PROGRESS REPORT

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Phase I RADIATION AND RADIOISOTOPE STUDIES WITH
LABORATORY AND DOMESTIC ANIMALS

Subsection: Radiation Effects on Reproduction and
Embryogenesis

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~~Phase I:~~ Radiation and Radioisotope Studies With
Laboratory and Domestic Animals;

~~Subsection:~~ Radiation Effects on Reproduction and
Embryogenesis

R. J. DuFrain and A. P. Casarett

I. Background Summary of Activities Performed Under AEC Contract.

1. Introduction

The human population is presently, and will continue to be, exposed to ionizing radiation from medical and industrial usage, from background sources, and from fallout and nuclear accidents. Of particular scientific and public interest is the effect of this radiation on reproduction and embryogenesis--both from irradiation of parents and from irradiation of early fertilized ova. There has been considerable concern over danger from total body incorporation of tritium and from the incorporation into bone of radioactive strontium.

Numerous reports have described effects such as abnormal embryo development or decreased litter size after exposure of experimental animals to ionizing radiation. Little is known, however, of the sequence of events which lead to these endpoints--of the relative importance of decrease in number of ova or sperm, impaired fertilizing ability, aberrant embryo development and implantation failure.

The experiments which have been done over the past four years and those which are proposed for the future are directed at understanding and quantifying certain effects of radiation on reproduction and embryogenesis which occur during preimplantation development. Our system for the culture

of preimplantation mouse embryos in vitro has proven to be a successful method for study of this problem.

2. Objectives

The long term objective of these experiments has been to characterize the response of the preimplantation mammalian embryo to ionizing radiation delivered to the embryo itself or to parental generations. More specifically, it has been, first, to study the morphologic development of preimplantation embryos which have been exposed to ionizing radiation, and to identify the radiosensitive events or stages in the developmental process. Secondly, it has been to study the effect of maternal and paternal irradiation on embryo development to blastula stage. Thirdly, it has been to compare the effects from external radiation with those from radionuclides incorporated in or near the reproductive tissues or embryos. Fourthly, it has been to correlate radiation-induced morphologic abnormalities or developmental delay in the preimplantation period (as observed in vitro) with failure of implantation or subsequent development.

Minor objectives have been to establish suitable systems for culture of preimplantation embryos and for subsequent implantation into foster mothers; to determine the G_1 , S and G_2 times of the mitotic cycle of two- and four-cell embryos in order to characterize periods of interphase sensitivity; to justify the use of the culture system by comparing the radiation response of embryos developed in vitro with those developed in vivo; to compare the effects of acute and chronic irradiation on preimplantation response; and to establish a technique for fusion of embryos in order

to characterize the contribution of irradiated blastomeres to the development of the zygote.

3. Main Research Assomplishments

The experiments in this project are of two general types:

a) Fertilized embryos are irradiated in vitro and their development to blastula is followed. Radiation is given at selected stages of embryo development from one-cell to blastula, acutely from external sources (X- or gamma-radiation) or chronically either from external sources (gamma-radiation) or from radionuclides (^{90}Sr - ^{90}Y , ^3H) incorporated into the culture medium. Observations of cleavage times and morphologic development are made at intervals throughout the four-day culture period. Radiosensitivity is generally determined by survival to blastocyst stage, with other endpoints used as appropriate. Other embryos are exposed in vivo to the same treatments, and blastocyst development is determined.

b) Radiation is given to parents for one or more generations prior to mating. The embryos are then studied from two-cell to blastula in the culture system, with particular emphasis on the development from morula to blastula (since cavitation requires genomic expression). Parents are irradiated with external acture radiation, by continuous exposure to tritium incorporated into drinking water, or by a single injection of ^{90}Sr - ^{90}Y . Radionuclide distribution, organ dose, gonadal effects and sperm parameters are measured in certain experiments to supplement studies of effects on embryos.

In conjunction with these major studies, new techniques have been developed for embryo culture, for embryo fusion and for non-surgical transfer of cultured blastocysts into foster mothers.

Our results to date include the following:

a) The LD_{50} values for acute exposure to x-rays of embryos which develop from two-cell to blastula in vitro are as follows: one-cell (3 hours post-coitus), 175 rads; one-cell (13 hours post-coitus), 255 rads; two-cell, 390 rads; four-cell, 570 rads; morula, 325 rads. These values were obtained with embryos from random-bred Swiss albino mice, cultured in Brinster medium.

b) Comparable LD_{50} values for embryos irradiated and developed to blastula in vivo are 150 rads, 200 rads, 420 rads, 640 rads and 340 rads. It is apparent that there is little difference in the response to acute radiation of the embryos in vivo and in vitro, as measured by this system.

c) The LD_{50} values for chronic exposure of embryos which develop and are irradiated from two-cell to blastula in vitro are as follows: cobalt-60 (external), 100 rads/day, 259 rads total effective dose; tritium oxide in culture medium, 450 μ Ci/ml, giving a radiation dose of 115 rads/day, 298 rads total effective dose; ^{90}Sr - ^{90}Y in culture medium, 0.94 μ Ci/ml, equivalent to 52 rads/day, 135 rads total effective dose. Effective doses are based on radiation exposure during the 62-hour time in vitro from start of culture at late two-cell to beginning of blastocyst expansion. A biphasic dose-response curve was obtained in all of the in vitro chronic radiation experiments, indicating either an unusually high sensitivity of a fraction of the population (5-20%), or a sequential,

multiple event phenomenon in the radiation response. All of these experiments were done with embryos from random-bred Swiss albino mice, cultured in modified Whitten medium.

d) Comparable LD₅₀ values for chronic exposure of embryos which develop and are irradiated from two-cell to blastula in vivo are: cobalt-60 (external), 220 rads/day, 414 rads total effective dose; tritium oxide, concentration in body water 900 μ Ci/ml, equivalent to 230 rads/day, 433 rads total effective dose. Effective doses are based on radiation exposure during the 45-hour time in vivo from start of irradiation at late two-cell to beginning of blastocyst expansion.

e) The survival curves for beta radiation from tritium and for chronic gamma radiation from cobalt-60 were compared by a weighted regression analysis for exposures both in vivo and in vitro. There was no significant difference between the response to the two types of irradiation. It is, therefore, concluded that the RBE for tritium, relative to cobalt-60, is one for this experimental system.

f) A system has recently been developed for culture of embryos from the pronuclear one-cell stage, using DuFrain medium, a modification of Whitten medium. With this system, the LD₅₀ values for pronuclear irradiation of embryos from BLOSJ F₁ females mated with random-bred Swiss albino males are 1135 rads and 110 rads for development to two-cell and expanded blastocyst, respectively.

g) Based on serial observations of embryos developing in vitro, it has been determined that the major effect of chronic irradiation during

preimplantation development from two-cell to blastula is failure of development from morula to blastula. An experiment with tritium has confirmed this observation for in vivo development. Results from an experiment in which embryos are given a single, acute x-ray exposure at the two-cell stage suggest that the same effect occurs with acute irradiation. Irradiation at the pronuclear one-cell stage has indicated that blastocoel formation is a radiosensitive process. It is suggested that this marked radiosensitivity is related to the need for expression of the genome of the zygote.

