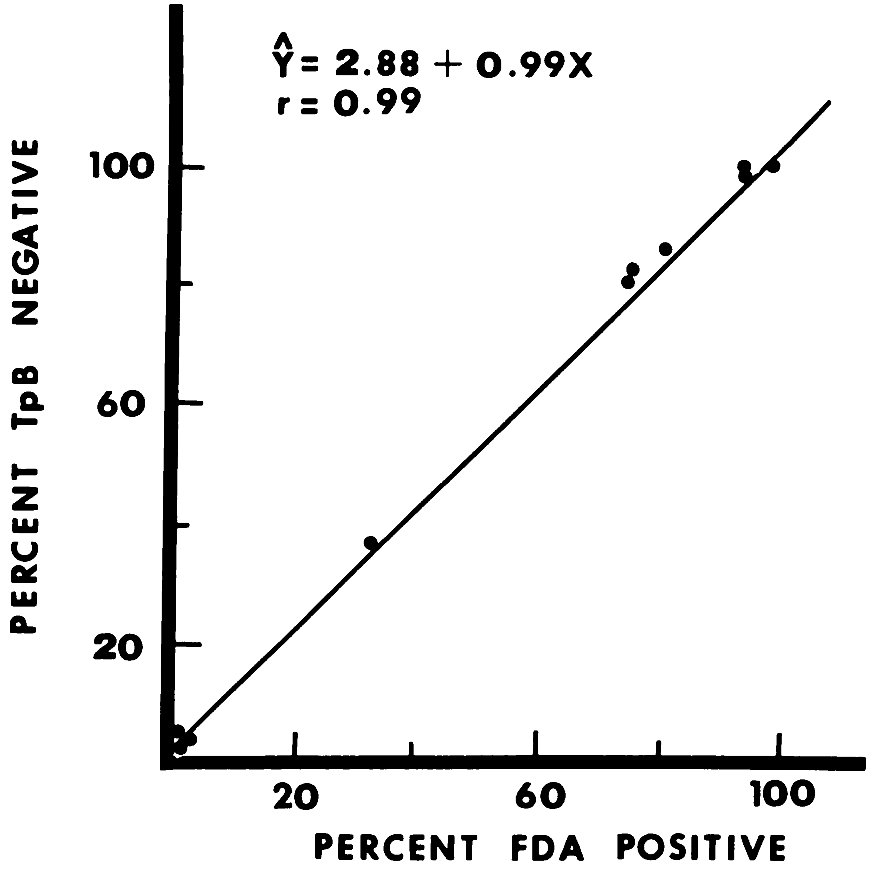


Figure 1. Statistical correlation between TpB and FDA.

exposed to near-ultra-violet light (n=5). Eighty percent of one-celled hamster embryos which stained viable by both dyes, proceeded to the two-cell stage 24 hr later, in vitro (Table 2). This was comparable to normal controls, not incubated in either dye. Embryos which did not fluoresce and took up TpB did not cleave. The dyes were not embryotoxic at the concentrations used.

Preliminary experiments determined that embryos incorporated ^{3}H-uridine maximally at concentrations of 2.8 μM for hamster embryos (Figure 2) and 5.6 μM for squirrel monkey ova (not shown). Uptake into the acid-soluble pool increased continuously. Without knowledge of the size of the endogenous precursor pool (estimated by others in the mouse embryo (Clegg and Pikó, 1977), but not possible with the sparse material available to us), this should have equilibrated the external and internal pools (Tasca and Hillman, 1970; Daentl and Epstein, 1971; Epstein, 1975). Ribonuclease or NaOH digestion for 1 hr after culture reduced uridine incorporation to that of background levels (Figure 3). Actinomycin D treatment for 1 hr prior to culture plus concomitant incubation of precursor and actinomycin D reduced ^{3}H-uridine incorporation more than 50%. Embryos which were morphologically degenerate had a reduced capacity to synthesize RNA to 38% of controls. In studies with ^{3}H-leucine, incorporation by monkey ova, likewise, plateaued at 0.4 μM (as assessed by autoradiography). TpB exclusion and FDA uptake correlated well with morphology and capacity to synthesize RNA in autoradiographs (Table 3). Grain counts of unfertilized and fertilized oocytes that stained TpB-positive and FDA-negative remained at background levels. Those which stained TpB-negative and FDA-positive showed much higher grain densities (25 ± 3 and

TABLE 2

Validation of Vital Dyes: *In Vitro* Development of Hamster Embryos from the One- to Two-Cell Stage

Treatment	No. Cultured	No. Developed to Two-Cell Stage After 24 hr (%)
Control (no dyes)	18	15 (83.3)
TpB^-/FDA^+	15	12 (80)$^{N.S.}$
TpB^+/FDA^-	15	0 (0)*

$^{N.S.}$Not significantly different from control group.

*Significantly different from control and TpB^-/FDA^+ groups ($p<0.05$).

