AN ABSTRACT OF THE THESIS OF

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Title: THE INFLUENCE OF IONIZING RADIATION, PHOTOPERIOD, AND ENVIRONMENTAL TEMPERATURE ON CELL PROLIFERATION IN THE INTESTINAL EPITHELIUM OF THE ROUGH-SKINNED NEWT (TARICHA GRANULOSA).

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Kinetics of cell division in the intestinal epithelial proliferative cells (cell nests) of the rough-skinned newt, Taricha granulosa, were studied using tritiated thymidine autoradiography and the mitotic arresting properties of colcemid. Percent labeled mitoses (PLM) curves were drawn from the autoradiographic data from two separate experiments in which the newts were maintained at room temperature (22-23°C). In those experiments, groups of newts were intraperitoneally injected with tritiated thymidine at times ranging between 5 and 58 hours before they were killed. Each newt in both experiments was intraperitoneally injected with colcemid five hours before it was killed.

Data from both experiments were very similar and the PLM curves were used to estimate cell cycle phase durations in the cell nests. The DNA synthetic phase was estimated to be 41 hours long and the sum of the G_2 phase duration and one-half the duration of mitosis was

approximately seven hours. The duration of the entire cell cycle was considered to be longer than 100 hours and the duration of the G_1 phase longer than 50 hours.

Extremely low mitotic indices in the intestinal cell nests of newts maintained at low environmental temperatures (4°C) and in newts which had been exposed to 1000 R X-irradiation precluded attempts to analyze the cell cycles in those animals. Studies were initiated, however, to characterize the effect of environmental temperature, photoperiod, and X-irradiation on the mitotic index of the cell nests.

In two separate experiments, groups of newts acclimated to room temperature were killed, five hours after intraperitoneal injection with colcemid, at three-hour intervals over a 24-hour period. A diurnal periodicity in the mitotic activity within the intestinal cell nests was present; peak and nadir mitotic indices occurred in those animals killed in early afternoon hours and in early morning hours, respectively.

An environmental temperature of 4°C with a light-dark cycle resulted in diminished mitotic activity in the intestinal cell nests. There was more than a two-fold difference between overall mitotic indices and the mitotic indices at room temperature. With an environmental temperature of 4°C and complete darkness, the overall mitotic index was nearly four-fold less than it was at the same temperature with a light-dark cycle.

Newts subjected to a dark and cold (4°C) environment for 2 months were warmed to room temperature over a two-day period and the mitotic indices in their intestinal cell nests were determined over a 27-hour period thereafter. A diurnal periodicity was evident with a peak mitotic index at 1600 hours. Intestinal cell nest mitotic activity was significantly greater than in newts acclimated to room temperature.

Ionizing radiation had a disruptive effect on mitotic activity in the intestinal cell nests of newts. An intraperitoneal injection of tritiated thymidine was determined to have an initial, slightly depressive effect on mitotic activity in the cell population but the mitotic index returned to normal or slightly higher levels within the 27-hour period.

Whole-body exposure of newts to 100 R X-irradiation essentially stopped mitotic activity in the intestinal cell nests for 32 hours and exposure to 200 R stopped the mitotic activity for 56 hours. Recovery of mitotic activity in the cell nests was in evidence in data from both groups of animals after both those time periods. Whole-body exposure of newts to 1000 R X-irradiation severely depressed mitotic activity in the intestinal cell nests for at least 78 hours and resulted in diminished numbers of the cell nests although the mature cells of the intestinal cells appeared to be relatively radioresistant.

The Influence of Ionizing Radiation, Photoperiod, and Environmental Temperature on Cell Proliferation in the Intestinal Epithelium of the Rough-Skinned Newt (*Taricha granulosa*)

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INTRODUCTION

General Remarks

Research concerning the kinetics of cell proliferation began with the observation by Howard and Pelc (1953) that DNA synthesis occurred within a limited period during interphase in the reproductive cycle of cells. Since that time, many refinements have been made in analyzing cell cycles and the kinetics of many normal cell populations as well as tumors have been characterized.

The study of the kinetics of cellular proliferation is currently being most actively applied to growth characteristics of tumors in order to take advantage of knowledge that cells are more susceptible to therapeutic agents during some phases of the cell cycle than others (Pelc and Howard, 1955; Lajtha et al., 1958a, 1958b; Painter and Robertson, 1959; Dewey and Humphrey, 1962; Terasima and Tolmach, 1963). Cell cycle data for tumors and for normal tissues will be necessary for effective formulation of therapeutic doses and schedules (Frindel and Tubiana, 1971; van Putten, 1974). The importance of protecting normal proliferative cells by considering their cell cycle status in the application of therapy schedules was stressed by Frindel (1975), Maruyama, Lee and McMillin (1975), and Valeriote (1975).

Several Japanese researchers have demonstrated X-ray induced and temperature-dependent variations in cycle time in intestinal epithelial cells of fish, and this investigation was designed to reveal whether similar temperature and radiation effects could be observed in the intestines of urodele amphibians. Hyodo (1965a) and Hyodo-Taguchi and Egami (1969) concluded that both survival times and intestinal epithelial morphologic changes in fish after whole-body X-irradiation were temperature dependent. Intestinal epithelial cell labeling with tritiated thymidine was shown to be considerably slower at 4°C than at 22°C (Hyodo, 1965b), and Hyodo-Taguchi (1970) demonstrated that radiation-induced perturbations of parts of the intestinal epithelial cell cycle in fish were also temperature dependent. Recovery by fish from split-dose whole-body radiation, as manifested by cellular repopulation of intestinal epithelium and hematopoietic organs, was shown to be slowed by lower environmental temperatures (Egami and Etoh, 1966; Etoh and Egami, 1967; Egami, 1969).

Originally, the objective of this study was to determine the duration of the cell cycle phases in cells of the proliferative compartment of the newt intestine and to observe any changes in the cell cycle which could be induced by ionizing radiation or by changes in environmental temperature. Taricha granulosa was chosen for these experiments because it is easily maintained in captivity and the intestinal morphology and epithelial cell replacement mechanisms in urodele amphibians are unique. Interesting diurnal

fluctuations in mitotic indices of the intestinal cells were observed during preliminary studies, so the protocol was expanded to include an investigation of the effects of physical stimuli on the diurnal mitotic rhythm. The results of this investigation, therefore, include (1) a cell cycle analysis of the proliferative cells in the intestine of the newt at 22°C, (2) an analysis of the diurnal rhythm of the mitotic and DNA synthesis indices at 22°C, and (3) an analysis of the changes in the mitotic index rhythm induced by changes in environmental temperature and the light-dark cycle and by X-irradiation.

Kinetics of Cell Division

Existing literature concerning the kinetics of cell division, both in vitro and in vivo, is very extensive and quite varied in scope. The following discussion includes those literature citations which are pertinent to the methods or the results of this investigation.

The Cell Cycle

The life cycle of a cell (cell cycle) includes events that occur between the midpoint of the mitotic division from which a cell originates to the midpoint of its mitotic division into two daughter

cells; the time interval between those points is defined as the generation time (Baserga, 1971). The following includes a brief discussion of the cell cycle as well as definitions of terms used in this study and the symbols that will be used for each term. They were used by Lipkin (1971) and, with various modifications, are found throughout the literature concerning cell cycle analysis.

The cell cycle is divided into four phases: (1) mitosis, M, during which division of the cell occurs, (2) a postmitotic and pre-DNA synthesis time interval called G_1, (3) S phase, during which DNA synthesis occurs, and (4) a post-DNA synthesis and premitotic time interval called G_2 (Howard and Pelc, 1953). Symbols which will be used to indicate the duration of these four phases are T_M, T_{G1}, T_S, and T_{G2}, and the sum of these intervals (generation time or cell cycle time) will be indicated by T_C. Three additional parameters for which symbols will be used throughout this thesis are the thymidine labeling index (LI), the mitotic index (MI), and percent labeled mitoses (PLM). The thymidine labeling index is defined as the decimal fraction of a cell population which has incorporated tritiated thymidine (^{3}H-TdR) during a given time period as shown by histological autoradiography. The mitotic index is the decimal fraction of a cell population that was undergoing mitosis at the moment of tissue fixation. The percent labeled mitoses is the decimal fraction (X 100) of cells in mitosis which have incorporated tritiated thymidine prior to mitosis (Lipkin, 1971).

The term "cell nest(s)" (Patten, 1960) will be used in reference to the intestinal epithelial proliferative cells of the newt. Each animal constitutes an individual observation and a sample mean mitotic (or labeling) index describes the mean index from the cell nests of a group of individual animals killed at a specified time. The term "set of mean mitotic (or labeling) indices" refers to all the sample mean indices from an experiment, and the overall mitotic (or labeling) index is the arithmetic mean of the set of mitotic (or labeling) indices.

Mitotic Inhibition by Colchicine and Colcemid

The alkyloid, colchicine, was isolated during the nineteenth century from the corm, seeds, and the flowers of the Autumn crocus, Colchicum autumnale. Its cytological effects have only been elucidated, however, since the third decade of the twentieth century. Ludford (1945), in his review on the mode of action of the drug, described the research which led to the identification of its mitosis-inhibiting properties. He also cited empirical evidence that colchicine inhibits mitosis by inhibiting formation of the mitotic spindles. Puck and Steffen (1963) presented experimental results from which they concluded that colcemid, a derivative of colchicine, blocks only cells that enter mitosis after its addition

to a culture of HeLa cells. They also concluded that no part of the cellular life cycle except the metaphase-anaphase period was affected by the drug.

More specific investigations concerning the kinetics of mitotic arrest by colchicine and its derivative, colcemid, were made by Taylor (1965), Hampton (1966), and Kleinfeld and Sisken (1966). Taylor (1965) used tritium-labeled colchicine with cultures of human cells in his experiments. As a result of his study, he postulated the existence of a set of cellular sites to which colchicine is bound. Correlation of intracellular radioactivity and the appearance of blocked mitoses led him to suggest that complexing of three to five percent of those sites in a cell was sufficient to arrest mitotic activity. The inhibition of mitosis by colchicine is of limited duration and he suggested that this was due to the reversibility of the binding reaction. The reversibility of the mitotic arrest by colcemid, a derivative of colchicine, was documented by Kleinfeld and Sisken (1966) with the use of time-lapse cinemicrographic techniques on cultured HeLa cells. They found that cells in which the mitotic division had been blocked for up to five hours were able to resume the process after removal of the drug. Their observations indicated no effect on cells during interphase, a normal progress through prophase, but mitotic arrest during metaphase due to the formation of deformed or shrunken mitotic spindles. The reversibility of colcemid-induced mitotic arrest in mouse thymic

cells was demonstrated by Borum (1973). She also noted that prolonged treatment with the drug resulted in death of the cells. Hampton (1966) likened the mitotic arresting properties of colchicine to those of a 3 Krad X-ray exposure. He observed, however, that many of the radiation-induced changes in intestinal epithelial crypts were not present in animals injected intraperitoneally with a colchicine dose at 0.04 mg/g body weight. The distribution of ^{14}C-labeled colchicine in organs of mice four hours after subcutaneous administration was investigated by Back, Walaszek and Uyeki (1951). They reported high concentrations of radioactivity in the spleen and kidneys; lower concentrations in the intestines and liver; and no radioactivity in the blood, brain, muscle, and heart.