h) Delay in cleavage from the four- to eight-cell stage is produced by x-irradiation given one to three hours before expected cleavage. In preliminary experiments, delays of 1.4, 1.8, 2.0 and 2.9 hours were found after radiation doses of 25, 50, 100 and 300 rads, respectively. Cleavage times were also determined for the division from two- to four-cell stages, for embryos irradiated at the pronuclear stage. A linear increase in cleavage time was found for doses up to 600 rads, with a delay of 0.8 minutes per rad.

i) Tritium oxide was incorporated into drinking water of C57BL/6J mice for two generations. Drinking water concentrations of 10 and 25 $\mu\text{Ci/ml}$ resulted in average body water concentrations of 7.1 and 17.8 $\mu\text{Ci/ml}$, respectively. Males of the first generation had a 23% and 56% reduction in testis weight and a 18% and 89% reduction in numbers of motile sperm for the 10 and 25 $\mu\text{Ci/ml}$ levels, respectively. Males of the second generation had a 25% and a 58% reduction in testis weight and a 60% and

89% reduction in numbers of motile sperm at the same dosage levels. Females of the first generation had an ovarian weight reduction of 20% and 47% at the 10 and 25 $\mu\text{Ci/ml}$ levels, respectively; the second generation females had reductions of 87% and 91%. The second generation females were not fertile. Males of the first generation mated with nonirradiated Swiss albino females fertilized 24% (10 $\mu\text{Ci/ml}$ level) and 47% (25 $\mu\text{Ci/ml}$ level) fewer ova; second generation males fertilized 26% (10 $\mu\text{Ci/ml}$ level) and 36% (25 $\mu\text{Ci/ml}$ level) fewer. Of the fertilized two-cell embryos from these matings which were cultured, there was a decrease of 7% (10 $\mu\text{Ci/ml}$) and 30% (25 $\mu\text{Ci/ml}$) in development to blastula of embryos sired by first generation males and a decrease of 3% (10 $\mu\text{Ci/ml}$) and 31% (25 $\mu\text{Ci/ml}$) of embryos sired by second generation males.

j) An intraperitoneal injection of 28 μCi of ^{90}Sr - ^{90}Y to young adult male mice gave a total dose (over a 28-day period) of 142 rads. More than half of this dose was received in the first 5 days after injection, and was from radionuclides localized in soft tissue in the vicinity of the scrotum. This dose resulted in a maturation depletion of the testes which is characteristic of an acute radiation exposure. Depressed sperm counts at 5 and 6 weeks after injection were associated with decreased mating success. The proportion of two-cell embryos which developed to blastula was reduced when mating occurred between 10 and 72 days after injection.

k) The technique for embryo fusion has been established and a preliminary radiation experiment has been completed. These preliminary results indicate that irradiation with 500 R of one member of a fusion pair

of eight-cell embryos results in a decrease in descendant cells from the irradiated embryo as counted at the blastocyst stage. Tritiated thymidine labeling and autoradiography were used to identify irradiated and unirradiated cells following fusion and subsequent development.

1) The cell cycle of the four-cell stage embryo developing in vitro has been characterized by tritiated thymidine labeling. The duration of the cell cycle in vitro of the four-cell stage embryo is approximately $14\frac{1}{2}$ hours in our system. The G_1 phase is short (less than 20 min.) or nonexistent, the S phase lasts about 9 hours and the G_2 phase is about 5 hours.

m) Embryos have been x-irradiated at four different times during the four-cell stage and their development subsequently monitored periodically. This study has shown that the 2-hour period immediately following cleavage is the most radiosensitive part of the cell cycle. At 2 to 4, and 4 to 6 hours after cleavage there is a progressive decrease in radiosensitivity. At 9 to 10 hours the radiosensitivity was similar to that at 4 to 6 hours.

Abstracts of Relevant Papers

Kirkpatrick, J. and Casarett, A. (1970) Radiation Response of the Preimplantation Mouse Embryo In Vitro. Radiation Res. 43: 231.

Casarett, A. and Kirkpatrick, J. (1970) Radiation-Induced Cleavage Delay of the Preimplantation Mouse Embryo In Vitro. Radiation Res. (Abstract)

Morgan, R. and Casarett, A. (1972) Effects of Tritium Oxide Upon Development of the Preimplantation Mouse Embryo. Radiation Res. 51: 503.

Parker, E. and Casarett, A. (1972) Effects of ^{90}Sr - ^{90}Y on Reproduction in the Male Mouse. Radiation Res. 51: 503.

DuFrain, R. and Casarett, A. (1973) Response of the Pronuclear Mouse Embryo to X-irradiation In Vitro. Rad. Res. 55: 560-561.

Casarett, A. and Morgan, R. (1973) The Relative Biological Effectiveness of Tritium on Preimplantation Mouse Embryos. Health Physics (Abstract).

DuFrain, R. and Casarett, A. (1974) Variations in Relative Radiosensitivity of Mouse Embryos Irradiated at Selected Times of the Cell Cycle. Rad. Res. (Abstract).

Casarett, A. and Kirkpatrick, J. Differential Sensitivity of Preimplantation Stages of Mouse Embryogenesis In Vivo and In Vitro (in preparation).

DuFrain, R. and Casarett, A. A System for the Culture of One-Cell Mouse Embryos from Random-Bred Swiss Albino Mice (in preparation).

II. Current Work (1973-74)

Foreword

During the past year, the entire experimental set-up has been moved from the Radiobiology Laboratory, 5 miles off campus, to a larger facility on the seventh floor of the new Multicategorical Research Wing of the Veterinary College. Extreme delays have been encountered in reestablishing an animal colony, setting up and recalibrating radiation sources (280 kVp X-ray machine plus a new Cesium-137 therapy source), and arranging conditions suitable for embryo culture. A minimum of experimental work has been completed this year as indicated by the three sections of the research summary. We expect now that all equipment and experimental procedures will be reestablished to our satisfaction by early fall, when we will proceed with the experiments outlined in our proposal.

The following sections detail the three experimental approaches which have been completed this year.

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Cell Cycle Stage Determination for the Four-Cell Mouse Embryo

R. J. DuFrain and A. P. Casarett

*and effects of irradiation delivered at different times of the four-cell stage cell cycle.*I. Introduction

Estimation of the length of the four-cell stage duration in our laboratory has been accomplished by three different methods, yielding times of 13.9, 14.7 and 15.4 hours. From these values, we have assumed a $14\frac{1}{2}$ hour cell cycle duration for this stage in vitro in our system. To further characterize the four-cell stage, we monitored the uptake of tritiated thymidine at various times after observed cleavage.

II. Methods and Materials

Embryos were obtained from immature BLU:Ha (ICR) mice by hormonally induced superovulation. Female mice with copulation plugs at 20 hours after the HCG injection were identified and subsequently sacrificed at 46 and 48 hours after the HCG injection. All embryos flushed from the oviducts in a single day (2 hour period) were pooled in a single embryological watch glass that contained approximately 3 ml. of Whitten culture medium (see Table I). Any abnormal or degenerate embryos, as judged by gross microscopic morphology, were discarded, and the remaining embryos were washed in two changes of fresh culture medium. During all handling of embryos subsequent to flushing from the oviducts extreme care was taken to maintain the temperature of the culture medium at 37°C and the pH at approximately 7.2. These requirements were met by keeping the watch glass containing the embryos on a heating pad and under a gas mixture with 5% CO_2 at all times other than during actual manipulations.