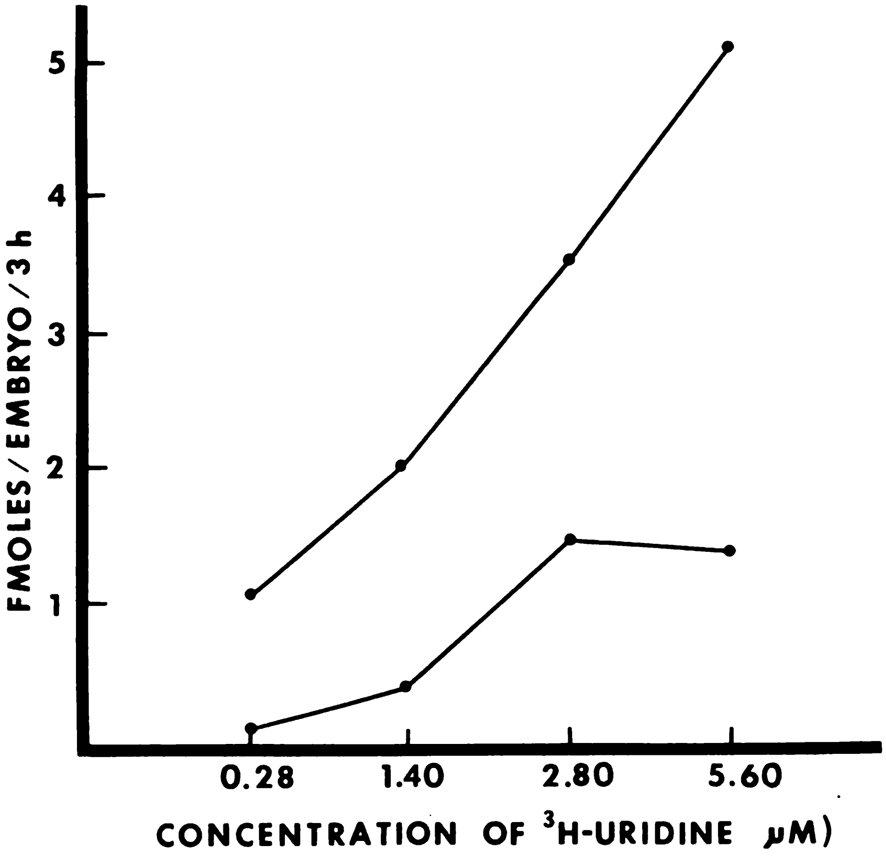


Figure 2. Effect of ^{3}H-uridine concentration on incorporation and uptake by embryos. Twenty to forty embryos were examined at each point. The upper line indicates uptake, while the lower represents incorporation.

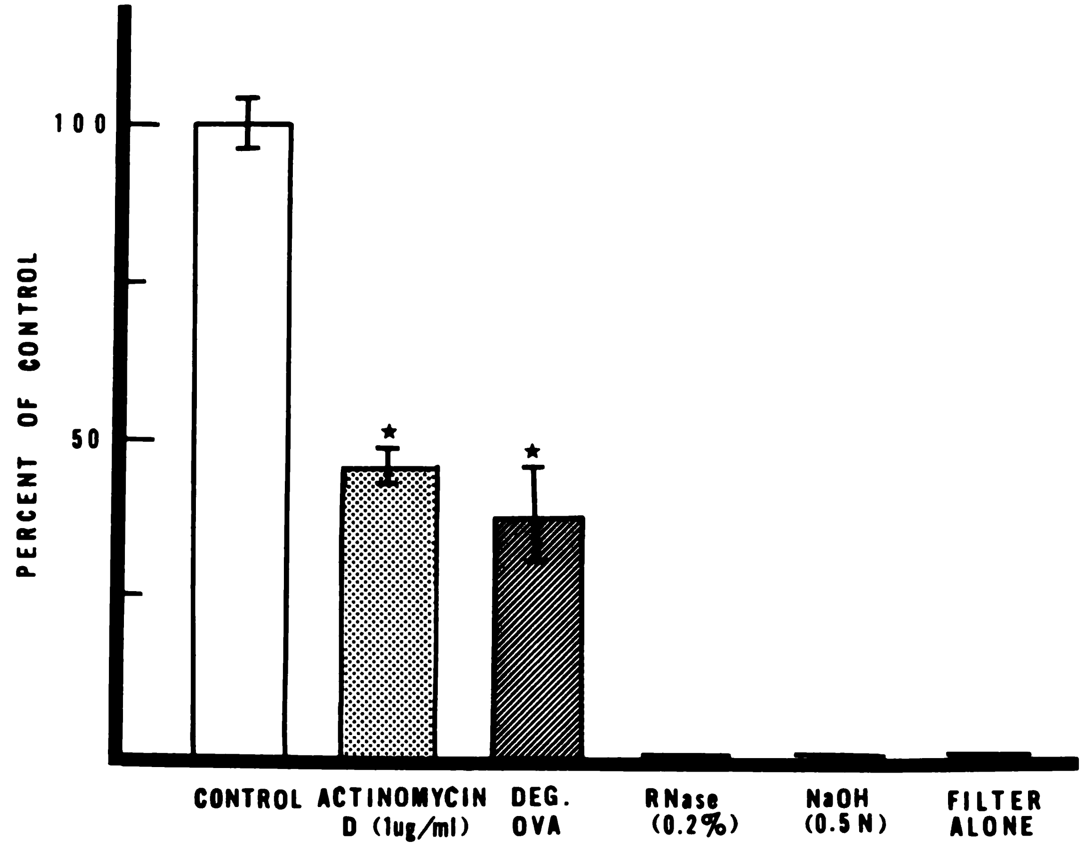


Figure 3. ^{3}H-Uridine incorporation in two-cell hamster embryos with respect to various treatments. Twenty to sixty embryos were examined in each group. Values are expressed as mean ± S.E. *Significantly different from control ($p<0.05$).

TABLE 3

Vital Dye Assays: Correlation with ^{3}H-Uridine Incorporation in Squirrel Monkey Ova

Cell Stage	N	Morphologically Normal (%)	TpB Exclusion (%)	FDA Uptake (%)	Grains/1000 μm^2	
					TpB$^-$/FDA$^+$	TpB$^+$/FDA$^-$
Unfertilized	23	8 (34.8)	8 (34.8)	8 (34.8)	25±3	2±0.5[1]
Fertilized one-cell	14	12 (85.7)	12 (85.7)	11 (78.6)	39±3	2±2[1]

[1] Grain counts reduced to background levels. Values are mean ± S.E.

39 ± 3 grains/1000 μm^2), respectively. Both vital dyes correlated well with ^{3}H-leucine incorporation by squirrel monkey oocytes (Table 4). Use of a suboptimal freezing procedure reduced viability as judged by TpB and FDA, and the number of silver grains in the autoradiographs to background levels, as compared to viable controls (Figures 4A, B).

^{3}H-Uridine incorporation and uptake both decreased in oocytes recovered from squirrel monkeys 36 hr after HCG administration, compared to oocytes recovered 16 hr after HCG (Table 5). Incorporation of ^{3}H-uridine increased at in vitro fertilization in squirrel monkey oocytes from 25 to 39 grains/1000 μm^2 and again after the second cleavage division from 44 to 70 grains/1000 μm^2 (Figure 5). ^{3}H-Leucine incorporation by squirrel monkey oocytes decreased after a 21 hr maturation period in vitro (from 329 ± 20 to 164 ± 20 grains/1000 μm^2). Grain counts remained the same at in vitro fertilization (118 ± 40 grains/1000 μm^2), with a nonsignificant elevation by first cleavage (to 220 ± 25 grains/ 1000 μm^2) (Figure 6).

In the steroid uptake experiments, correlative RNA studies were performed. Incorporation of ^{3}H-uridine into RNA increased by the morula stage in embryos from hamsters which ovulated naturally from 1.5 to 16.1 femtomoles (fmoles)/embryo/3 hr (Table 6). Uptake increased significantly at the 8-cell and morula stages over previous stages. Superovulated ova showed similar increases in incorporation at the 4-cell, morula, and blastocyst stages, and in uptake at the 4-cell and morula stages. There were no differences in incorporation or uptake of ^{3}H-uridine between embryos from naturally- or superovulated animals, except in the case of uptake by two-cell embryos.