Tritiated Thymidine Incorporation and Metabolism

Nearly all recent investigations of cellular kinetics include the use of tritiated thymidine (^{3}H-TdR) as a biochemical label for the analysis of DNA metabolism. Comprehensive reviews have been published on the subjects of thymidine metabolism (Cleaver, 1967) and tritium labeling of molecules (Feinendegen, 1967), although the behavior of tritiated thymidine in vivo has not yet been completely characterized.

One of the first studies showing the incorporation of thymidine into DNA was performed by Reichard and Eastborn (1951). They used ^{15}N-labeled thymidine injected into rats with subsequent analysis of purines and pyrimidines from DNA and RNA to show the DNA specificity of thymidine incorporation. About the same time, Howard and Pelc (1951) demonstrated ^{32}P incorporation into nuclei of cells of Vicia faba seedlings grown in solutions of $NaH_2{}^{32}PO_4$.

Friedkin, Tilson and Roberts (1956) used ^{14}C-labeled thymidine in an investigation with chick embryos. They showed the DNA specificity of the thymidine precursor and also used thymidine-^{14}C uptake to measure the rate of DNA synthesis in the embryos. One of the first uses of tritium-labeled thymidine was by Taylor, Woods and Hughes (1957) in which chromosomes duplicating in the presence of tritiated thymidine were shown, by autoradiography, to have incorporated the label.

Exogenous thymidine is apparently incorporated into DNA following a series of phosphorylations resulting in thymidine monophosphates, diphosphates, and triphosphates (Friedkin, 1959; Jacobson and Prescott, 1964). The thymidine triphosphate loses two phosphoric acid groups and, with three other deoxynucleotides, is incorporated into the DNA molecule (Baserga and Malamud, 1969). Thymidine not incorporated into the DNA molecule is catabolized into nonutilizable products which, with nonincorporated thymidine,

is fixative soluble and is removed from the tissue by subsequent histotechnical processes (Baserga and Kisieleski, 1963).

Extensive reviews of the use of tritiated thymidine in in vitro systems have been written. The book by Mitchison (1971) contains information on methods and results with prokaryotic, lower eukaryotic, and mammalian cells; while the chapter by Nachtwey and Cameron (1968) includes detailed methods for use with eukaryotic cells. Methodology for use with exponentially growing mammalian cell cultures was presented by Stanners and Till (1960) and includes mathematical treatment of data. Other in vitro cellular kinetics investigations will be reviewed when their results are considered appropriate to this literature survey.

Among the first in vivo cellular proliferation studies in which tritiated thymidine was employed were those by Hughes et al. (1958); Leblond, Messier and Kopriwa (1959); MacDonald and Mallory (1959); Cronkite et al. (1959); and Pelc (1959). Those investigators all listed limitations of the methods as well as possible further uses of tritiated thymidine in cell kinetics studies. Cronkite et al. (1959) listed a number of basic assumptions regarding the use of tritiated thymidine including the following:

1. There is no exchange of the tritium label on thymidine.
2. Once incorporated into DNA, the thymidine base is not exchanged.
3. DNA turnover results only from cell death or from mitosis.

4. The reutilization of large portions of the DNA molecule is not likely.
5. The reutilization of tritium-labeled material is not significant.
6. Once DNA synthesis has occurred in a cell, the cell is destined for division.
7. The distribution of tritiated thymidine throughout the body is initially uniform and it is either incorporated into DNA or degraded.
8. Tritiated thymidine is available for only a fraction of the total DNA synthesis time.
9. No radiation injury to the cells occurs from the tritium label in their DNA fraction.

The results of a number of investigations have been published since the list was proposed and many of the above assumptions have been shown to be valid.

The DNA specificity of thymidine was shown rather conclusively by Baserga and Kisieleski (1963) although Bryant (1966) presented evidence that tritium from ^{3}H-TdR was incorporated into proteins of the mouse. The origin of the protein-incorporated tritium was postulated to be the catabolic products of ^{3}H-TdR (Bryant, 1966) and the effect on subsequent autoradiography has been termed minimal (Baserga and Malamud, 1969).

The question of whether or not the radioactive label of DNA is available for reutilization after the catabolism of labeled DNA was discussed by Hamilton et al. (1959). Evidence indicates that the catabolic products, including thymidine, are reutilized. Diderholm, Fichtelius and Linder (1962) determined that cells of skin grafts received by a host two days after a series of three daily tritiated thymidine injections did become labeled. Bryant (1963) reported the reutilization of DNA thymidine from carbon tetrachloride necrotized liver cells by intestinal cells and spermatogonia of mice. Thymidine reutilization was also reported to have occurred in the bone marrow and spleen beginning one day after a single intravenous injection and in the thymus three days postinjection. It was suggested that the salvage pathway is largely localized to single organs of the hematopoietic system, however, and that this contribution is not significant in autoradiographic results (Feinendegen et al., 1973).

Nuclear labeling by DNA precursors in excess of that necessary for division by mitosis (Pelc, 1958; 1959; 1971; Pelc and Appleton, 1965) has led to a hypothesis concerning the existence of metabolic DNA. According to Pelc (1971, 1972), this metabolic DNA may be synthesized within and lost from the nucleus of a cell without a change in the amount of genetic DNA in the cell. It is considered responsible for the incorporation of ^{3}H-TdR into the nuclei of

cells showing no mitoses and for the excess labeling of mitotic cells. The false labeling would complicate experimental results in which the synthesis index and grain counts (discussed later) are used as parameters (Pelc, 1971). Evidence refuting the existence of metabolic DNA was presented by Burholt, Schultze and Maurer (1973). In mouse jejunal crypt cells, at least, all cells which incorporated tritiated thymidine eventually underwent mitotic division.

Evidence of late incorporation of labeled DNA precursors into DNA (i.e., up to 48 hours after ^{3}H-TdR injection) led Potten (1971, 1973) to postulate the existence of a long-lived DNA precursor pool in the mouse skin. That precursor pool was considered to be connected with stimulus-responsive cells, called G_o cells (discussed later) specific to certain tissues of the body. The G_o cells were thought to accumulate the DNA precursors due to differences in their cell membranes (Potten, 1971).

That tritiated thymidine is available for incorporation into essentially all cells after in vivo administration has been documented (Leblond, Messier and Kopriwa, 1959; Rubini et al., 1960; Pelc and Appleton, 1965). The site of administration of the ^{3}H-TdR was considered important, and Pelc and Appleton (1965) favored intravenous injection over intraperitoneal injection, especially if grain counts over labeled nuclei were to be made.

Potten, Hagemann and Reiland (1972) have reported inconsistencies in ^{3}H-TdR incorporation into DNA in mice. They indicated that two to six percent of the mice they injected did not uniformly incorporate the DNA precursor into the nuclei of all tissues.

The time of availability of labeled DNA precursors in vivo has been debated. The time period between injection and degradation by the liver of labeled thymidine was considered to be approximately one hour (Leblond et al., 1959). This was the same value given for ^{3}H-TdR availability in man (Cronkite et al., 1959) and labeling of proliferative cells of human bone marrow was found to be nearly complete within ten minutes (Rubini et al., 1960). Labeling of the intestinal epithelium of mice was considered complete in 10 to 20 minutes (Quastler and Sherman, 1959) after intraperitoneal injection. In mammalian systems, a single injection is generally considered a "pulse label" of 30 to 40 minutes duration (Thrasher, 1966; Baserga and Malamud, 1969).

Whether or not the ^{3}H-TdR incorporated into the DNA molecules of living cells is hazardous to the cells is still a controversial issue. Hughes et al. (1959) reported chromosomal defects caused by the label if high specific activity (860 Ci/mole) ^{3}H-TdR was used. Cronkite et al. (1959), however, reported no chromosomal abnormalities with doses sufficient to produce adequate autoradiographs. Recently, Payne and Shaw (1971) reported a radiation effect from ^{3}H-TdR if 88 μCi was orally administered in mice. The

effect was in the form of reduced label incorporation into the DNA of proliferating cells of the intestine and testes. They considered the effect similar to that from whole-body exposure to 25 R of X-irradiation. Based upon cinemicrography on human epithelial cancer cells in vitro, Kasten (1975) described the following morphologic effects of excess tritiated thymidine on cells: increase in cell size, small, dense nucleoli, and increased activity of cellular membranes.

Histological Autoradiography

Radioactive labels incorporated into cells, can be detected by liquid scintillation spectrometry and by autoradiography. Autoradiography offers the best means of specific localization of the radioactive label at the cellular level.

Two relatively recent reviews were written by Baserga (1967) and Baserga and Malamud (1969) on histological autoradiography as a means of locating radioactive material incorporated into tissues. Both reviews presented comprehensive information about tissue processing, photographic emulsions used, and techniques which could be employed.

Although the photochemical processes occurring in the photographic emulsion during exposure and during subsequent development

are beyond the scope of this paper, the following is a brief summary of the processes as discussed in the two above-mentioned reviews. Energy from the ionizing radiation or from light in photography produces a "latent image" (i.e., aggregate of reduced silver atoms) by the liberation of bromine from the silver bromide crystals in the gelatin matrix of the photographic emulsion. Subsequent immersion in a weak reducing agent (developer) reduces silver bromide ions of the emulsion, especially those immediately adjacent to the site of the latent image. The fixer bath of the final step of development removes the unreduced silver halide crystals and a transparent gelatin containing aggregations of metallic silver remains. Those aggregates show up, with the light microscope, as black "silver grains" (Baserga, 1967).

A prime concern in histological autoradiographic technique is the proximity of photographic emulsion to the tissue sections. Because latent images are formed as a result of radiation energy absorbed by silver halides in the emulsion, that is especially important for a low energy beta emitter such as tritium. Several methods and variations of each method have been used to achieve the tissue-emulsion proximity; each method has advantages and disadvantages. The stripping-film method of Pelc (1956) was criticized because of a relatively high background level and low

sensitivity, a tendency for air bubbles to become trapped between the emulsion and the section, and the relative difficulty of the method (Kopriwa and Leblond, 1962).