Table I

Whitten Medium

<u>Constituent</u>	<u>Amount Per Liter</u>
Sodium Lactate, 60%	3.7 ml
Phenol Red, 0.5%	0.2 ml
Sodium Chloride	5.140 g
Sodium Pyruvate	0.035 g
Glucose	1.000 g
Potassium Chloride	0.360 g
Calcium Lactate Tetrahydrate	0.480 g
Potassium Phosphate Monobasic	0.160 g
Magnesium Sulfate Septahydrate	0.290 g
Sodium Bicarbonate	1.400 g
Penicillin "G", 1625 i.u./mg	0.080 g
Streptomycin Sulfate	0.050 g
Bovine Serum Albumin, Crystallized	3.000 g

Embryos were labeled in culture in a special medium (Mintz, 1964) that allowed satisfactory uptake and incorporation of nucleotides. No apparent harm comes to the embryos from the short periods of culture in this medium. The composition of the media for labeling with ^3H -thymidine and for the "cold" wash are given in Table II. The "cold" chase medium contained excess thymidine to dilute internal pools of labeled thymidine rapidly. The medium for labeling was prepared and stored at 2X normal concentration without the radioactive thymidine. Immediately before using the ^3H -methyl-thymidine (New England Nuclear) was diluted to the proper concentration (4X the working concentration) with redistilled water, mixed with the 2X concentrate and then added to a like volume of fetal calf serum. The "cold" medium was prepared at normal strength and added to the fetal calf serum immediately before use.

The labeling of embryos took place in small (1 ml) agglutination titer-board wells (Linbro Plastics) that had been sterilized with alcohol and ultraviolet radiation. The embryos were placed in the labeling medium for 20 minutes and followed by a 20 minute period in the "cold" medium. Following this the embryos were washed twice in fresh Whitten medium and replaced in culture until time for slide preparation. All media used in these experiments were covered with sterile medical fluid and equilibrated with the 90:5:5 gas before use.

From the embryos selected at 46 to 48 hours after the HCG injection, cleaving and four-cell embryos were isolated at various times for the next 12 hours. At the end of the 12 hour period all groups of embryos were subjected to a 20 minute pulse in the labeling medium, a 20 minute chase in

Table II

Media for Isotope Labeling

(From Mintz, 1964)

<u>Constituent</u>	<u>Amount per Liter</u>	
	Labeling	"Cold"
Sodium Chloride	5.000 g	5.000 g
Potassium Chloride	0.400 g	0.400 g
Calcium Lactate Pentahydrate	0.530 g	0.530 g
Magnesium Sulfate Septahydrate	0.250 g	0.250 g
Sodium Phosphate Dibasic - $2H_2O$	0.140 g	0.140 g
Glucose	1.000 g	1.000 g
Penicillin "G" 1625 I.U./mg	0.080 g	0.080 g
Streptomycin Sulfate	0.050 g	0.050 g
Sodium Lactate	3.7 ml	3.7 ml
Thymidine	----	0.484 g
Thymidine (Methyl - H^3)	0.204 g (3 μ Ci/ml)*	----

Media are mixed 1:1 with fetal calf serum immediately before use

* Final Specific Activity

medium with non-radioactive thymidine and two 20 minute washes in fresh culture medium. Following the second medium wash the embryos were replaced in culture and left for 14 hours. After the 14 hour period of culture, embryos that had divided to the eight-cell stage were selected for slide preparation.

Slides were prepared from the labeled embryos by the air drying method of Tarkowski (1966). Embryos were transferred to an embryological watch glass containing 1% sodium citrate solution and left for 15 minutes.

Individual embryos were placed on microscope slides in small drops of the sodium citrate solution and 3 to 4 drops of freshly prepared acetic ethanol (3 parts alcohol : 1 part glacial acetic acid) were dropped on the embryo to fix and flatten the embryo on the slide. After drying, the slides were washed for 5 minutes in ice cold 5% TCA to remove any acid soluble label present in the embryos. The slides were then washed in redistilled water and allowed to dry.

In a completely dark room the slides were coated with NTB2 Nuclear Track Emulsion (Kodak, Rochester, N. Y.) and allowed to dry before being placed in a light tight dessicating slide box for the exposure period. Following the seven day exposure, the slides were developed in a darkroom following the procedure outline in Kodak Technical Pamphlet No. 64 and listed in Table III. Once through the last wash, the slides were stained with a 4% Giemsa stain solution (Fisher). After 30 minutes in the stain, the slides were destained in acetic acid, dehydrated through xylene, and coverslips were mounted with Harleco Synthetic Resin (Harleco Stain Co.).

Table III

Development of NTB-2 Coated Slides

Step	Solution	Time	Temperature
1. Develop	Kodak D-19 1:1	4 minutes	15°C
2. Stop	Distilled water	10 seconds	15°C
3. Fix	Kodak Fixer	5 minutes	15°C
4. Wash	Distilled water	30 minutes	15°C
5. Transfer to staining Procedure			

* Kodak Technical Pamphlet #64; Kodak Materials For Nuclear Physics and Autoradiography.

All slides were scored on a Zeiss Universal microscope using the oil immersion objective for a total magnification of 1250X. Grains developed directly over the nucleus or touching the nuclear membrane were counted for each nucleus of every embryo. A background count was made on each slide near each embryo. The size of the area counted for the background determination was equal to the area of the whole blastomere nucleus.

III. Results

Table IV contains a summary of the results of the labeling experiment. Table V is the summary of the analysis of variance for the labeling at the various times. Figure I shows graphically the incorporation of tritiated thymidine as a function of time after the observed cleavage.

IV. Discussion

The conclusion based on initial observation is that the rate of label incorporation and thus the rate of DNA synthesis differs significantly at different times in the S phase of the cell cycle.

The presence of unlabeled cells after labeling at 0 and 1 hour following cleavage suggests that a G_1 phase was present, but so short that the present system could not detect its length. The presence of unlabeled cells at 9, 10, and 11 hours after cleavage and the dramatic drop in label incorporation were strong indicators that a G_2 phase existed for the four-cell stage cell cycle in the preimplantation mouse embryo cultured in vitro.