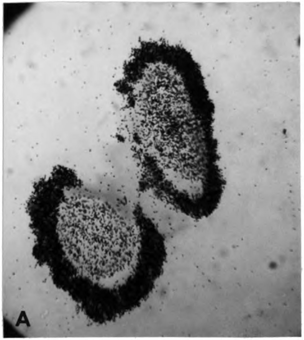
TABLE 4

Vital Dye Assays: Correlation with ^{3}H-Leucine Incorporation in Squirrel Monkey Ova

Treatment	N	Morphologically Normal (%)	TpB Exclusion (%)	FDA Uptake (%)	Grains/1000 μm^2
Control	14	14 (100)	14 (100)	14 (100)	359 ± 21
Suboptimal Freezing Procedure	14	0 (0)	0 (0)	0 (0)	5 ± 2[1]

[1] Grain counts reduced to background levels.

Values are mean ± S.E.



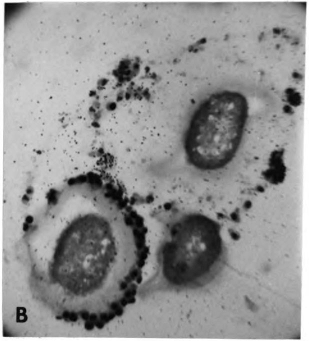


Figure 4a. Autoradiograph of unfertilized control oocytes from a squirrel monkey. Oocytes were incubated for 3 hr in medium supplemented with ^{3}H-leucine (x250).

4b. Autoradiograph of unfertilized monkey oocytes which were suboptimally frozen. Oocytes were incubated for 3 hr in medium supplemented with ^{3}H-leucine. Note the dramatic decrease in silver grains and TpB staining of the ooplasm (x250).

TABLE 5

^{3}H-Uridine Incorporation with Respect to the Time of Oocyte Collection in the Squirrel Monkey

Treatment	Incorporation[1]	Uptake[1]
16 hr after HCG	3.81±0.54 (8)[2]	68.41±8.93 (8)
36 hr after HCG	1.07±0.14 (4)*	3.28±1.00 (4)*

[1] Femtomoles ^{3}H-uridine/oocyte/3 hr ± S.E.

[2] Number of trials in parentheses; 1-5 oocytes per trial.

*Significantly different from respective group at 16 hr ($p<0.05$).

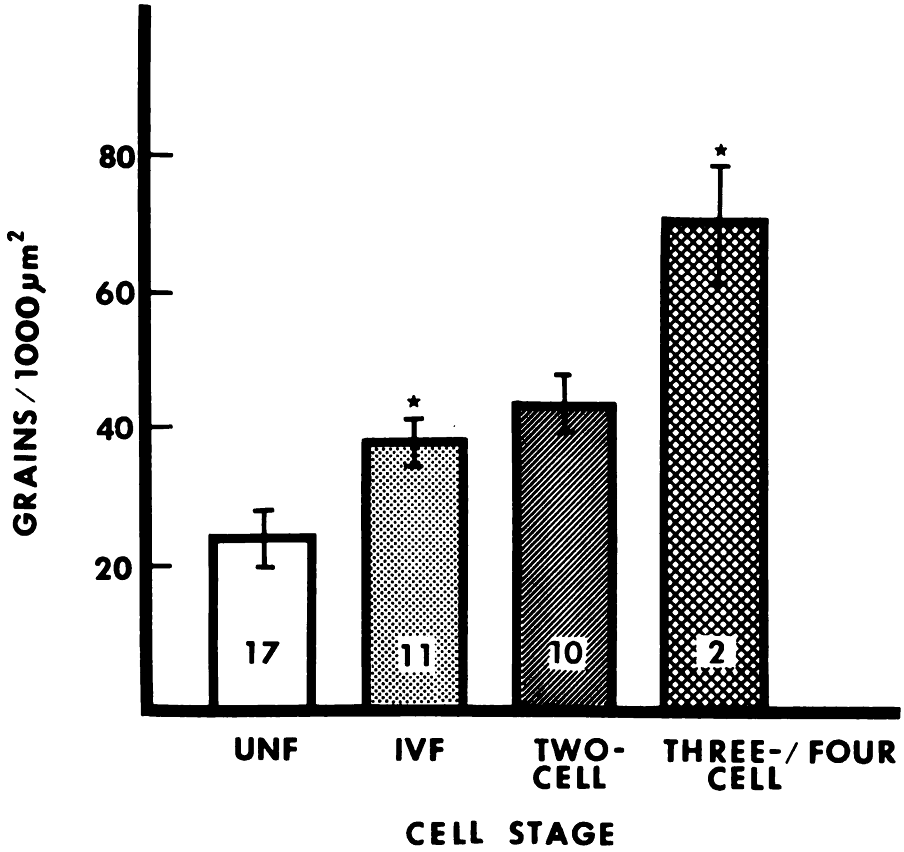


Figure 5. ^{3}H-Uridine incorporation by early embryos of squirrel monkeys, fertilized in vitro. Autoradiographic assessments were done on a per-cell basis. Number of ova at base of bars. Values are expressed as mean ± S.E. *Significantly different from previous cell stage ($p<0.05$).