The technique most widely reported in recent literature is the dip-coating method which gained popularity with the commercial availability of photographic emulsions in gel form. Before photographic emulsion in gel form became available, investigators resorted to scraping the emulsion from 35 mm film and melting it as described by Belanger (1952). The dip-coating method was described in detail by Kopriwa and Leblond (1962) and was the basis for their critique of the stripping film method. Basically, the method consists of: (1) preparation of microscope slides by the usual histotechnical methods, (2) dipping the slides into melted photographic emulsion, (3) exposure in a cold, dry state, and (4) developing with photographic developing techniques. The last three steps must be performed in a dark or safe-lighted room. Three disadvantages of the dip-coating method, according to Kopriwa and Leblond (1962), were: (1) nonuniform thickness of the emulsion layer, (2) occasional artifactual silver grains along irregularities in the tissue, and (3) nonuniform sensitivity of commercial photographic emulsions. Despite those disadvantages, numerous researchers have reported routine use of that method for large numbers of autoradiographs and have submitted their versions of

the technique (Joftes, 1959; Kopriwa and Leblond, 1962; Baserga, 1967; Baserga and Malamud, 1969).

Baserga and Nemerhoff (1962) developed a dipping technique by which two layers of emulsion separated by a layer of celloidin could be applied to a microscope slide. That technique reportedly was useful for differentiating between tritium and carbon-14 labeled compounds incorporated into the same tissue. The less energetic beta particles of tritium produced latent images in the first emulsion layer and carbon-14 beta particles were detected in the second emulsion layer which was applied after development of the first.

Procedures to which the tissue sections are subjected must be chosen carefully to protect the reliability of autoradiographs. Baserga and Kisieleski (1963) reported on the effects of treatment of smears containing labeled Ehrlich ascites cells with perchloric acid, trichloro-acetic acid, deoxyribonuclease, and ribonuclease. The results of those treatments on cells labeled with tritiated thymidine, cytidine, and leucine were generally predictable in removing the cytoplasmic or nuclear label, dependent on the label and treatment. They also quantitated the amount of radioactivity removed by Carnoy's solution and methanol when used as fixatives. Each fixative removed approximately the same amount of radioactivity, and they concluded that the fixative-soluble precursor was that which had not been incorporated into proteins or nucleic acids.

Thurston and Joftes (1963) investigated in detail the types of histological stains that may be used with liquid emulsion autoradiography. They classified several stains according to suitability for use and whether they were best used before dipping, after development, or partially at both times during the procedure.

One consideration to be used in examining autoradiographs is background consisting of silver grains over cells that are not actually labeled. That background may be caused by a variety of factors including cosmic radiation; processing artifacts such as static electricity, pressure effects, and chemical artifacts; and tritium metabolized and incorporated into other compounds within cells (Quastler and Sherman, 1959). Thrasher (1966) discussed methods of background determination. He routinely used a method whereby the number of silver grains in the emulsion above nuclei, in an obviously unlabeled population of cells as well as in the progenitor population, were counted. Comparisons of the counts were made to determine the number of grains at which a cell of the progenitor population could be considered labeled. That method can be used to compensate for artifactual silver grains over nuclei. It was noted by Quastler and Sherman (1959), however, that careful technique resulting in good preparations produced a small number of background silver grains. Those were only considered a problem in cases where the label was very weak.

Another factor to be considered when using autoradiography is the identification of populations of cells in which the parameters are being measured. Leblond and Walker (1956) established that there are several distinct populations of cells in an organism and that those cells proliferate at different rates. Quastler and Sherman (1959) speculated that a population of progenitor cells could consist of two or more subpopulations of cells which, while morphologically similar, could proliferate at different rates. They pointed out that parameter values obtained by autoradiography were average values for a cell population.

Cell Cycle Phase Durations

Numerous methods for determining the duration of the phases of the cell cycle exist and were reviewed in detail by a number of authors including Leblond (1959), Baserga (1965), Thrasher (1966), and Nachtwey and Cameron (1968).

The process of mitosis has been widely studied because mitotic figures are readily observable by light microscopy if they are present in a tissue. Cinemicrographic procedures such as those described by Sisken (1964) were used to define the duration of cell cycle phases, particularly the phases of the mitotic process. If the population of cells in question can be readily defined, the percent dividing cells or mitotic index (MI) for a fixed and stained tissue or cell culture may be obtained when a

statistically significant number of cells is available for examination (Leblond and Walker, 1956). The mitotic index of a tissue is not an indicator of the duration of mitosis; However, it might be used for comparisons of proliferative activity of two or more populations of cells (Bertalanffy and Leblond, 1953; Leblond, 1959) or for comparing the effects of chemical or physical agents on cell proliferation (Leblond and Walker, 1956).

The use of colchicine allowed measurement of the daily mitotic rate in the epidermis of rats by Storey and Leblond (1951). Colchicine was administered to a group of rats which were killed six hours postinjection and enumeration of colchicine-blocked mitotic figures was done microscopically. Groups of rats were treated in that manner over four consecutive six-hour periods and the mean mitotic indices from each period were summed to produce the fraction of cells undergoing mitoses per day (epithelial renewal rate).

The same technique was used to estimate the renewal rates of cells in the lungs of rats, guinea pigs, and mice (Bertalanffy and Leblond, 1953). In that investigation, the turnover time (time for renewal of a number of cells equal to the whole population) was calculated by dividing the fraction representing the renewal rate into one. A method by which the turnover time of a cell population could be calculated was proposed by Leblond and Walker (1956). By that method, the colchicine treatment time divided by

the mitotic index at the end of that time produced a maximum estimate of turnover time. Because the turnover time is also approximately equal to the duration of mitosis divided by the normal mitotic index, a rough estimate of mitotic duration may be determined (Leblond and Walker, 1956). The relationships of parameters derived using colchicine technique were summarized by Leblond (1959) with the equation $m = \frac{Pt}{q}$ where m equals mitotic duration, P equals normal mitotic index, t equals time in hours between colchicine injection and sacrifice, and q equals the mitotic index after treatment with colchicine.

Radioactively labeled DNA precursors have also been used to estimate the duration of mitosis. The data of Howard and Pelc (1953) correlated with their estimate of the mitotic duration although their estimate apparently was not based on those data. From the percent labeled mitosis (PLM) curve, Quastler and Sherman (1959) estimated the duration of mitosis as well as the durations of the DNA synthetic (S) phase, the G_1 phase, and the G_2 phase of the cell cycle. After a pulse label of tritiated thymidine, there was a brief period during which no labeled mitotic figures were evident in autoradiographs; that cohort contained cells which were in the G_2 phase at the time of labeling. The time from administration of ^{3}H-TdR to the time at which labeled mitotic figures appeared was considered the minimum duration of G_2. From that point, the percent labeled mitoses increased at a rapid rate nearing 100 percent.

The time period during which the increase occurred was considered to be the duration of mitosis (T_M). After a time period during which nearly all of the mitotic figures were labeled, the number began to decrease, theoretically at the same rate at which the increase occurred. The time period from the end of increasing labeled mitotic figures to the end of decreasing labeled figures was considered the length of the S phase (T_S). The percent labeled mitoses after a time began to increase for a second time to a point of maximum labeled figures. The time period from the first maximum to the second was termed the generation time (T_C). The duration of the G_1 phase (T_{G1}) was calculated by subtracting the sum of T_{G2}, T_M, and T_S from T_C (Quastler and Sherman, 1959). Slightly more reproducible estimates of S phase duration were made by using the time span between the point of 50 percent labeled mitoses on the ascending leg of the curve to the corresponding point on the descending leg. The time from the administration of the label to the time when 50 percent of the mitotic figures were labeled was considered to include $T_{G2} + 1/2\ T_M$ (Lesher, Fry and Kohn, 1961; Lesher, Fry and Sacher, 1961).

Stanners and Till (1960) used essentially the same methodology to determine the cell cycle phase durations in cultured L-strain mouse cells. They determined T_{G2} using a percent labeled metaphase curve but preferred using grain counts of metaphases for determining T_S. That method was considered to be less sensitive than the PLM

curve to synchronizations within the cell culture. The number of autoradiographic silver grains over cells in metaphase would remain at zero for a time equal to the G_2 period. An increasing number of grains over those cells would occur for a time equal to the duration of the S phase. Stanners and Till also calculated T_M to be equal to MI x $T_C/0.693$ derived from the equation $MI = e^{aT_M} - 1$ where $a = 0.693/T_C$. In that case, T_C represented the culture doubling time of an asynchronous culture in exponential growth. Stanners and Till derived a complex mathematical formula relating T_S to the cell labeling index (LI). Those formulae all used the culture doubling time for T_C which, due to reproductively inactive cells in a culture, are not strictly equivalent (Nachtwey and Cameron, 1968).

Variations on the techniques discussed above exist, but most investigators use mitotic inhibiting agents and/or radioactively labeled DNA precursors. Fry, Lesher and Kohn (1961a) used the unlabeled mitotic figures as well as the labeled figures to estimate the length of generation time in the crypt cells of intestinal epithelium. They also proposed estimating mitotic time in the same cells by using the formula $T = G\bar{I}$ where T equals mitotic time, G equals generation time, and $\bar{I}$ equals the mitotic index. The same authors later proposed the formula $T = M/b$ where $b = \Delta N/t$. That was based on the relationship $Z = M \times t/T = \Delta N$.

In the above formula, Z was the total number of mitoses completed during a time period (t) and equaled the increment in labeled cells, ΔN. M was the index of labeled mitotic figures and T was the duration of mitosis. One limitation to the method was that it could only be applied for a time period (t) during which the rate of division of labeled cells was constant and no labeled cells divided. In mouse intestinal epithelial crypts, this period extended between two and seven hours after injection of tritiated thymidine (Fry, Lesher and Kohn, 1961b).

Puck and Steffen (1963) presented a method by which durations of cell cycle phases could be estimated in a culture of HeLa cells. Using simultaneous addition of tritiated thymidine and colcemid, they counted total number of mitoses, the number of labeled mitoses, and the number of labeled cells. By means of mathematical equations, they related the durations of the cell cycle phases to each other as well as to the culture doubling time (corrected for dead cells). Substituting the experimentally derived values into the equations and using graphic representations of the data enabled them to estimate the time cells spend in each phase of the cell cycle. A method combining tritiated thymidine and colcemid was used by Kollmorgen, Trucco and Sacher (1967) with cultures of Chinese hamster cells. In their experiment, they altered times between administration of tritiated thymidine and

colcemid. Their data was subjected to the mathematical treatment formulated by Puck and Steffen (1963) and the resultant cell cycle durations compared favorably with durations derived by other methods.