From this data we concluded that the G_1 phase is short or nonexistent, the S phase lasts for about 9 hours and the G_2 phase is about 5 hours in our system. These values appear to be compatible with the existing literature on preimplantation mouse embryo cell cycle stages as reported by

Table IV. Summary of Labeling Index for Cells Labeled at Various
Times During the Four-Cell Stage

Time After Observed Cleavage	Nuclei Observed (% Labeled)	Grains per Labeled Nucleus $\pm$ S.E.M.	Range Grains per Nucleus	*
Immediately	55 (83.6)	37.3 $\pm$ 25.9	0 - 91	a
1 hour	85 (92.9)	36.1 $\pm$ 20.7	0 - 89	a
3 hours	62 (100)	60.2 $\pm$ 31.2	7 - 121	b
5 hours	64 (100)	69.2 $\pm$ 29.1	15 - 135	c
7 hours	48 (100)	93.3 $\pm$ 50.7	28 - 227	d
9 hours	27 (88.9)	59.3 $\pm$ 29.7	0 - 114	b
10 hours	87 (93.0)	11.3 $\pm$ 7.3	0 - 37	e
11 hours	92 (85.9)	9.2 $\pm$ 7.7	0 - 42	e
5-8 cell Embryos	116 (86.2)	12.8 $\pm$ 12.3	0 - 65	

* Rows not followed by the same letter are significantly different
p < 0.01

Table V. Summary of Analysis of Variance for Labeling at Various
Times after Cleavage to the Four-Cell Stage

Source of Variation	d.f.	Sum of Squares	Mean Square	F
Between Times	7	366211.83	52315.98	77.31 **
Within Times	482	326186.92	676.70	
Total		692380.75		

** $p < 0.01$

Figure I. ^3H -Thymidine Incorporation by Four-Cell Embryos During 20 Minute Pulses at Various Times in the Cell Cycle

Samoshkina (1968), Gammow and Prescott (1970) and Barlow, Owen, and Graham (1972).

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A. Effects of Irradiation Delivered at Different Times of the Four-Cell Stage Cell Cycle.

R. J. DuFrain

I. Introduction

Variation in radiosensitivity at different stages of preimplantation development in the mouse were reported by Brenneke in 1937 (1) and later confirmed in many subsequent investigations (2,3,4,5,6). Russell and Montgomery (6) found a fivefold variation in the radiosensitivity of embryos irradiated as little as four hours apart. They proposed that the variation in response was caused by irradiation at different times of the cell cycles of the blastomeres. These investigators did not attempt to identify the specific stages of the cell cycle that were either sensitive or resistant to radiation induced damage. Because of the inherent variability of mating and development in vivo and the failure of interperitoneal

³H-thymidine injections to label blastomere nuclei (7), more specific localization of the sensitive or resistant times were not possible.

By using hormonally induced superovulation, timed matings, and large scale culture, it was possible to study the effects of irradiation delivered at different times after observed cleavage to the four-cell stage. This combination of techniques, when combined with periodic observation, allowed monitoring of development through the hatched blastocyst stage. It was also possible to make a qualitative assessment of the radiation induced cleavage delay, when irradiation was delivered at different times of the four cell stage.

II. Methods and Materials

Several hundred two-cell embryos were obtained by flushing the oviducts of thirty to forty superovulated (7 I.U.'s each hormone) five week old BLU:Ha (ICR) mice 44 to 48 hours after the HCG injection. The embryos were washed twice in fresh Whitten culture medium (see Table I) and grossly abnormal embryos were discarded. The embryos were maintained in an embryological watch glass in approximately three ml of culture medium. The medium in the watch glass was covered with a layer of sterile medical fluid and the watch glass and contents were kept at 37°C in a humidified 90% N₂, 5% O₂, 5% CO₂ atmosphere.

At 49, 51, and 52½ hours after the HCG injection, four-cell embryos were removed from the pool of embryos, placed in separate watch glasses, and kept under the same conditions. Four-cell embryos from each group were randomly assigned to different exposure levels at this time. All

embryos to be irradiated with a given exposure were placed in microdrops of medium in 35mm plastic (Falcon) petri dishes. The microdrops had previously been covered with medical fluid and equilibrated with humidified gas at 37°C. Petri dishes were sealed in plastic boxes after gas re-equilibration, and irradiations (and sham irradiation of the controls) were carried out using a 280 kVp Picker X-ray machine with aluminum and copper filters. Irradiation was given to two-cell embryos at $53 \pm \frac{1}{2}$ hours after the HCG injection; to four-cell embryos within two hours after cleavage to the four-cell stage; to four-cell embryos 2 to 4 hours after cleavage to the four-cell stage; and to four-cell embryos 4 to 6 hours after cleavage to the four-cell stage. Irradiation of the four-cell embryos at 9 to 10 hours after cleavage to the four-cell stage took place at $59 \pm \frac{1}{2}$ hours after the HCG injection. The four-cell embryos for this group had been selected at 50 hours after the HCG injection. Eight-cell embryos were irradiated $69 \pm \frac{1}{2}$ hours after the HCG injection. They had previously been cultured in vitro from 48 hours after the HCG injection.

After irradiation, embryos were removed from the microdrops and placed 10 per well in the depressions of a Microtest tissue culture plate (Falcon). Each depression (well) contained a drop of fresh culture medium covered with a drop of sterile medical fluid and had been previously equilibrated at 37°C with 90% N₂, 5% O₂, 5% CO₂ gas. By using the Microtest plates, which had 60 depressions, it was possible to place all embryos from a single day's work in one container and eliminate the possibility of partial loss of the test embryos. Along with the Microtest plate containing the embryos, a 10 milliliter beaker with culture medium and two 60 mm petri

dish bottoms filled with distilled water were sealed in the re-equilibrated plastic box. The beaker of culture medium allowed rapid visual determination of the integrity of the seal (pH changes in bicarbonate buffered medium with phenol red indicator indicate a loss of CO₂ from the atmosphere by changing to a bright pink color) and the distilled water maintained the humidity in the plastic box.

Observations of the development of the embryos were made at 12 hour intervals through 192 hours (8 days) after the HCG injection. At each observation the number of blastomeres per embryo or the developmental stage reached for each embryo was recorded. Observations were made with a Zeiss inverted phase microscope at 100X magnification. During the observation the box was removed from the incubator, embryos were rapidly scored, and the box was replaced in the incubator. It was not necessary to open the sealed box to make an observation. Thus, the seal was maintained throughout the culture period. In this experiment all embryos were scored using the classification in Table VI. If the embryos did not fit into one of these classifications they were judged to be abnormal and scored as such.

After each experimental series had been completed, a chronological assessment of development for each well was made and the maximum number of embryos in the individual well reaching each stage was recorded. These values were then used to compute the survival data.

The experimental design used in these experiments also allowed for an estimation of the delay in the first cleavage following irradiation. The delay was determined by computing the average number of cleaved

Table VI. Embryo Classification for Normal Preimplantation
Mouse Embryos

<u>Stage</u>	<u>Description</u>
2-cell	Two blastomeres within the zona pellucida.
3-cell	Three blastomeres within the zona pellucida. (Only normal if found between 46 and 60 hr after HCG.)
4-cell	Four morphologically normal blastomeres contained within the zona pellucida.
5- to 16-cell	More than four normal blastomeres within the zona pellucida, but all five or more have distinct cell membranes and have not started to fuse.
Morula	Blastomeres within the zona pellucida have started to fuse, but no sign of blastocoel forming.
Early Blastula	Blastocoel has formed and is distinct within the cell mass inside the zona pellucida. Blastocoel is less than 50% of the volume of the embryo.
Blastocyst (expanded)	The volume of the blastocoel must be more than 50% of the total volume of the embryo.
Hatching	Signs of a break in the zona pellucida or protrusion of the embryo outside of the zona, but the embryo is not free from the inside of the zona pellucida.
Hatched Blastocyst	Embryo is completely free from the zona pellucida.

embryos per well at 84 hours after the HCG injection for each exposure level delivered to each stage of the four-cell period. By this time all control embryos had divided and were beyond the four-cell stage. A regression analysis of the mean number of cleaved embryos per well against radiation exposure for each irradiation time was carried out and the regression coefficients were compared by an analysis of covariance (8). This procedure allowed a qualitative evaluation of the differences in cleavage delay.