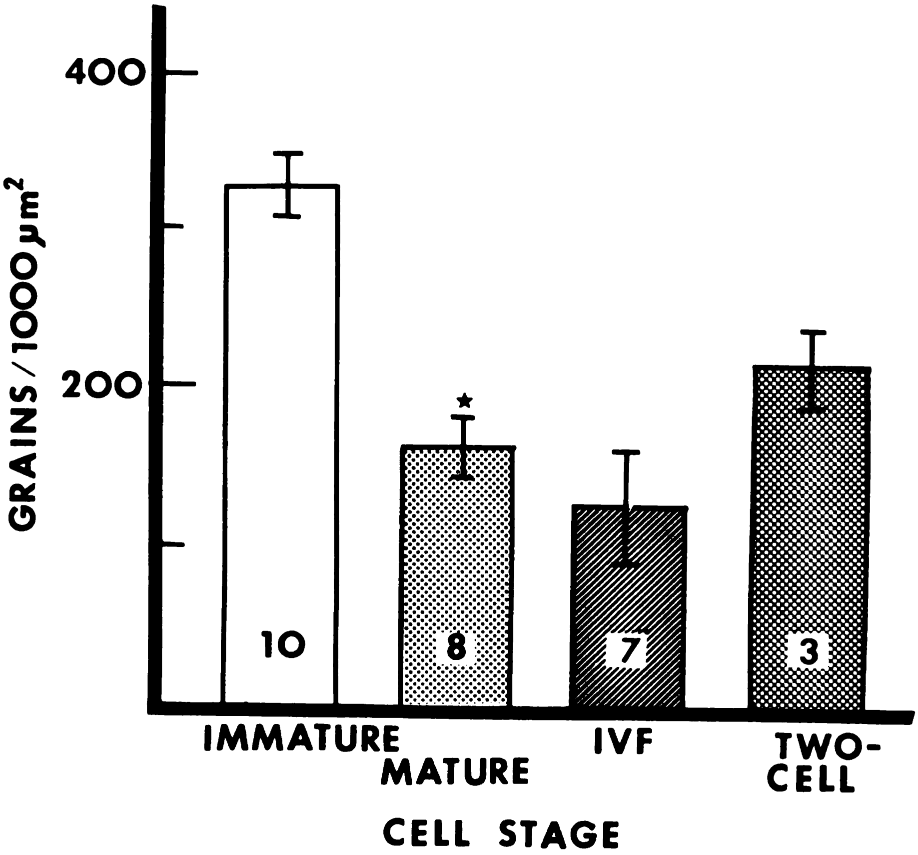


Figure 6. ^{3}H-Leucine incorporation by early embryos of squirrel monkeys, fertilized *in vitro*. Autoradiographic assessments were done on a per-cell basis. Number of ova at base of bars. Values are expressed as mean ± S.E. *Significantly different from previous cell stage ($p<0.05$).

TABLE 6

Incorporation and Uptake of ^{3}H-Uridine by Preimplantation Hamster Embryos: Effect of Superovulation

Cell Stage	N[1]	Natural Ovulation		N[1]	Superovulation	
		Incorporation	Uptake		Incorporation	Uptake
Two-cell	45	1.48±0.71[2]	4.50±0.10	80	1.17±0.30	3.30±0.45**
Four-cell	40	3.60±0.80	6.17±1.17	80	3.04±0.55*	8.80±1.95*
Eight-cell	50	3.47±0.87	10.90±1.36*	60	4.80±0.76	13.80±1.42
Morulae	25	16.05±0.95*	37.15±1.15*	10	11.70±2.02*	36.00±5.13*
Blastocysts	20	26.70±5.40	113.00±37.9	15	24.47±1.30*	53.41±11.43

[1] Total number of embryos. Number per replicate: 10-20 (2-cell), 10 (4-cell), 5-10 (8-cell), 5 (Morulae and blastocysts).

[2] Femtomoles ^{3}H-uridine/embryo/3 hr ± S.E.

*Significantly different from previous cell stage ($p<0.05$).

**Significantly different from respective group of naturally-ovulated ova ($p<0.05$).

Steroid uptake by hamster and squirrel monkey ova was validated by autoradiography. Hamster two-cell embryos containing ^{3}H-estradiol (^{3}H-E_2) possessed 252±30 grains/1000 μm^2 (n = 11 trials) (Figure 7A), whereas background levels were 65±6. The use of the nonradioactive competitors estradiol and progesterone (P) abolished uptake of ^{3}H-E_2 and -P, respectively, to that of background levels (Figure 7B). Unfertilized oocytes from squirrel monkeys showed grain counts of 122±28 grains/1000 μm^2 over background (n=3). The ratio of uptake of steroids as determined by autoradiography to that by liquid scintillation counts (below) was equivalent between two-cell hamster embryos and unfertilized oocytes of squirrel monkeys.

E_2 uptake increased with development in hamsters ovulating naturally (from 2200 to 4000 to 5700 CPM/embryo) (Table 7). There was no increase seen in E_2 uptake by the superovulated group over the same stages. E_2 uptake declined dramatically at the morula stage. P uptake increased at the 4-cell stage but remained constant at the 8-cell stage in naturally-ovulated hamsters. In the superovulated group, a significant increase was not seen until the 8-cell stage, and P uptake then remained constant. The superovulatory regimen reduced uptake of both steroids at all stages analyzed.

E_2 uptake by squirrel monkey ova fertilized *in vitro* in the groups treated with 4- or 5-days of FSH was augmented compared to unfertilized controls, although the increase was not statistically significant (Table 8). The group treated with FSH for 5 days showed increased E_2 uptake by unfertilized oocytes when compared to the 4-day group. P uptake increased with fertilization in both FSH groups.

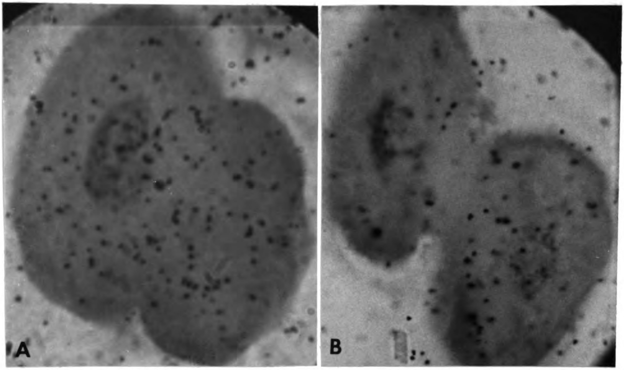


Figure 7a. Autoradiograph of a two-cell hamster embryo which was sectioned and incubated for 2 hr in medium supplemented with ^{3}H-estradiol-17β (x900).

7b. Autoradiograph of a two-cell hamster embryo which was sectioned and incubated for 2 hr in medium supplemented with ^{3}H-estradiol-17β and washed for 1.5 hr in a 1000-fold excess of nonradioactive estradiol-17β. Note the vast reduction in silver grains (x900).

TABLE 7

Uptake of ^{3}H-Estradiol 17β and ^{3}H-Progesterone by Preimplantation Hamster Embryos: Effect of Superovulation

Cell Stage	Estradiol 17β		Progesterone	
	Natural	Superovulation	Natural	Superovulation
Two-cell	2195±146[1] (3)[2]	1244±111 (6)**	1136±239 (3)	777± 80 (6)
Four-cell	3939±316 (3)*	1208±179 (4)**	2129± 12 (2)*	941±165 (4)**
Eight-cell	5707±467 (4)*	1824±257 (5)**	2270±333 (3)	1372±172 (5)[+]
Morulae	---	200± 11 (3)*[+]	---	1000± 23 (4)

[1] Units are CPM/embryo/2 hr ± S.E.