Double labling techniques for cell cycle analysis have also been utilized. One such method involves the use of tritiated thymidine and ^{14}C-thymidine and utilizes the dual emulsion layer autoradiographic technique previously discussed (see page 17). The duration of S phase, for example, was estimated from the relationship $H/C = \Delta t/ts$, where H equals the total number of tritium labeled cells, C equals the total number of ^{14}C-labeled cells, Δt equals the time interval between injections of the isotopes, and ts equals the S phase duration (Thrasher, 1966). Yet another double labeling study (Sharav, Brin-Erb and Sciaky, 1973) utilized two groups of animals where one group of animals received a single injection of tritiated thymidine and the other group received two successive injections with a time interval (Ti) between injections. Ts (DNA synthesis time) was derived using the equation $Ts = LI_1/(LI_2-LI_1) \times Ti$, where LI_1 equals the labeling index in tissues of singly injected animals and LI_2 equals the labeling index in tissues of animals receiving two injections. T_g (generation time) was calculated with the equation $T_g = (Ts/LI_1) \times 100$.

It has long been realized that not all cells of a population progress through the cell cycle at the same rate. Quastler and Sherman (1959) noted that the descending leg of their PLM curve was not exactly the inverse of the ascending leg. They speculated that some variability was inherent in the M and G_2 phases and estimated the standard deviation of the S phase to be 1.5 to 2 hours with an S phase duration of approximately 7.5 hours. Stanners and Till (1960) recognized individual cell variation in the lengths of the G_2 phase as indicated by the slope of the ascending percent labeled metaphase curve. Puck and Steffen (1963) also took this variance into consideration in their mathematical formulas. Measuring the generation time of cultured HeLa cells, cinemicrographically, they showed a mean generation time of 20.5 hours with a standard deviation of 1.5 hours.

Detailed analysis of the distribution of cell values for mitosis and for phases of the mitotic process were made by Odartchenko et al. (1964). Using bone marrow erythroblasts of dogs injected with tritiated thymidine as their in vivo model, they concluded that: (1) T_{G2} variation was greater than T_M variation, (2) the duration of mitosis did not differ markedly from one cell to another (35 ± 4 minutes in their model), and (3) no effect of tritiated thymidine incorporation was evident on their system. Relationships between phases of the cell cycle and distribution of phase durations were also investigated by Sisken

and Morasca (1965). They used combined tritiated thymidine and cinemicrographic techniques on human amnion cell cultures proliferating exponentially. They found the T_{G2} and prophase time distribution to be similar to that of the generation time which led the investigators to conclude that a mechanism exists whereby a cell progressing through one phase at a relatively slow rate may progress through another phase at a compensatingly faster rate.

The existence of a fifth phase of the cell cycle, G_o, was suggested by Quastler (1963). G_o was considered to be a phase outside the cell division cycle into which a daughter cell could pass immediately after it emerged from mitosis. During G_o, a "decision" was made regarding subsequent differentiation or continued proliferation of the cell (Quastler, 1963). Evidence for the G_o phase was presented in a review by Epifanova and Terskikh (1969) along with evidence for another quiescent stage of the cell cycle. The other nonproliferative phase was considered to occur after S phase and before mitosis. The same "decision" would be made for a cell at that time; but, if the cell differentiated, a mature cell with duplicated chromatin would result.

A problem exists in resolution of the G_o phase from the G_1 phase. Burns and Tannock (1970) separated the inclusive G_1 phase into G_o and "true" G_1. True G_1 was defined as the length of time that continually proliferating cells would spend in G_1. Calculation of phase durations by Burns and Tannock (1970) are based on the

assumption that T_C, T_{G1}, T_S, T_{G2}, and T_M are constant. Burns (1975) presented methods of calculating rate constants for cells progressing into G_o from the C phase (cell cycle) and out of G_o into the C phase. He included a mathematical characterization of progression of cells into a Q phase which was defined as a nonproliferative compartment (made up of cells which have lost the capacity to divide). The rate constants were then used to calculate the mean duration of the G_o phase.

Cells in the G_o phase were reported by Potten (1971) to be responsible for late incorporation of tritiated labels into mouse epidermal cells. Potten observed, however, that rapidly proliferating cell populations did not contain cells in G_o phase. That observation was confirmed for mouse jejunal crypt cells by Burholt, Schultze and Maurer (1973). Izquierdo and Gibbs (1974) suggested that, if a G_o phase exists in cells of hamster cheek pouch epithelium, it is considerably shorter than the turnover time of the cell population.

In recent years, computerization of data has become increasingly more useful. Nachtwey and Cameron (1968) presented a description of computer simulated PLM curves and computer generated correction factors to be used with cell cycle analysis data. Computerized methods of analyzing the PLM curves have been described by Barrett (1966), Steel and Hanes (1971), Steel (1972), Gilbert (1972), Ashihara (1973), and Steward (1975).

Proliferative Activity in Cell Populations

The purpose of normal cell replication by means of the cell cycle in an adult organism is the constant renewal of cell populations in which cells die or are lost. Some tissues have definitive, proliferative areas (progenitor populations) of cells; while the stem cells of other populations remain undefined. The relationships of progenitor cells to the specialized cells of a tissue were reviewed by Thrasher (1966). Those relationships in the gastro-intestinal tract will be discussed subsequently.

Groups of tissues of ectodermal, endodermal, and mesodermal origin were described by Leblond and Walker (1956) on the basis of mitotic activity observed within the tissues. In their review of the literature, the authors cited tissue turnover times ranging from 0.2 days for cat and rabbit lymphocytes to 133 days for human erythrocytes. The cell populations were classified into three groups on the basis of mitotic activity. The three groups of cell populations were (1) those with no detectable mitoses, (2) those with infrequent mitoses, and (3) those with abundant mitotic figures. The third group was divided into four classes, three of which included surface epithelium with the fourth consisting of mesenchymal tissues (Leblond and Walker, 1956). Leblond, Messier and Kopriwa (1959) classified the cell populations of mice and rats on the basis of

the labeling index observed eight hours after an injection of tritiated thymidine. They classified the cell populations into three groups: (1) stable cell populations with labeling indices of 0 to 0.2 (2) growing cell populations with labeling indices between 0.1 and 0.8 and (3) renewing cell populations with labeling indices from 1.4 to 6.7; values were listed as percents. The first group included the three types of muscle cells and neurons while the third group consisted primarily of epithelial cells.

The data used for those general classifications would not necessarily permit more specific classification. Bertalanffy (1964) noted that data for the same kind of tissue varies between species and also varies according to the technique used. A discrepancy exists, for example, in the data of Pelc (1958, 1959). Although regarding the seminal vesicle of a rat as nondividing tissue, he observed substantial incorporation of tritiated thymidine by the cells of that tissue. He attributed this excess incorporation to metabolic DNA (discussed on page 11).

Control of Cell Proliferation. The control of cell replication within a proliferating cell population is not clearly understood. Leblond and Walker (1956) speculated about factors that influenced mitotic activity in cell renewal systems but limited their brief discussion to factors including diurnal variations, seasonal variations, hormonal influences, nutrition, temperature, and

irritation of tissues. Cameron (1975) suggested different levels of mitotic control dependent on anatomical level of organization. These were (1) general control over all proliferating cell populations which included nutritional status, age, endocrine influence, and influence of body temperature; (2) control over specific cell populations or tissues by biochemical means including specific hormones, bacterial endotoxins and antigens; and (3) cell-to-cell control within a cell population including loss of contact inhibition and wound hormones. Cameron proposed that the control mechanisms could all operate simultaneously.

Bullough (1962) reviewed the literature regarding control of mitotic activity published prior to 1961. He listed a number of factors which may influence mitotic activity including nutrition, respiration, and hormones. Generally, a poorly nourished animal would exhibit a reduced mitotic rate, at least in some tissues. That was shown by McManus and Isselbacher (1970) who studied intestinal crypt cell proliferation in groups of starved and fed rats. Other factors which contribute to the physiological state of an animal have an effect on mitotic rates within certain tissues. Lesher, Fry and Kohn (1961) showed that the generation time in intestinal crypt cells increased as mice became older. Lesher, Walburg and Sacher (1964) investigated the proliferative activity in duodenal crypts of germ-free and conventional mice and observed that the proliferation rate was slower in the crypts of germ-free mice.

Though not a new concept, Castor (1972) cinemicrographically observed contact inhibition of mitosis in cell cultures. A hypothesis to explain the phenomenon included reduced uptake of serum macromolecules from the culture medium as the mitotic-limiting factor. An in vivo manifestation of that concept was noted by Fisher and Maibach (1972) who reported that physical occlusion of stripped epidermis in psoriasis patients had a mitotic inhibiting effect.

Hormonal influence on the proliferative rate of certain tissues was shown by Bullough (1962). An inhibitory effect was attributed to glucocorticoids, adrenalin, and noradrenalin. Androgens and estrogens were considered stimulatory but data of Epifanova (1963) indicated that the effect of injections of sex hormones were due to increased blood concentrations epinephrine and adrenocorticotrophic hormones. Bullough (1962) postulated that the mitotic rate in a tissue was not stimulated; it was inhibited by tissue-specific mitotic inhibitors. He suggested that an increase in mitotic rate was ultimately due to a reduction in concentration or to neutralization of the inhibitors. According to his thesis, such would be the case with hormonal influence on cell proliferation such as the stimulatory effects of androgens and estrogens. Bullough (1962) also speculated that a group of compounds called "chalones" (discussed later), were tissue-specific mitotic inhibitors.

Control of mitotic activity in epidermis was discussed on a molecular basis by Gelfant and Candelas (1972). Their control model was based on an assumption that synthesis of a specific protein is required before cells can become mitotic and that nondividing but reproductively viable cells contain a long-lived messenger molecule which is complexed with a repressor molecule. Induction of mitosis occurs when the function of the repressor is interfered with by some mechanism. They speculated that chalones may be the repressor molecules. Cyclic changes in cellular concentrations of enzyme proteins were related to stages of the cell cycle by Klevecz (1975). Glycolytic enzymes, for example, exhibited peak concentrations during late G_1 or early S and during S or G_2. Klevecz suggested that the periodic synthesis of enzymes, characteristic of many eukaryotic cells, were important for timing of the cell cycle.