III. Results and Discussion

A. Cleavage Delay

The data presented in Table VII represent the compiled summary of the mean number of cleaved (beyond four-cell stage) embryos per well 84 hours after the HCG injection. By this time, all of the control embryos from each of the four groups had cleaved and were beyond the four-cell stage of development (the value was thus 10 for all unirradiated embryos, as there were 10 embryos per well). The lower portion of each column summarizes the data that were used for the regression analyses. A summary of the analysis of regression coefficients by covariance is presented in Table VIII. This analysis shows that the regressions were not the same ($p < 0.005$). Figure II is a graphic representation of the best fitting straight regression lines through a fixed point (the control or unirradiated values). This type of representation aids in visualizing the differences between the groups of embryos.

The information presented in these tables and the graph indicate that early four-cell embryos have a greater cleavage delay than embryos

Table VII. Summary of Cleaved Embryos per Well at 84 Hours
After the HCG Injection

Group	0 - 2 Hours	2 - 4 Hours	4 - 6 Hours	9 - 10 Hours
0	10	10	10	10
25	9.40	9.40	9.17	10.0
50	8.75	9.50	9.20	9.67
75	7.67	9.40	9.00	10.0
100	7.75	9.75	10.0	9.00
150	9.50	9.75	9.0	9.00
200	10.0	8.50	9.25	9.00
250	6.50	8.25	9.60	10.0
300	4.00	7.50	9.75	7.67
350	6.50	7.50	7.80	7.67
400	4.67	7.00	8.60	6.80
500	--	7.33	8.60	6.33
600	--	--	--	7.14
Σx	1900	2400	2400	3000
Σx^2	516250	766250	766250	1162500
Σy	84.67	103.88	109.97	112.28
Σy^2	695.3039	912.6364	1012.2739	991.4552
Σxy	12410.5	18955	21360.25	23188.00
x	172.73	200	200	230.769
y	7.6973	8.6567	9.1642	8.6370
n	11	12	12	13
b	-0.011774	-0.006362	-0.002214	-0.002342
r	.774	.930	.559	.852

Table VIII. Summary of the Comparison of Regressions by Analysis
of Covariance

Time	d.f.	Σx^2	Σy	Σy^2	d.f.	Reduced SS
0 - 2	10	188068.1818	-2214.31818	43.575818	9	17.504396
2 - 4	11	286250.0	-1821.0	13.381867	10	1.797444
4 - 6	11	286250.0	-633.75	4.490492	10	3.087386
9 - 10	12	1162500.0	-2722.76923	21.701477	11	15.324297
Residuals for Individual Regression					40	37.715523
Single Regression 44		1923068.182	-7391.83741	83.149654	43	54.737110
Differences for Homogeneity of Regressions					3	17.023587

F = 6.02*** 3 and 40 degrees of freedom

*** Significantly different $p < 0.005$

Figure II. Average Number of Cleaved Embryos per Well

84 Hours After HCG Injection for Irradiation at

0 to 2, 2 to 4, 4 to 6, and 9 to 10 Hours After

Cleavage to the Four-Cell Stage In Vitro

irradiated two hours further into the four-cell stage. Embryos irradiated later than four hours after the cleavage have less delay than the embryos irradiated earlier. From this type of analysis, no quantitative evaluation of the exact extent of the cleavage delay could be made, but it was quite apparent that the delay was also dose dependent.

Tables IX through XIV contain the survival data for embryos irradiated at two-cell, various four-cell and eight-cell stages. In Table XV the control values for subsequent development are presented to show that all of the four-cell groups of embryos were equally able to develop to the stages observed (that is, there was no difference in culturability of the different groups). Two-cell stage embryos did not develop with the same efficiency as embryos observed to cleave in vitro. Tables XVI through XX contain summaries of the logistic analyses of survival for development to each of the five selected stages. Figure III is a graphic representation of the data presented in Tables XVI through XX.

Radiosensitivity of development to the morula stage was found to be well above the exposures used in these experiments. Morgan (9) also found that in vitro embryonic development to the morula stage was radio-resistant over a similar range of exposure ionizing radiation. His study, however, used chronic irradiation from tritium in the culture medium, or chronic irradiation with gamma rays from an external cesium source. These findings using the in vitro culture system support the contention of Russell and Montgomery (6) that embryos irradiated during cleavage continue to

Table IX. Survival to Selected Endpoints of BLU:Ha (ICR) Mouse Embryos Irradiated at the Late Two-Cell Stage. Irradiation Within Two Hours of the Expected Cleavage to the Four-Cell Stage.

(Numbers in parentheses give percentage development to that stage)

Exposure (Roentgens)	Embryos	Number Reaching Morula	Number Initiating Cavitation	Number Reaching Blastocyst	Number Initiating Hatching	Number Completing Hatching
0	200	179 (89.5)	171 (85.5)	170 (85.0)	140 (70.0)	111 (55.5)
25	80	63 (78.8)	57 (71.3)	52 (65.0)	29 (36.3)	12 (15.0)
50	80	69 (86.3)	66 (82.5)	65 (81.3)	41 (51.3)	25 (31.3)
75	80	66 (82.5)	62 (77.5)	51 (63.8)	19 (23.8)	8 (10.0)
100	110	89 (80.9)	83 (75.5)	60 (54.5)	32 (29.1)	14 (12.7)
150	80	61 (76.3)	51 (63.8)	38 (47.5)	15 (18.8)	10 (12.5)
200	100	72 (72.0)	58 (58.0)	66 (36.0)	10 (10.0)	6 (6.0)
250	100	77 (77.0)	61 (61.0)	26 (26.0)	5 (5.0)	3 (3.0)
300	110	71 (64.5)	41 (37.3)	19 (17.3)	2 (1.8)	1 (0.9)
350	120	84 (70.0)	37 (30.8)	19 (15.8)	2 (1.7)	2 (1.7)
400	110	77 (70.0)	37 (33.6)	19 (17.3)	2 (1.8)	1 (0.9)
500	100	58 (58.0)	16 (16.0)	7 (7.0)	0 (0.0)	0 (0.0)

Table X. Survival to Selected Endpoints of BLU:Ha (ICR) Mouse Embryos Irradiated Within Two Hours of Cleavage to the Four-Cell Stage. (Numbers in parentheses give percentage development to that stage.)