[2] Number of replicates in parentheses: 5-10 embryos per replicate.

*Significantly different from previous cell stage ($p<0.05$).

**Significantly different from respective group of naturally-ovulated ova ($p<0.05$).

[+] Significantly different from 2-cell stage ($p<0.05$).

TABLE 8

Uptake of ^{3}H-Estradiol 17β and ^{3}H-Progesterone by Unfertilized and In Vitro Fertilized Ova from Squirrel Monkeys: Effect of Ovulation Regimen

Cell Stage	Estradiol 17β		Progesterone	
	4 days FSH	5 days FSH	4 days FSH	5 days FSH
Unfertilized	1159±182[1] (10)[2]	2453±511 (11)**	668± 58 (6)	649±121 (11)
Fertilized one-cell	2200±488 (7)	2998±954 (6)	1685±189 (5)*	1361±122 (6)*

[1] Units are mean CPM/ovum/2 hr ± S.E.

[2] Number of ova in parentheses; 2-3 ova per replicate.

*Significantly different from unfertilized group ($p<0.05$).

**Significantly different from respective group treated with 4 days of FSH ($p<0.05$).

In a separate experiment (where the specific amount of incorporated radioactivity was known), steroid uptake was analyzed for embryos fertilized *in vitro* from squirrel monkeys treated with 4 days of FSH. There was a trend for increased E_2 uptake with *in vitro* fertilization and first cleavage (Figure 8), although this was not statistically significant (from 0.59 ± 0.07 to 0.87 ± 0.17 to 1.20 ± 0.40 picomoles (pmoles)/embryo/2 hr). P uptake increased at *in vitro* fertilization (from 0.21 ± 0.02 to 0.49 ± 0.05 pmoles/embryo/2 hr), and then remained constant at the two-cell stage (at 0.38 ± 0.10 pmoles/embryo/2 hr).

Preliminary experiments were run to determine the viability of hamster ova and squirrel monkey oocytes after a 4 hr incubation in modified TC-199 in the oxygen monitor. Of 115 ova, 100% were morphologically normal, excluded TpB and fluoresced brightly with FDA following culture. Linear regression analysis proved oxygen consumption by such ova to be 4- to 5-fold above baseline levels (n = 5 trials). Immature oocytes from squirrel monkeys and mature hamster ova did not differ in their oxygen consumption (4.85 ± 1.84 (n = 4 trials, 9-10 oocytes/trial) and 5.51 ± 0.92 nL O_2 consumed/ovum/4 hr (n = 8 trials, 50-200 ova/trial), respectively.

Preliminary experiments assessed the viability of hamster ova and squirrel monkey oocytes subsequent to a 4 hr culture in PBS for the studies of $^{14}CO_2$ production. Control ova in PBS alone (n=10) and ova in PBS + radioglucose (n=15) all excluded TpB and FDA after the culture period. Unfertilized oocytes from squirrel monkeys incubated in 1.80×10^{-4} and 5.56×10^{-4}M U-^{14}C-glucose incorporated 10.4 ± 2.4 (n = 4

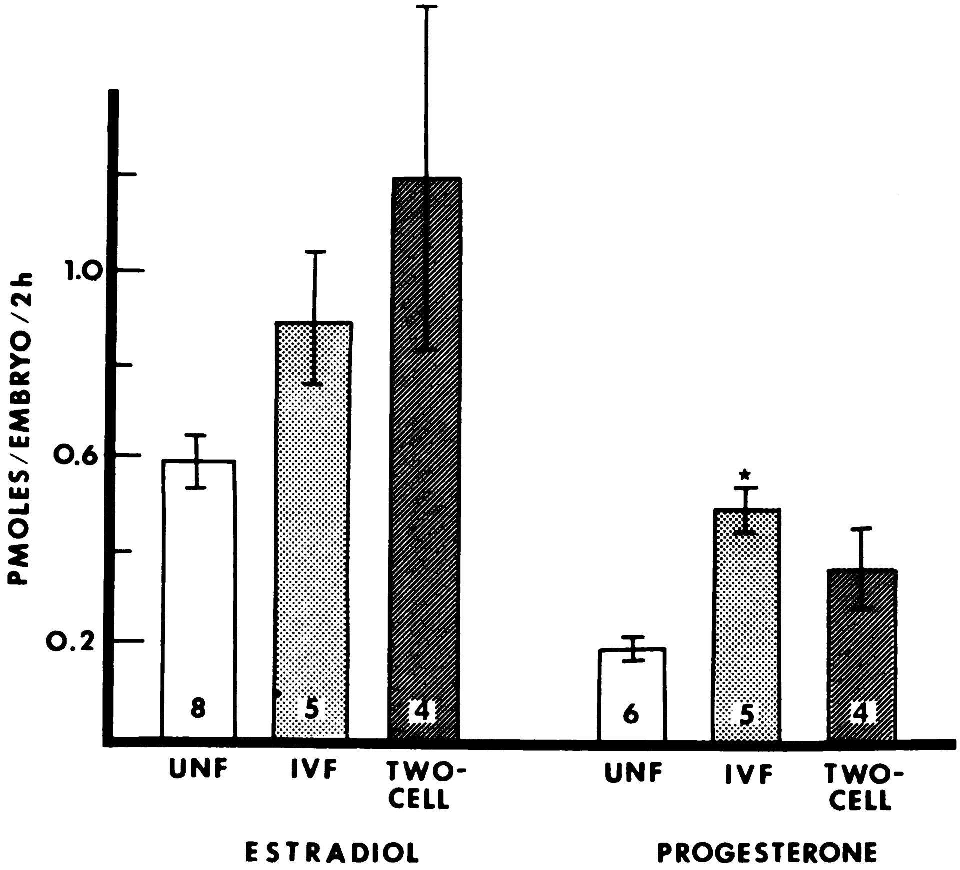


Figure 8. Steroid uptake by early embryos of squirrel monkeys, fertilized in vitro. Number of ova at base of bars. Values are expressed as mean ± S.E. *Significantly different from previous cell stage ($p<0.05$).

trials, 9-10 oocytes per trial) and 41.9±2.8 picogram (pg)-atoms glucose carbon/oocyte/4 hr (n = 4 trials), respectively. From the experiments on oxygen consumption and $^{14}CO_2$ production, an estimated molar ratio of $^{14}CO_2$ produced from universally-labelled glucose to total oxygen consumption was calculated at 0.19 for the unfertilized squirrel monkey oocyte.