Investigations of biochemical control of cell replication have been conducted by numerous researchers. Lieberman and Short (1972) determined that an infusate comprised of L-triiodothyronine amino acids, glucogon, and heparin stimulated DNA synthesis in cells of an intact liver. Baserga (1972) discussed the role of nonhistone, chromosomal proteins in stimulation of cell division. Those proteins (nuclear acidic proteins) were considered a part of the mechanism by which the genome of a nondividing cell was activated for subsequent cell division.

Cohen (1972) described an "epidermal growth factor" which stimulated proliferation of epidermal tissue and chemically was a single-chain polypeptide. Extracts of intestine and liver from adult Pleurodeles were found to have an inhibitory effect on mitotic activity in corresponding tissues in larva of the same animal, a salamander (Brugal, 1973). Tutton (1973) isolated a heat labile factor from homogenized intestinal crypt cells of the rat which was found to have an inhibitory effect on proliferative activity in crypts and was tissue-specific in that it had no effect on cells of colonic crypts, esophagus, or skin.

Literature regarding chalones was reviewed by Elgjo (1972), who described them as tissue-produced inhibitors of cell proliferation. They were determined to be water soluble, thermo-labile, and nondializable. Chalones are apparently tissue-specific for a type of tissue (e.g., epidermal chalones may also inhibit mitosis in the squamous epithelia of the gingiva, esophagus, and cornea) but are not necessarily species-specific. Molecular weights of the compounds may range from 4,000 to 45,000 and they are easily destroyed by trypsin. Interruption of the cell cycle by those compounds is considered to be by regulation of the passage of cells from the G_1 phase to the S phase but a G_2 block has also been attributed to epidermal chalones (Elgjo, 1972).

Tumor Growth. Experimental evidence indicates that the cell cycles of tumor cells are not necessarily shorter than

those of many other cells of normal mammalian tissues. Therefore, increased individual cell proliferation rates are not characteristic of tumor growth (Baserga, 1965; Oehlert, 1973; Mendelsohn, 1975).

Baserga et al. (1962), from research with human patients, confirmed the hypothesis that tumor cells do not necessarily proliferate faster than cells of normal tissues. They sampled primary and metastatic solid tumors as well as a strip of abdominal skin after injecting the patients with tritiated thymidine. Great variation was found in the label incorporation rates of different kinds of tumors but incorporation of tritiated thymidine by the epidermal cells was constant between patients. Solid tumor cell proliferation rates, it was observed, also did not increase (Baserga, 1965).

Mendelsohn (1975) reviewed some of the growth characteristics of solid tumors and noted that most of them exhibited cellular kinetics similar to those of normal tissues in which cell renewal occurs. T_{G2} values for tumor cells were reported to be generally longer than those of normal tissues. A linear relationship was shown to exist between T_S and T_C in cells of tumors, tissue cultures, and many normal tissues. That relationship was considered true enough so that T_C values could be predicted from it if T_S values were known. T_{G1} and T_C were shown to be related by a curvi-linear relationship. It was acknowledged, however, that

the validity of both those relationships could be nullified in cell populations where a long G_o phase exists. The data of Brown (1970) showed that there was a substantially longer generation time for normal hamster cheek pouch epithelium than for chemically induced carcinomas in the same tissue. Two different methods of calculating generation time were used for the two cell populations (Brown and Oliver, 1968; Brown, 1970) however, and probably accounted for the difference. According to Potten (1971) and Burns (1975), the type of PLM curve obtained for the normal hamster cheek pouch epithelium would indicate the existence of a G_o phase in the cell cycle of those cells. If the G_o phase was not present in the chemically induced carcinoma, a discrepancy in the calculated T_c would result. Investigating the mitotic cycle of intraperitoneal Erlich ascites tumors in mice, Baserga (1963) estimated an 18-hour generation time for the cells with a T_S of 11 hours. He also determined that the growth rate of the tumor declined with age and that this decline was due to a decrease in the fraction of dividing cells rather to an increased length of the generation time of the cells. Simpson-Herren, Sanford and Holmquist (1974) also reported an age-related decline of the growth rate for transplanted and metastatic Lewis lung carcinomas in mice. The decrease in those tumors was attributed to an increase in the length of the generation times.

The review by Oehlert (1973) advanced the concept that tumor growth is due primarily to an increase in the ratio of

intermitotic to postmitotic cells in a tissue. A similar observation was made by Terz and Curutchet (1975) on the cell kinetics of normal mucosa and of an epidermoid carcinoma of the maxillary antrum in a human patient. That was essentially the same trend observed in wound-reparative regeneration; so, Oehlert (1973) postulated that carcinogenesis must be due to a disturbance in the division mechanism whereby there are more mitoses contributing cells to the proliferative pool than to the pool of cells which ultimately differentiate. That concept was further substantiated by the data of Flaxman and Chopra (1972), who showed that normal epidermal cells and cells of psoriatic epidermis proliferated at essentially the same rates in vitro.

Epithelial Renewal. The "steady state" system for renewal of cell populations was discussed by Leblond and Walker (1956). In that system, the number of cells in a population doesn't change; therefore, the same number of cells must leave the population as are produced by mitosis. Cell renewal systems for the epidermis, gastric mucosa, intestinal mucosa, and seminiferous epithelium were illustrated by those authors. They suggested the use of cell labeling with DNA precursors, such as ^{32}P-labeled phosphorus-containing compounds, ^{14}C-adenine, and ^{14}C-formate, to facilitate observation of cellular migratory patterns. Hughes et al. (1958) used tritiated thymidine with autoradiography for that purpose and elucidated the migration of cells within renewal

systems in many tissues of the mouse with emphasis on the gastrointestinal tract. The classification scheme of Leblond, Messier and Kopriwa (1959), in which cell populations were classed as stable, growing or renewing, was based on the same technique.

Kinetics of intestinal epithelium were described in detail by Quastler and Sherman (1959). Dividing the cell renewal system into compartments (progenitor and functional) and subcompartments (M, G_1, S, and G_2), they formulated mathematical relationships between the compartments based on parameters such as number of cells, time spent in the compartment, and birth and death rates. The migratory patterns of cells from the progenitor through the functional compartments were traced by radioactive labeling and autoradiography. A study by Schultze et al. (1972) indicated that the jejunal crypt epithelium was, indeed, a steady state epithelial renewal system. Double-labeling techniques were used to determine a constant flux of 7.15 percent cells per hour through the system. They estimated the proliferative pool size in the crypts to be 72 and 77 percent in two separate determinations. Evidence was presented by Burholt, Schultze and Maurer (1973) which indicated that every jejunal crypt cell incorporating a label eventually underwent mitosis. The same double-labeling technique as that used by Schultze et al. (1972) was employed.

Further investigations of jejunal crypt cell kinetics were made by Kovaks and Potten (1973). They characterized the crypt

cell population and indicated that only about half of the crypt cells were proliferative and that Paneth cells numbered about 50 cells per crypt. A zone of cells at the border between the Paneth cells and the proliferative cells remained in question. Potten, Kovaks and Hamilton (1974) suggested that that group of cells could be Paneth cell or goblet cell precursors.

Effect of Ionizing Radiation. The effects of ionizing radiation on individual cells constitutes an academic discipline unto itself. This review, therefore, includes a discussion primarily of radiation effects on the cell cycle as those effects are manifested in epithelial renewal systems.

Results of an investigation concerning the effect of X-irradiation on the mitotic index in tissues of mice were reported by Knowlton and Hemplemann (1949). Studying the adrenal gland, lymph node, jejunum and epidermis, they observed an immediate (within a few hours) decline of the mitotic index to a value approaching zero after exposure to 325 R X-irradiation. With lower exposure doses, the decline was less severe. A detailed review of information concerning the effects of radiation on morphology and division of cells was contributed by Carlson (1954).

The radiosensitivity of dividing cells was recognized to be high at a time immediately preceding mitosis (Knowlton and Hempelmann, 1949; Carlson, 1954). Howard and Pelc (1953) confirmed the delay of cells in G_2 (four-hour delay) from 140 R X-ray

exposure in Vicia faba meristematic cells and also reported a ten-hour delay of cells in G_1. A later report was submitted by the same authors (Pelc and Howard, 1955) in which they reaffirmed the DNA synthesis inhibiting qualities of moderate X-ray exposure doses. Inorganic ^{32}P incorporation was used as an indicator of DNA synthesis in both studies. For ^{32}P incorporation by cells of the thymus, Ord and Stocken (1958) observed the same presynthetic radiosensitivity in rats. A dose-response relationship was shown in which ^{32}P incorporation decreased rapidly between 25 and 200 R exposure and decreased linearly, but not as rapidly, between 200 and 3200 R exposure. Phosphorus-32 incorporation was measured two hours after the X-ray exposure.

Essentially the same kind of dose-response relationship was shown by Lajtha et al. (1958b) for human bone marrow cells in vitro with ^{14}C-formate as the DNA precursor. The radiation doses, however, were considerably higher for that system, ranging from 1000 to 10,000 rads. They suggested, as did Ord and Stocken (1958), that the biphasic dose-response curve was indicative of two separate types of biochemical lesions induced by radiation. Lajtha et al. (1958a) found that 200 to 300 rads would not perturb DNA synthesis if administered after human bone marrow cells in vitro had entered the S phase. The same dose administered in G_1, however, prevented about 50 percent of the cells from entering the S phase during a 17 to 22 hour period following

irradiation. Irradiation of the human bone marrow cells with doses greater than 500 rads during G_1 directly inhibited DNA synthesis. Similar results were reported by Brown (1970), who observed a reduction of T_{G1} in hamster cheek pouch epithelial cells induced by 500 to 1000 rad doses.

DNA synthesis in the cells of the thymus, spleen, and small intestine of rats was measured after whole body irradiation with exposure doses of 50 to 800 R (Nygaard and Potter, 1959). The resulting decrease in radiochemically detected ^{14}C-thymidine incorporation was dose-related and incorporation of the DNA precursor reached minimal levels in all three tissues for each exposure dose within eight hours after irradiation. No recovery was noted in the thymus and spleen during a 24-hour period; but, for all but the 400 and 800 R groups, DNA synthetic activity was slightly above the control level by 24 hours postexposure. The same type of investigation was performed by Sherman and Quastler (1960) but with a wider range of radiation doses, a longer observation period, and tritiated thymidine as the DNA precursor. They also found a rapid initial decrease in thymidine incorporation and minimal values for tritiated thymidine incorporation which were related to the X-ray dose which the animals received. The initial decrease was attributed to blockage of DNA synthesis. For larger doses (3,000 rads), it was observed that cells which resumed DNA synthesis did not necessarily divide. That resulted

in large misshapen cells occupying the original sites of the crypts two days postirradiation.