Exposure (Roentgens)	Embryos	Number Reaching Morula	Number Initiating Cavitation	Number Reaching Blastocyst	Number Initiating Hatching	Number Completing Hatching
0	60	60 (100)	59 (98.3)	59 (98.3)	58 (96.7)	47 (78.3)
25	50	48 (96.0)	47 (94.0)	43 (86.0)	27 (54.0)	15 (30.0)
50	40	39 (97.5)	38 (95.0)	36 (90.0)	21 (52.5)	10 (25.0)
75	50	50 (100)	46 (92.0)	37 (74.0)	13 (26.0)	6 (12.0)
100	30	29 (96.7)	27 (90.0)	15 (50.0)	9 (30.0)	5 (16.7)
150	40	39 (97.5)	35 (87.5)	16 (40.0)	2 (5.0)	1 (2.5)
200	30	30 (100)	20 (66.7)	10 (33.3)	0 (0.0)	0 (0.0)
250	40	39 (97.5)	23 (57.5)	4 (10.0)	1 (2.5)	1 (2.5)
300	30	29 (96.7)	18 (60.0)	7 (23.3)	0 (0.0)	0 (0.0)
350	40	38 (95.0)	14 (35.0)	3 (7.5)	1 (2.5)	1 (2.5)
400	30	30 (86.7)	9 (30.0)	2 (6.7)	0 (0.0)	0 (0.0)

Table XI. Survival to Selected Endpoints of BLU:Ha (ICR) Mouse Embryos Irradiated Between 2 and 4 Hours After Cleavage to the Four-Cell Stage. (Numbers in parentheses give percentage development to that stage.)

Exposure (Roentgens)	Embryos	Number Reaching Morula	Number Initiating Cavitation	Number Reaching Blastocyst	Number Initiating Hatching	Number Completing Hatching
0	50	50 (100)	50 (100)	50 (100)	48 (96.0)	41 (82.0)
25	50	50 (100)	50 (100)	50 (100)	45 (90.0)	19 (38.0)
50	40	39 (97.5)	38 (95.0)	37 (92.5)	28 (70.0)	14 (35.0)
75	50	50 (100)	50 (100)	46 (92.0)	24 (48.0)	13 (26.0)
100	40	40 (100)	39 (97.5)	34 (85.0)	26 (65.0)	14 (35.0)
150	40	39 (97.5)	36 (90.0)	25 (62.5)	15 (37.5)	10 (25.0)
200	40	40 (100)	35 (87.5)	23 (57.5)	12 (30.0)	6 (15.0)
250	40	40 (100)	30 (75.0)	13 (32.5)	1 (2.5)	1 (2.5)
300	40	39 (97.5)	32 (80.0)	20 (50.0)	5 (12.5)	2 (5.0)
350	40	39 (97.5)	27 (67.5)	10 (25.0)	0 (0.0)	0 (0.0)
400	40	39 (97.5)	31 (77.5)	15 (37.5)	1 (2.5)	0 (0.0)
500	30	28 (93.3)	15 (50.0)	6 (20.0)	0 (0.0)	0 (0.0)

Table XII. Survival to Selected Endpoints of BLU:Ha (ICR) Mouse Embryos Irradiated Between 4 and 6 Hours After Cleavage to the Four-Cell Stage. (Numbers in parentheses give percentage development to that stage.)

Exposure (Roentgens)	Embryos	Number Reaching Morula	Number Initiating Cavitation	Number Reaching Blastocyst	Number Initiating Hatching	Number Completing Hatching
0	70	68 (97.1)	68 (97.1)	67 (95.7)	67 (95.7)	57 (81.4)
25	60	56 (93.3)	54 (90.0)	53 (88.3)	49 (81.7)	31 (51.7)
50	50	45 (90.0)	44 (88.0)	44 (88.0)	40 (80.0)	26 (52.0)
75	60	57 (95.0)	56 (93.3)	53 (88.3)	38 (63.3)	26 (43.3)
100	40	39 (97.5)	39 (97.5)	37 (92.5)	33 (82.5)	26 (65.0)
150	50	46 (92.0)	46 (92.0)	45 (90.0)	35 (70.0)	24 (48.0)
200	40	38 (95.0)	36 (90.0)	32 (80.0)	18 (45.0)	13 (32.5)
250	50	47 (94.0)	47 (94.0)	37 (74.0)	19 (38.0)	11 (22.0)
300	50	47 (94.0)	41 (82.0)	34 (68.0)	18 (36.0)	12 (24.0)
350	50	44 (88.0)	42 (84.0)	32 (64.0)	12 (24.0)	6 (12.0)
400	50	46 (92.0)	41 (82.0)	26 (52.0)	13 (26.0)	5 (10.0)
500	40	40 (100)	39 (97.5)	22 (55.0)	7 (17.5)	5 (12.5)

Table XIII. Survival to Selected Endpoints of BLU:Ha (ICR) Mouse Embryos Irradiated Between 9 and 10 Hours After Cleavage to the Four-Cell Stage. (Numbers in parentheses give percentage development to that stage.)

Exposure (Roentgens)	Embryos	Number Reaching Morula	Number Initiating Cavitation	Number Reaching Blastocyst	Number Initiating Hatching	Number Completing Hatching
0	110	110 (100)	110 (100)	110 (100)	105 (95.5)	89 (80.9)
25	60	60 (100)	60 (100)	60 (100)	54 (90.0)	45 (75.0)
50	60	60 (100)	60 (100)	60 (100)	56 (93.3)	44 (73.3)
75	60	60 (100)	60 (100)	60 (100)	54 (90.0)	46 (76.7)
100	60	60 (100)	60 (100)	57 (95.0)	42 (70.0)	29 (48.3)
150	60	60 (100)	60 (100)	60 (100)	48 (80.0)	30 (50.0)
200	50	50 (100)	49 (98.0)	41 (82.0)	34 (68.0)	23 (46.0)
250	60	60 (100)	59 (98.3)	58 (96.7)	37 (61.7)	24 (40.0)
300	60	57 (95.0)	55 (91.7)	46 (76.7)	25 (41.7)	17 (28.3)
350	60	59 (98.3)	56 (93.3)	46 (76.7)	24 (40.0)	16 (26.7)
400	60	60 (100)	58 (96.7)	38 (63.3)	22 (36.7)	13 (21.7)
500	50	50 (100)	44 (88.0)	21 (42.0)	2 (4.0)	2 (4.0)
600	70	64 (91.4)	50 (71.4)	18 (25.7)	4 (5.7)	3 (4.3)

Table XIV. Survival to Selected Endpoints of BLU:Ha (ICR) Mouse Embryos Irradiated at the Eight-Cell Stage. (Numbers in parentheses give percentage development to that stage.)

Exposure (Roentgens)	Embryos	Number Reaching Morula	Number Initiating Cavitation	Number Reaching Blastocyst	Number Initiating Hatching	Number Completing Hatching
0	80	80 (100)	80 (100)	80 (100)	60 (75.0)	50 (62.5)
50	50	50 (100)	50 (100)	48 (96.0)	34 (68.0)	22 (44.0)
75	50	50 (100)	50 (100)	43 (86.0)	30 (60.0)	22 (44.0)
100	50	50 (100)	49 (98.0)	41 (82.0)	28 (56.0)	14 (28.0)
150	50	50 (100)	50 (100)	44 (88.0)	24 (48.0)	13 (26.0)
200	50	50 (100)	48 (96.0)	31 (62.0)	13 (26.0)	6 (12.0)
300	50	50 (100)	50 (100)	36 (72.0)	12 (24.0)	5 (10.0)
450	50	50 (100)	46 (92.0)	26 (52.0)	1 (2.0)	1 (2.0)
600	50	50 (100)	43 (86.0)	15 (30.0)	1 (2.0)	0 (0.0)
750	60	60 (100)	53 (88.3)	17 (28.3)	0 (0.0)	0 (0.0)

Table XV. A Comparison of Development of the Controls from All Four-Cell Groups. (Numbers in parentheses give percentage development to that stage.)