The addition of insulin at concentrations of 10 nM and 1 μM increased 2-deoxyglucose uptake by unfertilized oocytes from squirrel monkeys over controls, but not to a significant extent (from 13.95±2.40 to 15.64±3.78 to 18.84±1.22 femtomoles (fmoles)/oocyte/3 h, respectively) (Figure 9). There was no change of 2-DG uptake at fertilization (Table 9). All ova used in the 2-DG experiments were viable by TpB and FDA prior to and subsequent to the culture period. The uptake of 2-deoxyglucose by fertilized and unfertilized ova classified as degenerate by morphology and vital dyes was reduced to background levels (Table 9).

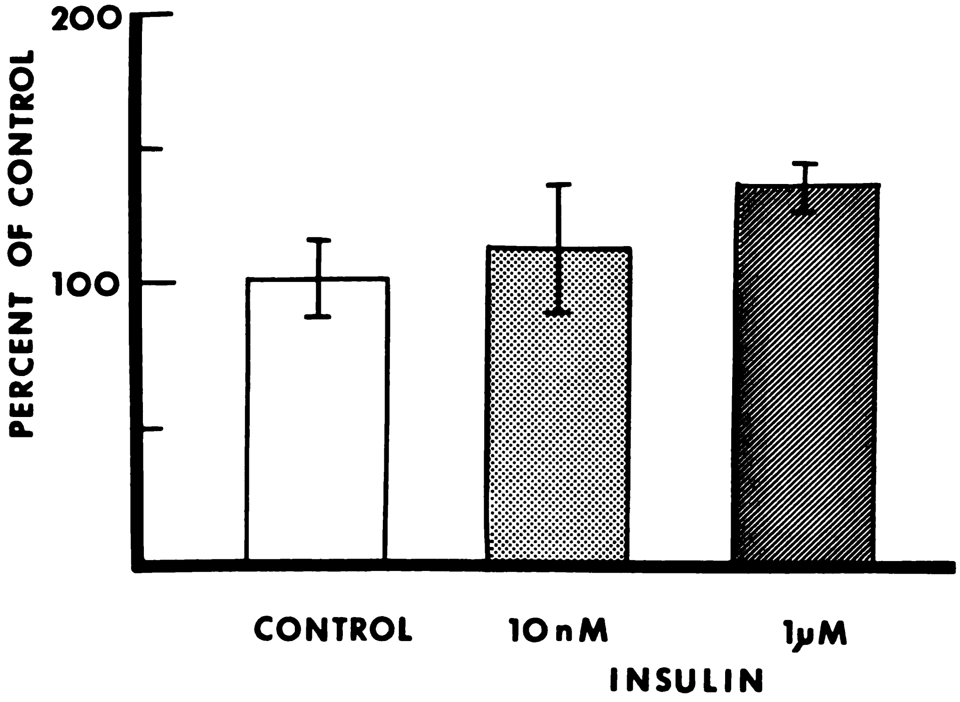


Figure 9. 2-Deoxyglucose uptake by unfertilized oocytes from squirrel monkeys: Effect of insulin. Four to five trials were run per group, with 4-10 oocytes per trial. Values are expressed as mean ± S.E.

TABLE 9

2-Deoxyglucose Uptake by Squirrel Monkey Ova that are Unfertilized, *In Vitro* Fertilized or Degenerate

Cell Stage	Percent Viable by Vital Dyes	2-DG Uptake[1]
Unfertilized	100 (5)[2]	14.63±1.20
Fertilized one-cell	100 (2)	16.67±2.60
Degenerate	0 (3)	---[3]

[1] Femtomoles 2-DG/ovum/3 hr ± S.E.

[2] Number of trials in parentheses; 3-10 ova per trial.

[3] Scintillation counts reduced to background levels.

DISCUSSION

The vital dyes trypan blue (TpB) and fluorescein diacetate (FDA) were validated for use with hamster and primate ova in the present studies. Exclusion of TpB and positive fluorscence with FDA by ova in various treatments correlated well with ^{3}H-uridine and -leucine incorporation and stability and development *in vitro*. Such correlations have also been shown for embryos and granulosa cells of other mammals (Campbell, 1979; Mohr and Trounson, 1980; Peluso *et al*., 1982). Over a sufficiently long period of culture, particularly in groups with intermediate viability, FDA appears a better predictor of viability than either TpB or purely visual assessments of morphology. However, as there exists such a high statistical correlation between TpB and FDA, it would be unnecessary to utilize both together in the future. Although FDA may be a more active indicator of viability, the assay also requires expensive fluorescence equipment and is more time-consuming than TpB. Therefore, TpB should suffice for most quick assessments of embryo viability *in vitro*. Nevertheless, both vital dyes are excellent indicators of viability of primate embryos produced by *in vitro* fertilization.

The decrease in ^{3}H-uridine incorporation by oocytes at 36 hr after HCG administration to squirrel monkeys is consistent with the decrease seen with intrafollicular maturation in mammalian oocytes (Baker et al., 1969; Bachvarova, 1974; Wassarman and Letourneau, 1976). Autoradiographic analysis showed an increase in the capacity of squirrel monkey ova to incorporate ^{3}H-uridine after in vitro fertilization. There was a further increase again after the second cleavage division. However, the amount of ^{3}H-uridine incorporated was several-fold lower than in similar autoradiographs of two-cell hamster embryos. This difference may be due to appreciable differences in precursor pools or membrane permeabilities to precursors (Clegg and Pikó, 1982). Nevertheless, relative RNA synthesis remains quite low in the early primate embryo. This observation compares with the low synthesis of RNA detectable at early stages in the mouse (Knowland and Graham, 1972). In this species, major increases in RNA synthesis do not occur until the 8-cell and morula stages, with further increases at the blastocyst stage (Monesi and Salfi, 1967; Ellem and Gwatkin, 1968). The decreases in ^{3}H-leucine incorporation with oocyte maturation and the low levels at fertilization and first cleavage are qualitatively similar to that demonstrated for other mammalian species (Brinster, 1971a; Schultz et al., 1978, 1979; Chen et al., 1980). These attenuated levels of relative protein synthesis correlate with the observed low number of polyribosomes up to the morula and blastocyst stages of primate embryos fertilized in vivo (Enders and Schlafke, 1981) and in vitro (Yorozu et al., 1983). Again, as the size of the precursor pool for leucine is not known in primate ova, absolute measurements could not be made. Apparent increases in protein synthesis occur late in preimplantation development, primarily at 8-cell and blastocyst stages in the mouse (Epstein and Smith, 1973; Abreu and Brinster, 1978; Schultz et al., 1979; Kaye et al., 1982) and at the blastocyst stage and beyond in embryos of domestic animals (Godkin et al., 1982; Janzen et al., 1982).