A series of experiments with rather conclusive results were performed by Terasima and Tolmach (1963). By irradiating synchronized populations of HeLa cells at different times during the cell cycle, they elicited several radiation-related responses. The best survival rate was exhibited by cells irradiated in early G_1 or in G_2, but survival was diminished considerably if cells were in late G_1 and early S phase or in mitosis at the time of irradiation. Cells irradiated during their DNA synthetic phases exhibited a delay in entering the next S phase, a longer S phase with decreased rate of DNA synthesis, and a slight delay in G_2. Irradiation of cells during the mitotic process produced a slight delay in G_1, a slightly longer S phase with a reduced rate of DNA synthesis, and a major delay during the following G_2 phase.

Using time-lapse cinemicrography, Marin and Bender (1966) followed the course of radiation-induced division delay in HeLa cells after 400 and 600 R X-ray exposures. The cells entered the first mitotic division after exposure with no delay, but the time between successive divisions was lengthened. Doida and Okada (1969) determined a radiation-induced block of the cell cycle to be in mid-G_2 for mouse leukemic cells in vitro. They suggested that the block occurred as a result of inhibition of protein synthesis. The radiation-induced prolongation of T_{G2}

was also reported for plant cells by Kovacs and Van't Hof (1971) who considered their data to be consistent with the protein synthesis inhibition hypothesis. Dewey (1975) investigated radiation-induced chromatid aberrations in cultured Chinese hamster cells and found that if the synchronized cells were irradiated at a time in G_2, ten minutes prior to prophase, no aberrations or division delays were observed. That point in the cell cycle was referred to as an "X-marker."

Radiation effects on the cell cycle similar to those described above have been investigated in in vivo systems, particularly in epithelial cells of the gastro-intestinal tract. The data of Lesher (1967) indicated the G_1 delay and the G_2 block if crypt cells in those phases were irradiated with 300 R of ^{60}Co gamma irradiation. Also, there was a reduced rate of DNA synthesis by the cells during S phase if they were in that phase during irradiation. Dose related G_1 and G_2 responses were determined by Lesher and Lesher (1970) in the same system with a graded series of exposure doses. In that study, G_1 delay and G_2 blocks were manifested as depressions of the synthetic and mitotic indices, respectively.

Sherman and Quastler (1960) described the movement of epithelial cells from crypts to villus tips after an acute exposure of mice to ionizing radiation. They observed that the migration of cells did not cease in the absence of proliferative

activity in the crypts and, in time, that led to denudation of the villi. Fry et al. (1969) conducted a species comparison study for susceptibility to gastro-intestinal radiation syndromes in six rodent species. They determined that, generally, the species with the longest transit times of cells from crypt to villus tips were the most radio-resistant based on survival time of the animals. The relationship between the lifespan of intestinal epithelial cells and survival of an animal was described as linear by Matsuzawa and Tsubouchi (1969). Those authors also considered the proliferative part of the recovery phase to consist of exponential growth of the crypt cell population after a sublethal radiation dose. The mitotic and synthetic indices of those rapidly proliferating cell populations were observed to be higher than control values between one and three days following X-ray exposure (Lesher and Lesher, 1970). The time when the compensatory proliferation occurred was dependent on the dose level. Hagemann, Sigdestad and Lesher (1972) suggested that the overproliferation of crypt cells could be used to reduce effects of subsequent radiation exposure if proper timing between exposures was employed. Lesher and Lesher (1970) discovered a partial synchronization of cell cycles in crypt cells during recovery phase. It was manifested by fluctuations in the synthetic and mitotic indices similar to observed diurnal fluctuations.

Mechanisms by which gastro-intestinal radiation death occurs remain controversial. Ultrastructural consequences of

X-ray exposure in undifferentiated but postmitotic cells of the gastro-intestinal tract were described by Quastler and Hampton (1962). Nuclear changes included enlargement and changes in shape one day after exposure. A decrease in number of cytoplasmic membranous organelles, such as mitochondria and cytoplasmic vesicles, was apparent after two to five days postirradiation. Depletion of ribosomes was also evident during the two to five day time period. Similar ultrastructural responses were observed by Goldfeder (1965). As a result of cell deaths with continued migration of cells from crypts to villus tips, the villi became shortened within four days postirradiation. It was suggested that interference with cell proliferation was not a sole consequence of irradiation of the intestine (Quastler and Hampton, 1962). Lushbaugh (1969) discussed research regarding the gastro-intestinal radiation syndrome. He concluded that previous speculation about loss of electrolytes and fluids from an irradiated and denuded intestinal wall were not valid. He cited evidence implicating bacterial proliferation inside the denuded gut and lesions of the intestinal capillary structure as contributing factors to death by radiation.

Effect of Temperature. Due to the thermo-control mechanisms of homeothermic animals, most of the research concerning temperature-related changes in the cell cycle has been done either with poikilothermic organisms or with mammalian cell cultures.

Héroux (1959) conducted an investigation of nonfreezing cold injury to the ears of rats. He found substantial differences in the response to cold injury by tissues of the first 3 mm from the edge of the ear and tissues 4 to 6 mm away from the edge. An increase in cellular metaphases during the first 21 days postexposure was reported in tissues located 1 to 3 mm from the edge of the ear. That effect was accompanied by corresponding decreases in prophases and telephases. Only a slight mitotic block was reported to have occurred during the first week in tissues located 4 to 6 mm from the edge of the ear.

In another study, Héroux (1960) found that skin temperatures of rats kept at 30°C for three months varied from 30.4°C on the ear to 35.3°C on the back. Estimating mitotic activity by the colchicine technique, it was found that the rate at which cells entered mitosis was slower and the duration of mitosis was longer in skin at lower temperatures. It was found, however, that the colchicine technique failed when rats were cold-acclimated. The failure was considered due to extreme slowing of the mitotic process.

In vitro studies of exponentially proliferating HeLa cells indicated that, after a temperature shift, the duration of each cell cycle phase gradually increased or decreased to a value characteristic for the new temperature (Rao and Engleberg, 1965). The G_1 phase was first to reach the new value if temperature

shifts were within the range of 30°C to 40°C. At temperatures between 26°C and 31°C, exponential growth was interrupted and an accumulation of cells in the mitotic phase occurred. It was concluded that mitosis was the most temperature sensitive phase of the cell cycle. Data refuting that conclusion was presented by Watanabe and Okada (1967). Investigating temperature effects on growth rate of mouse leukemic cells in vitro, they determined the G_1 and S phase cells to be the most sensitive to temperature changes. They presented a figure based on data of other authors which illustrated the relative effects of changes in the duration of the S and G_1 on the overall growth rate of cells as indicated by the generation time. In that figure, it was shown that changes in T_{G1} are the most influential in changing the T_C.

That survival of frogs after X-irradiation may be prolonged by lower environmental temperatures was shown by Patt and Swift (1948). For each exposure dose from 1000 to 9000 R, they exposed and kept one group of frogs at ambient temperatures (approximately 23°C) and exposed and kept another group at 5 to 6°C. They determined that survival was greatly enhanced in the latter group for each exposure; but, if frogs from the 6°C environment were transferred to a 23°C environment, they succumbed to radiation injury at a rate similar to that of a frog from the former group. They concluded that the prolonged survival at colder temperatures was due to a slower rate of development of the radiation damage rather than to repair processes.

A similar temperature-related response to X-rays was observed by Hyodo (1965a). Goldfish exposed to 8 kR survived for less than ten days if kept at 22°C but survived more than 100 days if kept at 4°C. Transfer of the fish from the cooler to the warmer environment resulted in death due to intestinal epithelial damage within ten days. The survival times and numbers of epithelial cells per intestinal cross-section were shown to be closely related. It was also noted that mitotic figures were frequently observed in the intestinal epithelium of nonirradiated fish kept at 4°C and at 22°C as well as in irradiated fish kept at 4°C, but were considerably less frequent in those fish irradiated and kept at 22°C.

In goldfish injected with tritiated thymidine, Hyodo (1965b) observed that: (1) in fish kept at 22°C, the LI, autoradiographic grain counts over nuclei, and PLM reached maximum values three hours after injection; (2) in fish kept at 4°C, the LI remained low at 24 hours and the PLM reached 20 to 80 percent after 44 hours; (3) at 4°C, the PLM was significantly lower in intestines of those fish receiving X-ray exposure to 8 kR.

A relationship between survival after exposure to 8 kR of irradiation and the intestinal epithelial cell kinetics in different temperature ranges was reported in the common goldfish by Hyodo-Taguchi and Egami (1969). They listed the durations of cell cycle phases as: T_M = two hours, T_{G1} = five hours, T_S = nine hours, T_{G2} = one to two hours, and T_C = 18 hours at 23 to

35°C. As environmental temperatures were lowered to 15°C and to 4 to 6°C, T_M values of four hours and 36 hours respectively; T_{G2} values of four hours and 44 hours respectively; and T_S values of 24 hours and "very long" respectively, were listed. Due to extreme increases in length of T_C, values for T_{G1} and T_C were not available for the two lower temperatures. Approximate survival times were listed as 15 days, 30 days, and "very long" for fish kept at 23 to 25°C, 15°C, and 4 to 6°C, respectively. Exposure of the goldfish to sublethal doses (1 and 2 kR) of X-irradiation produced a G_2 block in the proliferating cells of the intestinal epithelium (Hyodo-Taguchi, 1970). The block had a duration of 48 hours for a 1 kR exposure and 72 hours for a 2 kR exposure with a 25°C environmental temperature. With an environmental temperature of 15°C, the block lasted 72 hours and 96 hours for the 1 and 2 kR exposures, respectively.

Using split doses and fractionated doses of radiation on the small killifish (Oryzias latipes), Egami and Etoh (1966), Etoh and Egami (1967), and Egami (1969) demonstrated recovery of the intestinal epithelial cells from radiation injury. The recovery process was observed to be slower if fish were kept at lower environmental temperatures (Egami and Etoh, 1966) and it was concluded that radiation injury from fractionated and chronic irradiation accumulated at the lower temperatures due to much slower cell repopulation (Etoh and Egami, 1967). The recovery process was considered to consist of three phases: (1) rapid

recovery due to intracellular repair within the first three hours after exposure, (2) characteristic fluctuation of LI and MI (possibly due to partial synchronization of the cells) between three and 48 hours, and (3) repopulation of the epithelium at times longer than three days. The lengths of the phases were greater at lower temperatures (Egami, 1969; Hyodo-Taguchi and Egami, 1969).