Group	0-2 Hours	2-4 Hours	4-6 Hours	9-10 Hours
Embryos	60	50	70	110
Number to Morula	60 (100)	50 (100)	68 (97.1)	110 (100)
Number Initiating Cavitation	59 (98.3)	50 (100)	68 (97.1)	110 (100)
Number Forming Expanded Blastocyst	59 (98.3)	50 (100)	67 (95.7)	110 (100)
Number Initiating Hatching	58 (96.7)	48 (96.0)	67 (95.7)	105 (95.5)
Number Completing Hatching	47 (78.3)	41 (82.0)	57 (81.4)	89 (80.9)

Table XVI. Logistic Analyses of Development to the Morula Stage

Time of Irradiation	Logistic Estimate of E.D. ₅₀ Roentgen Exposure	Standard Error of Estimate (Roentgens)	Slope of Logistic Curve
2-cell (late)	688.68	75.54	-0.0346
4-cell 0-2 Hours	1090.55	478.25	-0.0034
4-cell 2-4 Hours	1954.19	1942.91	-0.002
4-cell 4-6 Hours	9051.49	44760.47	-0.0032
4-cell 9-10 Hours	n.a.	---	---
8-cell	n.a.	---	---

Table XVII. Logistic Analyses of Development through Initiation of Cavitation

Time of Irradiation	Logistic Estimate* of E.D. ₅₀ (Roentgen Exposure)	Standard Error of Estimate (Roentgens)	Slope of Logistic Curve	Fiducial Limits of E.D. ₅₀ Value (95%)
2-cell (late)	309.29 ^a	10.68	-0.0078	288.4-330.2
4-cell 0-2 Hours	306.67 ^a	14.68	-0.0110	278.3-335.0
4-cell 2-4 Hours	512.92 ^a	46.85	-0.0060	421.1-604.8
4-cell 4-6 Hours	1047.05 ^{ab}	375.67	-0.0027	310.7-1783.4
4-cell 9-10 Hours	753.93 ^a	62.07	-0.0066	632.3-875.6
8-cell	1456.37 ^b	432.84	-0.0025	608.0-2304.7

* The values followed by the same letter are not significantly different $p < 0.05$ by lsd (Steel and Torrie, 1960).

Table XVIII. Logistic Analyses of Development to the Expanded Blastocyst Stage

Time of Irradiation	Logistic Estimate* of E.D. ₅₀ (Roentgen Exposure)	Standard Error of Estimate (Roentgens)	Slope of Logistic Curve	Fiducial Limits of E.D. ₅₀ Value (95%)
2-cell (late)	179.50 ^a	10.06	-0.0082	160.1-199.5
4-cell 0-2 Hours	150.45 ^a	10.79	-0.0126	129.3-171.6
4-cell 2-4 Hours	268.37 ^b	15.52	-0.0077	237.9-298.8
4-cell 4-6 Hours	481.02 ^c	34.46	-0.0058	413.5-548.6
4-cell 9-10 Hours	468.24 ^c	17.14	-0.0077	434.6-501.8
8-cell	472.51 ^c	29.16	-0.0043	415.4-529.7

* The values followed by the same letter are not significantly different $p < 0.05$ by lsd or Tukey procedure (Steel and Torrie, 1960).

Table XIX. Logistic Analyses of Development through Initiation of Hatching

Time of Irradiation	Logistic Estimate of E.D. ₅₀ (Roentgen Exposure)	* lsd	** Tukey	Standard Error of Estimate (Roentgens)	Slope of Logistic Curve	Fiducial Limits of E.D. ₅₀ Value (95%)
2-cell (late)	61.31	a	a'	10.29	-0.0125	41.1-81.5
4-cell 0-2 Hours	36.86	b	a'	11.73	-0.0154	13.9-59.9
4-cell 2-4 Hours	124.77	c	b'	10.49	-0.0130	104.2-145.3
4-cell 4-6 Hours	234.35	d	c'	14.31	-0.0071	206.3-262.4
4-cell 9-10 Hours	302.76	e	d'	11.89	-0.0089	279.5-326.1
8-cell	199.28	f	c'	14.20	-0.0109	171.5-227.1

* The values followed by the same letter are not significantly different $p < 0.05$ by lsd procedure (Steel and Torrie, 1960).

** The values followed by the same letter are not significantly different $p < 0.05$ by Tukey procedure (Steel and Torrie, 1960).

Table XX. Logistic Analyses of Development through Completion of Hatching from the Zona Pellucida

Time of Irradiation	Logistic Estimate of E.D. ₅₀ (Roentgen Exposure)	* lsd	** Tukey	Standard Error of Estimate (Roentgens)	Slope of Logistic Curve	Fiducial Limits of E.D. ₅₀ Value (95%)
2-cell (late)	Below sensitivity -10.06	a	a'	22.92	-0.0101	-55.0-34.9
4-cell 0-2 Hours	Below sensitivity -32.41	a	a'	30.69	-0.0112	-92.0-27.7
4-cell 2-4 Hours	20.61	a	a'	24.16	-0.0088	-26.7-68.0
4-cell 4-6 Hours	143.23	b	b'	17.89	-0.0062	108.2-178.3
4-cell 9-10 Hours	239.83	c	b'	12.85	-0.0081	214.6-265.0
8-cell	116.23	b	b'	15.38	-0.0109	86.1-146.4

* The values followed by the same letter are not significantly different $p < 0.05$ by lsd procedure (Steel and Torrie, 1960).

** The values followed by the same letter are not significantly different $p < 0.05$ by Tukey procedure (Steel and Torrie, 1960).

Figure III. Preimplantation Survival of Embryos
Irradiated at Various Stages of Development

develop and it is chance as to whether a decidual reaction will be elicited by the embryo. Brenneke (1) first reported that the number of implantation sites exceeded the number of normally cleaving embryos following irradiation and he postulated that abnormally cleaving embryos (radiation damaged) could implant in the uterine wall (induce decidua formation).

The initiation of the process of blastocoel formation or cavitation was found to be more sensitive to irradiation. Two-cell and early four-cell embryos were sensitive, while later four-cell embryos and eight-cell embryos were less sensitive to this developmental arrest before initiation of cavitation. This may indicate some process occurring in the four-cell stage affects the subsequent ability of the embryo to initiate the process of cavitation.