RNA synthesis is augmented throughout preimplantation development in mammalian embryos (Epstein, 1975; Clegg and Pikó, 1982). Our values for the relative incorporation of ^{3}H-uridine in hamster embryos closely resemble those for the mouse (Daentl and Epstein, 1971). (Absolute values for ^{3}H-uridine incorporation (and RNA synthesis) cannot be given, as the size of the embryonic UTP pool is unknown for any mammalian species but the mouse (Clegg and Pikó, 1977).) However, few studies have attempted to assess steroid uptake by embryos (Smith, 1968; Bhatt and Bullock, 1974; Wu and Lin, 1982a). The present studies demonstrate definite uptake of E_2 and P by ova from hamsters and squirrel monkeys and changes with in vitro development. The classical mode of steroid action is by gene derepression and activation of RNA synthesis (O'Malley et al., 1973). One can therefore expect changes in ^{3}H-uridine incorporation through the use of various ovulatory regimens as compared to normal ovulatory cycles. This hypothesis was based on the observation that treatment with exogenous gonadotropins alters levels of endogenous steroids in several species (Greenwald, 1976; Schrams et al., 1979; Edwards et al., 1980). In the present study, varying the length of FSH treatment showed no appreciable effects on steroid uptake in embryos from squirrel monkeys. However, the superovulatory regimen given hamsters significantly decreased uptake of exogenous radiosteroids by the embryos at virtually all stages analyzed. Yet, there was no concomitant effect on relative incorporation of ^{3}H-uridine. Absence of effects of exogenously administered steroids on RNA synthesis of mouse embryos, in vitro, was demonstrated by Warner and Tollefson (1977, 1978). These investigators hypothesized that the effects of E_2 and P are directly or indirectly on the membrane to alter permeability and subsequent embryonic cleavage. This hypothesis is yet to be confirmed. Changes in embryonic steroid uptake and receptors may therefore not be mediated by alterations in RNA synthesis.

The apparent reduction in steroid uptake by superovulated hamster embryos may be attributed to saturation of embryonic receptors with augmented levels of endogenous steroids. (Steroid uptake, however, is not necessarily indicative of receptor number (Martel and Psychoyos, 1981; Logeat et al., 1982).) Such saturation of receptors would alter true uptake values of radiosteroids. This has been reported to occur in studies of steroid uptake at implantation sites in pregnant mice and rats (Sartor, 1977; Ward et al., 1978). (In preliminary studies in our laboratory, we administered ^{3}H-E_2 and ^{3}H-P to pregnant hamsters carrying embryos at varying stages of preimplantation development and demonstrated steroid uptake by the reproductive tract and embryos. Relative changes between the in vitro and in vivo studies were identical, suggesting in vivo saturation of steroid receptors.) An alternative hypothesis is an actual alteration in receptor sites such as occurs in down-regulation in the presence of prolonged, elevated levels of endogenous hormones (Savoy-Moore et al., 1980). Yet another hypothesis is a delay in receptor synthesis which was not detected as a concomitant change in RNA synthesis at the level investigated.

Although changes in uptake or receptor content may occur with superovulation, it is apparent that these changes are probably not severe enough to disturb ovum normality (Seidel, 1981). The changes apparently do not prevent normal implantation and post-implantation development as superpregnancy normally ensues. In fact, litter sizes in hamsters have been reported as large as 27 (Fleming and Yanagimachi, 1980). Therefore, steroids may rather play a role in effecting developmental changes of early preimplantation embryos and cleavage. However, nearing implantation, embryos possess aromatase activity and are probably able to synthesize their own steroids (Dickmann and Dey 1974; Shutt and Lopata, 1981; Sengupta _et al_., 1981; Gadsby _et al_., 1981; Hoversland _et al_., 1982; Wu and Lin, 1982b), and thereby reduce their uptake accordingly. This may be the case for hamster embryos at the morula stage in the present study.

The limited studies on oxygen consumption and $^{14}CO_2$ production exhibit rates several-fold higher than reports in the literature for mouse, rat, rabbit and rhesus monkey ova (which are extremely varied) (Fridhandler, 1961; Mills and Brinster, 1967; Brinster, 1971b; Magnusson _et al_., 1977). This may be due to the hormonal stimulation administered to our hamsters and monkeys to induce ovulation. It has been demonstrated that oxygen consumption increases with HCG-induced maturation in rat oocytes (Magnusson _et al_., 1981). Also, experiments with rhesus monkeys used ova collected at laparotomy, without ovulation induction, from a heterogeneous population, and were not assessed for viability prior to culture (Brinster, 1971b). Although the volume of the squirrel monkey occyte is 3-4 times that of the hamster ovum, the two were equivalent in their rates of oxygen consumption. This is contrary to previous measurements on rabbit and mouse embryos (Mills and Brinster, 1967), which were more direct. It is possible that either the sensitivities of the techniques differ, or that the mature hamster ovum maintains a higher metabolic activity than the immature primate oocyte. This may compensate for the smaller size of the hamster ovum. The mouse ovum, in fact, possesses LDH activity 80 times that of other mammalian oocytes, including the squirrel monkey (Brinster, 1967b). The ratio of CO_2 production to O_2 consumption of 0.19 for oocytes from squirrel monkeys was roughly 9-fold greater than that calculated for the unfertilized mouse oocyte (Brinster, 1967a), but still quite depressed. It appears that glucose oxidation is extremely low in early embryonic development. In the mouse oocyte, less than 5% of oxygen uptake is due to glucose oxidation, with the rest primarily due to pyruvate (Brinster, 1969).