Nieto and Johnson (1972) studied the turnover of DNA in tissues of goldfish as it was manifested by a loss of incorporated ^{125}I-deoxyuridine. DNA turnover in the fish was determined to be only slightly temperature-dependent in environments with temperatures ranging from 5°C to 37°C. At a near lethal 37°C, a slightly increased rate of cell turnover was discerned, but the increased rate was coupled with a decrease in the number of proliferating cells. In another study, goldfish were kept at temperatures between 6°C and 37°C, given single injections of tritiated thymidine, and killed one hour after injection (Garcia and Johnson, 1972). Results reported from that study included a rising LI in intestinal epithelial cells between 6°C and 20°C after which the LI decreased with increasing temperatures. Grain counts over labeled nuclei in the same tissue increased slightly over the temperature range used and little change was exhibited in the cellular generation times. The authors concluded that the duration of the S phase was most sensitive to temperature variations.

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Diurnal Rhythms. Diurnal rhythms in cellular proliferative activity apparently exist in most tissues of many animals. The mitotic index and the labeling index of cell populations, being most visually apparent in histological sections, have been frequently characterized for their diurnal rhythms. One of the first studies of animal tissues was performed by Carleton (1933), who investigated the daily rhythm of mitotic activity in skin sections from newborn mice and rats. Her results indicated maximum mitotic activity in the tissues from 2000 to 2400 hours and minimum activity at 1200 hours. She determined that the daily mitotic rhythm could be perturbed by varying the light-dark cycle to which the animals were exposed, and that the rhythm could be suppressed by exposure to constant light but not

by constant darkness. On a limited number of specimens, Cooper and Schiff (1938) studied mitotic activity in the epidermis of the prepuce circumcised from human infants. They discovered maximal mitotic activity at approximately 2200 hours and minimal activity at 1030 hours, essentially the same times given for the skin of mice and rats (Carlton, 1933).

Additional data, including the maximal (peak) and minimal (nadir) mitotic and DNA synthetic activities of cell populations as determined by several investigators, are presented in Table 1. Due to the variety of tissues listed and technical variations by the individual researchers, the data are not directly comparable but some general conclusions may be drawn. Generally, peak mitotic activity occurred between midnight and noon, while the least mitotic activity generally occurred in the early morning hours and late evening hours with lowest values during daylight hours.

Discrepancies in the data are present. For example, the mitotic and DNA synthetic activity in the cheek pouch epithelium of hamsters has not yet been well characterized. Also, Pilgrim, Erb and Maurer (1963) reported observing no diurnal fluctuations in mouse jejunal crypt epithelium, but later investigations by Sigdestad, Bauman and Lesher (1969) and Sigdestad and Lesher (1970) resulted in statistically significant peak mitotic activity in the same model. Dobrokhotov and Nikanorova (1962) reported

TABLE 1. Peaks and Nadirs of Mitotic Activity and Tritiated Thymidine Labeling Indices in Mammalian Tissues. (The Data were Generated by the Authors Listed in the Last Column.)

Tissue	Animal	Mitotic Index		Labeling Index		References
		Peak	Nadir	Peak	Nadir	
Corneal Epithelium	Rat	0600	2100	-	-	Gololobova (1958)
Corneal Epithelium	Rat	0600-1200	2000	1600-1700 & 0300	2200-0100	Scheving & Pauley (1967)
Cutaneous Epidermis	Rat	0600	1500-2400	-	-	Gololobova (1958)
Epidermis	Human	1400-1500 &2400-0400	1100-1300	-	-	Scheving (1959)
Epidermis Tongue Forestomach Esophageal Epithelium	Mouse	0530	Remainder of Day	1730	1130	Pilgrim, Erb, & Maurer (1963)
Esophageal Epithelium	Rat	0400-0700	1600-2200	-	-	Dobrokhotov & Kurdyumova (1962)
Adrenal						
Zona Glomerulosa	Rat	1900-2200	0100-1600	-	-	Dobrokhotov & Nikanorova (1962)
Zona Fasciculata & Zona Reticularis	Rat	0700-1300	1600-0400	-	-	Same
Parotid Gland	Mouse	-	-	2100	Remainder of Day	Burns et al. (1972)
Kidney	Mouse	-	-	0100-0500	0900	Burns et al. (1972)

TABLE 1. (Continued)

Tissue	Animal	Mitotic Index		Labeling Index		References
		Peak	Nadir	Peak	Nadir	
Small Intestine						
Crypt Epithelium	Rat	0600&1200	1500-2100	-	-	Gololobova (1958)
Jejunum	Mouse	None	None	None	None	Pilgrim et al. (1963)
Jejunum	Mouse	0200	1500	0200	1500	Sigdestad et al. (1969) & Sigdestad & Lesher (1970)
Duodenum	Mouse	-	-	0100	2100	Burns et al. (1972)
Cheek Pouch Epithelium	Hamster	0600&1200	2000	2200-0200	1200-1600	Brown & Berry (1968)
Cheek Pouch Epithelium	Hamster	0200-0400	1400	-	-	Gibbs & Casorett (1969)
Cheek Pouch Epithelium	Hamster	1200	2400	0600-0800	2000	Izguierdo & Gibbs (1972)
Cheek Pouch Epithelium	Hamster	1100&1500	2300	-	-	Møller et al. (1974)

Note: Times are listed in hours on a 24-hour clock. Two times separated by a hyphen signifies a broad peak or nadir. Two times separated by an ampersand signify two definite peaks or nadirs although not necessarily of the same magnitude.

peak mitotic activity in the different cell zones of the adrenal gland. The peaks in the zona glomerulosa were observed at times of day opposite those of the peaks for the zona fasciculata and zona reticularis. It was concluded by the authors that the differences were due to different hormonal control of the three cell populations.

Marked consistencies also exist in the data listed in Table 1. For example, the values for human epidermis (Scheving, 1959) closely parallel the values given by Cooper and Schiff (1938) (page 53). Also the data for rat corneal epithelium given by Gololobova (1958) and Scheving and Pauly (1967) are in close agreement. In fact, the data presented for all rat tissues from four separate investigations show similar diurnal fluctuations.

In an attempt to determine the most radiosensitive time of tumor cells, studies have been conducted to elucidate the diurnal fluctuations of mitotic activity in those tumors. Tahti (1956) combined his data from 20 human malignant tumors with the data of Voutilainen (1953) to graphically illustrate the mitotic activity in the tumors over a 24-hour period. The resulting curve was biphasic with maximal values at 0200 and 1200 hours and minimal values at 0800 and 1800 hours. It was noted that diurnal fluctuations of the mitotic activity varied considerably from one tumor to another. Mitotic activity during the 24 hours following X-irradiation of the tumors fluctuated in a diurnal

rhythm similar to the pre-irradiation rhythm although the times of maximum and minimum values had been altered by the radiation. Radiation treatment at 1200 hours (a period of maximal mitotic activity) caused a slightly greater depression in post-treatment mitotic activity than did radiation at 0800 hours (a period of minimal mitotic activity) (Tahti, 1956).

Echave Llanos and Nash (1970) analyzed the daily mitotic activity in two types of mouse hepatomas; one was a fast growing tumor and the other was characterized by slow growth. Mitotic indices were at maximal values at 0400 hours and at 1600-2000 hours in the fast growing tumor, and only one time of maximal activity at 1600 hours was observed in the slow growing tumor. Diurnal variation was significantly greater in the slow growing tumor although it had considerably less mitotic activity during the dark hours. The diurnal fluctuations in mitotic activity of the slow growing tumor were said to resemble those of normal immature liver cells. Both of those tumors are sublines of a spontaneous hepatoma which is transplantable in mice; the original tumor was transplanted in 1949.

Diurnal variations in mouse livers were reported by Halberg et al. (1958) whose experimental results included highest glycogen levels during the daylight hours. He found the peak mitotic activity in mouse liver to occur at 1200 hours and highest DNA synthetic activity to occur from 0400 to 0800 hours with minimal

values for both parameters at 2400 hours. Other diurnal variations in hepatocytes were determined by Chedid and Nair (1972). Ultrastructurally, differences in the relative amounts of smooth and rough endoplasmic reticulum were observed at different times of the day. Cytoplasm of the hepatocytes contained considerably more smooth endoplasmic reticulum at 2200 hours than at 1400 hours. This was paralleled by microsomal hexobarbitaloxydase activity. In blinded animals, no significant variation in either parameter was determined.

The mechanism of the diurnal fluctuations in mitotic and DNA synthetic activity has not been well defined. Pilgrim, Erb and Maurer (1963) using autoradiographic grain counts over tritiated thymidine labeled cells as a criterion, concluded that there was no change in the rate of DNA synthesis by cells. The cells did exhibit a diurnal fluctuation of DNA synthesis (Table 1). Chiakulas and Scheving (1966) using the same criteria, coupled with radiochemical analysis of the tissues, defined an accelerated rate of DNA synthesis in the epidermis of larval urodeles at certain times of the day. A similar conclusion was reached by Sigdestad, Bauman and Lesher (1969) regarding the crypt cells of mouse jejunal epithelium. The diurnal fluctuation in tritiated thymidine uptake by cells of rat corneal epithelium was attributed to a combination of increased numbers of cells in the S phase and an increased rate of DNA synthesis in the cells (Scheving and Pauly, 1967). Diurnal variation in mitotic

activity was attributed to changes in the duration of mitosis (Evenson, 1963) and Brown and Berry (1968) observed a slight increase in the duration of mitosis at times of high mitotic indices. Izquierdo and Gibbs (1972), on the other hand, concluded that there was no evidence that phase durational changes were responsible for circadian rhythms in hamster cheek pouch epithelium.

The diurnal rhythm of mitotic and DNA synthetic activity may be perturbed by physical and chemical agents. Carleton (1933) determined that the mitotic rhythm of cells of mouse epidermis could be eliminated if mice were exposed to continuous light. Continuous darkness did not affect the magnitude of the diurnal change in mitotic activity but did shift it by approximately four hours. By shifting the light-dark cycle to which the mice were exposed by six hours either way from 0600, the rhythm in the mitotic cycle of the epidermal cells was completely disrupted. Halberg et al. (1958) observed reversals in daily rhythms of mouse hepatic and pinnal epidermal cellular activities including mitosis, DNA synthesis, RNA synthesis, glycogen content (liver), and corticosterone concentrations (blood serum) with reversed light-dark cycles. They allowed more time for the shift in activity rhythms to occur than did Carleton (1933) however, with acclimation times ranging between eight and 23 days; the latter time was for pinnal epidermal mitoses. Carleton (1933) allowed

between five and 14 days with most trials ranging between eight and ten-day acclimation times. Sigdestad, Lesher and Scott (1975) demonstrated a complete reversal of daily rhythms in mitotic and DNA synthetic activity in mouse jejunal crypts after 35 days of exposure to a reversed light-dark cycle.