Formation of an expanded blastocyst required both initiation of blastocoel formation and maintenance of the enlarged blastocyst after its formation. Maintenance of the expanded blastocyst in the expanded condition was shown to be a property of the blastocyst not requiring new RNA synthesis or protein synthesis (10). The initiation of cavitation, however, requires previous synthesis of RNA (11). The findings of this investigation were that the formation of an expanded blastocyst was less radiosensitive than the maintenance of the blastocyst in the expanded condition. The radiation response (failure to form an expanded blastocyst) decreases with time of irradiation following cleavage to the four-cell stage, at least for the first six hours. After that decrease in the radiation response, the sensitivity remains approximately constant. This may mean

that the RNA synthesis necessary for synthesis of proteins involved in the maintenance of the expanded blastocyst occurs during the first six hours of the four-cell stage. The radiation responses of the late two-cell and early four-cell stage embryos are similar (sensitive). This indicates that damage to the DNA at these times would be manifested as depressed maintenance of the blastocyst in the expanded condition.

Hatching of the cultured mouse blastocyst from the zona pellucida was also found to show a variation in radiation response when irradiation was delivered at different times of the four-cell stage. The response curve shows that the pattern of radiosensitivity was the same for initiation of hatching and the completion of hatching from the zona pellucida. Therefore, both processes are considered in this report to be manifestations of the same genetic activity. Any differences between the initiation of the hatching process and completion of the escape from the zona pellucida were assumed to have been the result of the suboptimal (amino acid deficient) culture medium for studying hatching. Spindle and Pedersen (12) have shown that amino acids are necessary for optimal hatching of the embryo from the zona pellucida.

The response patterns of the hatching processes were different from the patterns associated with blastocyst formation and maintenance. The time of greatest radiosensitivity was the earliest part of the four-cell stage which was irradiated and the part of the least radiosensitivity was the latest part of the four-cell stage which was irradiated. If, as Graham (13) has claimed, cleavage after the four-cell stage determines the cell that will give rise to the inner cell mass, and the inner cell mass

is responsible for the process of hatching, as has previously been proposed (14,15). The findings presented here indicate that, as the genome of the blastomere destined to become the inner cell mass is completed (in the four-cell stage), the ability of irradiation to inhibit the hatching process is decreased. After cleavage to the eight-cell stage, the radioresistance drops again. This may mean that processes occurring during the eight-cell stage also influence the process of hatching, possibly by some interaction with the process or factor that occurs during the four-cell stage. Wilson (14) and Cole (15) have postulated that some inductive mechanism in the inner cell mass may cause the trophoblastic cells to weaken the zona pellucida at the point of initial fracture in the hatching.

Russell and Montgomery (6) found a fivefold difference in radiosensitivity of embryos irradiated at times differing by as little as four hours. The results presented here confirm their findings. Depending on endpoint selection, a three- to seven-fold difference in radiosensitivity was demonstrated in embryos irradiated within two hours of cleavage to the four-cell stage when compared with embryos irradiated four to six hours after the cleavage to the four-cell stage. From the general pattern of response, the least sensitive time to induction of developmental arrest was the middle of the four-cell stage cell cycle or the time of greatest incorporation of labeled thymidine into the nuclear DNA of the embryonic blastomeres. (See Section A.).

IV. Summary

The developmental progression through morula, beginning of blastocoel

formation, blastocyst maintenance in the expanded condition, initiation of hatching and completion of hatching was observed for embryos irradiated at the two-cell stage, four different times during the four-cell stage and at the eight-cell stage. Data obtained was analyzed using a logistic model to describe the radiation induced developmental arrest of the mouse embryos cultured in vitro. From this logistic model, various relative radiosensitivities were determined for irradiation delivered to different numbers of cells and for irradiation delivered at various times during a specific cell stage. A qualitative evaluation of cleavage delay was made by assessing the dose dependent reduction in cell number (relative to control values) at a specific time in the developmental sequence monitored. The results obtained were tentatively explained in terms of known or postulated molecular events that occur or have been postulated to occur during the preimplantation period of development of the mouse embryo.

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C. Studies of Allophenic Embryos Following Irradiation of One of the
Fused ^{mouse} Embryos.

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I. Introduction

Genetic mosaicism in mice can be produced by the fusion of two embryos during the cleavage period of the fertilized egg (1). The blastomeres of the eggs can be distinguished from each other by labeling one with tritiated thymidine (see Section A). The relative contribution of each embryo to the resulting blastocyst can be determined by autoradiography following slide preparation. When one of the embryos is irradiated prior to fusion, it is possible to determine the effect of this irradiation on the contribution of the embryo to the blastocyst. To date, only a preliminary experiment has been completed in which embryos were labeled, irradiated, and their contribution to the blastocyst assessed.

II. Methods and Materials

Embryos were collected 50 to 52 hours after the second hormone injection in the superovulation sequence as previously described (see Section B). Three hours after embryo collection all four-cell embryos were randomly assigned to one of two groups. One group was placed in labeling Mintz medium and the other group was placed in the Mintz medium without radioactive thymidine (For composition see Table II). After one hour in these media, the embryos were washed in culture medium (Table I), placed in culture and allowed to develop overnight (to the eight-cell stage).

At 20 hours after the labeling, half of each group of 8-cell embryos

were irradiated with 500 R of X-rays from a 280 kVp Picker X-ray machine with copper and aluminum parabolic filters. Controls were sham irradiated. After irradiation, each group of embryos were separately stripped of their zona pellucida with pronase by the method of Mintz (2). Fusions were then accomplished by placing the pairs of embryos to be combined in a Phytohemagglutinin (PHA) solution (3). After about 10 minutes in the PHA, the fusion was accomplished by pushing the embryos together with glass rods. The fused embryos were washed and transferred to fresh culture medium and allowed to develop for an additional 48 hours (to the blastocyst stage).

Slides were made from the fusion products by the air drying method of Tarkowski (4). Autoradiographs were prepared as previously described (See Section A). Blastomere nuclei with developed grains above them were scored as labeled if the number of grains exceeded the number above a equally sized portion of the cytoplasm.

III. Results

Before the radiation experiment was started techniques were standardized. The success rate on twelve trials was 90% survival ($227/251$) for 48 hours following fusion. In the radiation experiment, 96% ($44/46$) continued to develop for 48 hours.

The radiation experiment resulted in five different groups of fused blastocysts:

- a.) 1 labeled and irradiated + 1 unlabeled and unirradiated
- b.) 1 labeled and unirradiated + 1 unlabeled and irradiated
- c.) 2 labeled and irradiated
- d.) 2 labeled and unirradiated
- e.) 2 unlabeled and unirradiated

Table XXI

Effects of Radiation on Allophenic Embryos

Experimental Group	Number of Embryos	Mean Number of Cells per Embryo	Percent Labeled Cells	Percentage of Cells in the Blastocyst from Irradiated Embryo
a.	3	55 ± 3	30	30
b.	2	47 ± 16	61	39
c.	3	32 ± 6	100	-
d.	11	64 ± 14	100	-
e.	3	53 ± 8	0	-
a & b	5	52 ± 10	-	33

A summary of the preliminary results is given in Table XXI.

IV. Discussion

Conclusions for these results must be very tentative, but it appears that the irradiated embryos are contributing fewer cells to the blastocyst than are the unirradiated embryos. It was not apparent from these preparations whether the irradiated embryos were contributing to the inner cell mass of the blastocyst and thus the precursor of the fetus. To determine this will require reimplantation of the irradiated embryos, or postimplantation development in vitro.

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