The 2-deoxyglucose experiments open up an exciting area of quick assessments of ovum metabolism and viability. Degenerate oocytes from squirrel monkeys showed a greatly diminished uptake of 2-DG compared to oocytes assessed as viable by vital dye assays. The uptake of 2-DG by viable oocytes incubated with or without insulin was determined. Although immunologic cross-reactivity between insulin of New World primates and cattle is low, the hypoglycemic action of bovine insulin is still effective in most primates (Howard, 1983). Insulin normally enhances glucose uptake by most body tissues. Yet, pharmacologic concentrations of insulin were required to significantly increase 2-DG uptake beyond controls in rat thymocytes, in vitro (Segal and Ingbar, 1980). In our studies of early primate embryos, in vitro, 2-DG uptake was not insulin-sensitive. The lowest levels of insulin used here were still greater than 50 times the physiological levels in serum from squirrel monkeys (Davidson and Blackwell, 1968). (Insulin levels in follicular fluid from squirrel monkeys are not currently available.) Therefore, insulin effects at the oolemma may not be normally operational. In fact, previous work has shown that the addition of insulin to the culture medium had no effect on either the rate of oocyte maturation or in vitro fertilization in squirrel monkeys (Kuehl and Dukelow, 1979), or on embryonic development in the mouse (Brinster, 1965). The experiment on 2-DG uptake with in vitro fertilization of oocytes from squirrel monkeys is consonant with the large body of data showing low utilization of glucose by early embryos of most mammals.

The results of the present studies indicate that biochemical changes were detected in the primate ovum with in vitro fertilization, including augmented incorporation of ^{3}H-uridine and steroid uptake and diminished incorporation of ^{3}H-leucine. The embryos are viable and follow metabolically normal development comparable with preimplantation development of other mammalian species.

SUMMARY AND CONCLUSIONS

The present studies were designed to evaluate the viability and biochemical alterations of squirrel monkey ova fertilized and developed *in vitro*. In addition, the effects of ovulatory regimens on the above variables were determined. The following conclusions resulted from the data obtained:

Staining with vital dyes as an index of viability

1. Exclusion of trypan blue and uptake and fluorescence with fluorescein diacetate by ova from hamsters and squirrel monkeys was highly correlated with *in vitro* development and relative syntheses of RNA and protein.

Macromolecular synthesis in the early embryo

1. ^{3}H-Uridine incorporation and uptake by squirrel monkey oocytes were reduced at 36 hr after HCG administration compared to 16 hr.
2. Relative incorporation and uptake of ^{3}H-uridine both increased with embryonic development in the hamster. Superovulation had no effect on either variable during embryonic development in the hamster.
3. ^{3}H-Uridine incorporation increased at *in vitro* fertilization in squirrel monkey ova. Another increase occurred at the second cleavage division.

4. ^{3}H-Leucine incorporation decreased with ovum maturation, and thereafter remained constant to the two-cell stage, fertilized *in vitro*.

Steroid uptake by the early embryo

1. Uptake of both estradiol-17β and progesterone by embryos recovered from superovulated hamsters increased with embryonic development. The superovulatory regimen reduced uptake of both steroids at virtually all stages analyzed.
2. Uptake of both steroids was increased with fertilization *in vitro* and first cleavage in the squirrel monkey. Only the progesterone increase was statistically significant. Changing the ovulatory regimen for squirrel monkeys from 4 to 5 days of FSH prior to HCG administration had no appreciable affect on steroid uptake.

Metabolism of the ovum and early embryo

1. Oxygen consumption by immature oocytes from squirrel monkeys was similar to mature hamster ova.
2. Unfertilized squirrel monkey oocytes incorporated 41.9 picograms of glucose carbon over a 4 hr period.
3. Utilization of the above variables produced a molar ratio of $^{14}CO_2$ production from glucose to total oxygen consumed estimated at 0.19.
4. The uptake of 2-deoxyglucose by unfertilized oocytes from squirrel monkeys was not altered by the addition of insulin.
5. There was no change of 2-deoxyglucose uptake at *in vitro* fertilization in squirrel monkeys.

6. 2-Deoxyglucose may be used as a viability indicator of primate ova.

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APPENDICES

APPENDIX A

BIOCHEMICAL EVALUATION OF THE UNFERTILIZED HUMAN OOCYTE*

A pilot study was designed to investigate the effect of the time of oocyte recovery on ^{3}H-uridine incorporation. Due to the difficulty in obtaining patients and oocytes, only two oocytes were processed.

Protocol

Patients were administered 5000 I.U. HCG on day 12 of the menstrual cycle to induce ovulation. Laparoscopic procedures and egg collection were routine (Jones _et al_., 1982). Incubation and processing of human eggs was as described for oocytes from squirrel monkeys.

Data

Morphologic data on the human oocyte recoveries are recounted in Table 1 and Figure 1. Incorporation of ^{3}H-uridine was reduced by 35 hr following HCG administration, compared to 12 hr after (Table 2).

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*This work was approved by the Human Subjects Committee of Michigan State University, East Lansing and Edward W. Sparrow Hospital, Lansing, Michigan.

TABLE 1

Morphologic Data on Human Oocyte Recoveries

Variable	Patient No.	
	002	005
Ovary punctured	Right	Left
Follicle size by ultrasound scan	≈20 mm	≈20 mm
Volume of follicular fluid	<<6.8[1]	4.4
Compactness of cumulus cell mass	Loose	Loose
Oocyte		
Morphology	Oolemma pulled away from zona pellucida; ovum expanded in culture	Ovum started to shrink; expanded in cultured
Meiotic state	Germinal vesicle intact	Germinal vesicle broken down

[1] Follicular fluid was combined with the saline used to flush the cannula prior to volume measurements.

Figure 1. Photograph of a human oocyte recovered 12 hr after HCG administration. Note the intact germinal vesicle and nucleolus. (Phase-contrast, x600).

TABLE 2

^{3}H-Uridine Incorporation and Viability with Respect to the Time of Oocyte Collection in Humans

Treatment	N	TpB Exclusion		FDA Uptake		Grains/1000 μm^2 ooplasm[1]
		Ovum	Cumulus %	Ovum	Cumulus %	
12 hr after HCG (Patient 002)	1	1	≈40	1	≈40	21
35 hr after HCG (Patient 005)	1	1	≈60	1	≈60	--[2]

[1] Ova were incubated for 3 hr in 5.6 μM ^{3}H-uridine.

[2] Not statistically different from background levels.

APPENDIX B

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Full Papers

Peluso, J. J., England-Charlesworth, C. and Hutz, R. (1980). Effect of age and of follicular aging on the preovulatory oocyte. Biol. Reprod. 22, 999-1005.

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Asakawa, T., Ghosh, M., DeMayo, F. J., Chan, P. J., Ridha, M. T., Hutz, R. J., Dooley, V. D. and Dukelow, W. R. (1981). Reproductive test systems involving *in vitro* and xenogenous fertilization. Proc. Center for Environmental Toxicology, East Lansing, MI.

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APPENDIX C

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