An injection of tritiated thymidine was found to perturb the diurnal mitotic rhythm (Gibbs and Casarett, 1969, 1971; Møller, Larsen and Faber, 1974). Hamsters were injected intraperitoneally with 1 μCi/g body weight of tritiated thymidine (specific activity: 6.0 μCi/millimole) (Gibbs and Casarett, 1969) and mitotic activity in their cheek pouch epithelia was determined. Initially, an increase in mitotic activity was noted, but the mitotic index dropped to a level considerably below that of control values after two hours postinjection. By six hours postinjection, the mitotic index of the epithelial cells had risen to control values and nearly paralleled control values thereafter. The effect was not observed if nonradioactive thymidine was injected. Essentially the same effect of tritiated thymidine injection on the mitotic activity of the hamster cheek pouch epithelial cells was observed by Møller, Larsen and Faber (1974). They attributed the effect to a shortening of the G_2 duration in the cells as the effect appeared to be dependent upon the number of cells in G_2 at the time of injection. Izquierdo and Gibbs (1972), however, reported finding no effect of tritiated

thymidine on the mitotic index of hamster cheek pouch epithelium if doses of 0.5 μCi/g body weight were used. They used tritiated thymidine with a specific activity of 6.0 μCi/ millimole, the same as that of Gibbs and Casarett (1969) and Møller, Larsen and Faber (1974).

Another chemically induced perturbation of the circadian rhythm was investigated by Burns, Scheving and Tsai (1972). They determined that a single injection of isoproterenol would alter uptake of tritiated thymidine by cells of the kidney, parotid gland, and duodenal crypts in mice. Depending on the time of day that the injection was administered, the drug would either stimulate or inhibit tritiated thymidine uptake in those tissues. Isoproterenol is a drug which is generally administered orally for therapeutic relief of bronchial asthma or cardiac block.

X-irradiation was shown to alter mitotic rhythms in hamster cheek pouch epithelium (Gibbs and Casarett, 1971). The results of 100 R whole-body exposure produced perturbation in the mitotic daily rhythm similar to those of an injection of high specific activity tritiated thymidine except for the initial sharp rise in the mitotic index. In addition to the G_2 block produced by the radiation exposure (approximately four hours in length), the uptake of tritiated thymidine was also initially depressed.

Most investigators studying cell kinetics stress the need for recognizing diurnal rhythms in cellular activity when using

the percent labeled mitoses curve to determine durations of the cell cycle phases. Pilgrim, Erb and Maurer (1963) indicated that in several studies cellular generation times were deduced using the tritiated thymidine labeling index or the mitotic index. Those studies assumed an asynchronous cell population, an assumption which may not have been valid in light of the diurnal rhythms. Gibbs and Casarett (1969) emphasized the need for analysis of cytokinetics by means of more than one technique. Using a double labeling technique on hamster cheek pouch epithelium, they determined a T_S of five hours. The PLM curve for the same tissue after a single injection of tritiated thymidine indicated a T_S of approximately ten hours. They also determined a T_M of 53 minutes calculated from the mitotic index at 1000 hours and 77 minutes calculated from the mitotic index at 1300 hours. Sigdestad, Lesher and Scott (1975) deduced cell cycle times (T_C) of 13.1 hours if the PLM curve was started at 1500 hours and a T_C of 11.1 hours if the PLM curve was started at 0300 hours. Their model was the mouse jejunal epithelial crypt. T_S values ranging from 8.0 to 13.5 hours were deduced from PLM curves for hamster cheek pouch epithelium by Møller, Larsen and Faber (1974). They attributed the wide range to experimental error encountered by using a technique designed for asynchronous cell populations when the cell population being studied did, in fact, have a pronounced circadian rhythm. Izquierdo and Gibbs (1974)

investigated the effect of time of day on the PLM curve. They injected groups of hamsters with tritiated thymidine at 0800 hours and other groups at 2000 hours; these were considered times of high and low proliferative activity respectively. Durations of S and G_2 + 1/2M remained constant according to the PLM curves constructed from the cells of cheek pouch epithelium. They did find, however, that age distribution curves indicated nonuniform distribution of cells throughout the cell cycle. An integration of the mitotic index over a 24-hour period was proposed for determination of proliferative rate and turnover time. That method functioned independently of any circadian rhythm.

The effect of the circadian rhythm on whole-body radiosensitivity in Chinese hamsters was investigated by Lappenbusch (1972). He determined that hamsters were most radiosensitive at 1100 hours and least radiosensitive at 0100 hours. The $LD_{50/30}$ values listed for irradiation at the two times were 823 rads and 954 rads respectively. The radiosensitivity data was correlated with mitotic indices of bone marrow white blood cells, and it was found that the highest mitotic index occurred at the time of highest radiosensitivity and the lowest mitotic index occurred at the time of least radiosensitivity. There was no apparent parallel in magnitude of mitotic indices between bone marrow white blood cells and cheek pouch epithelium. Data of Gibbs and Casarett (1969, 1971) showed the highest mitotic activity in

cheek pouch epithelial cells to be between 0200 and 0500 hours with the least mitotic activity at 1400 hours.

Data regarding circadian rhythms in cell populations of poikilothermic vertebrates is not as plentiful as it is for mammals. Scheving and Chiakulas (1965) investigated diurnal rhythms in mitotic activity and DNA synthetic activity of epidermal cells from larvae of the urodele, Amblystoma tigrinum, (tiger salamander). They determined tritiated thymidine uptake to be rhythmic with statistically significant peaks at 0900 to 1200 hours and 0200 to 0400 hours. Radiochemical analysis of the tissues was used to quantitate the tracer uptake. Two peaks of mitotic activity were also observed in that tissue, and those peaks were at 1230 hours and 0230 hours which corresponded to the peaks of tritiated thymidine uptake. The investigators suggested that there were two subpopulations of cells in the tissue; one with an S to M time of nine to ten hours, and the other with an S to M time of 16 hours. Incorporating labeling indices and autoradiographic grain counts into their data, the same investigators (Chiakulas and Scheving, 1966) further confirmed the peak times of tritiated thymidine uptake in the same tissues. Their research resulted in data which indicated more DNA synthetic activity than could be accounted for by the mitotic activity. It was suggested that either the cells were synthesizing metabolic DNA or that not all cells which had synthesized DNA were to undergo mitosis during the 24-hour period studied.

A study of diurnal fluctuations in radiosensitivity of urodele amphibians was conducted by Lappenbusch (1970). Groups of Taricha granulosa (rough-skinned newt) were exposed to 1000 R of X-irradiation at three-hour intervals during the day. Survival times indicated that the newts were most radiosensitive at 2100 hours and least radiosensitive at 2400 hours. The time of highest radiosensitivity corresponded to a time of approximately five hours before a time of significantly high mitotic and DNA synthetic activity in urodele larval epidermis (0200 hours) reported by Scheving and Chiakulas (1965). The time of least radiosensitivity was immediately prior to that same peak. No significant differences in radiosensitivity were reported by Lappenbusch (1970) at times corresponding to the high proliferative activity at 1000 hours reported by Scheving and Chiakulas (1965).

The basic control of diurnal periodicity in cell populations is not understood, but a number of speculations based on empirical data have been made. Alov (1963) presented evidence for a correlation of mitotic periodicity with a variety of factors including the light-dark cycle, periodic functional and metabolic activity, and motor activity of animals. An inverse relationship between functional activity and divisions of the cell was said to exist; for example, the mitotic diurnal rhythm in the intestinal cells of mice was reversed when the mice were fed only at night instead of normal morning feeding. Motor activity of the organisms

was also considered antagonistic to cellular mitotic activity. In the opinion of Alov (1963), motor activity of the animal and functional activity of the cells are first altered by reversals of the light-dark cycle; and those influence the mitotic index secondarily.

The influence of photoperiod on animal behavior and function was reviewed by Farner (1961). He cited examples of gonadal function and the reproductive cycle in numerous organisms as they are influenced by light. In lower vertebrates a relationship between temperature and photoperiod was considered responsible for control of the reproductive cycle. The role of light as a synchronizing agent for cellular proliferative activity was discussed by Scharrer (1964). He suggested the possibility of light penetration of the brain of animals. An example of response to light by blinded animals was used. A system of neurosecretory cells was proposed which, in response to light or darkness, secreted neurohormones with regulatory powers over endocrine glands. The influence of the endocrine system would result in the observed circadian rhythms. An eye-hypothalmus-neuroendocrine pathway was proposed by Jöchle (1964) to explain the response of an organism to its environment. The existence of a nervous pathway leading from the eye via a tract in the optic nerve to the diencephalon and from there to the hypophysis was theorized by Hollwich (1964). He cited data including light-induced color

changes in frogs, light-induced recovery from anemia in the chicken, and physiological function such as water-balance in blind humans as evidence confirming his theory. The observation that hypoplastic hypophyses and corresponding underdevelopment of the sella turcica occurred in humans blinded at an early age was considered to constitute anatomical evidence for his theory.

The Experimental Animal

The rough-skinned newt (Taricha granulosa) is native to the Pacific Coast and is generally found in aquatic habitats from California to Alaska. It is an urodele (with tail) amphibian of the family Salamandriidae. The male of the species may be as long as 19 cm and have a body weight of 16 to 18 gm. Females are generally smaller; size and the shape of the tail are the most obvious distinguishing features between the sexes. Body colors range from a brown to black dorsal surface and a yellow-orange to reddish-orange ventral surface (Blair et al., 1968).

Intestinal Morphology of Urodele Amphibians

The intestinal epithelial renewal system of urodele amphibians is apparently unique in the vertebrate evolutionary scale. Patton and Andrew (1954) studied the system in nine species of salamanders. They utilized nitrogen mustard and colchicine as

research tools to determine the function of the submucosal "cell nests" which were observed in all specimens. Those aggregates of cells were observed to function in epithelial cell replacement. Mitotic activity of 0.57 percent was counted and some of the cells of the "cell nests" were seen to have differentiated into nidal (goblet) cells which, it was speculated, were able to migrate out into the epithelium. Due to the relatively low mitotic activity in the cell nests, they suggested that the nests represented a secondary means of epithelial cell replacement.

In cross-section, Patten (1960) observed the following morphologic features of urodele intestine:

(1) long folds of simple columnar epithelium containing goblet cells;

(2) no muscularis mucosa present;

(3) a combined lamina propria and submucosa; and

(4) inner circular and outer longitudinal layers of musculature.

Using tritiated thymidine and autoradiographic techniques, Patten (1960) elucidated the cell renewal function of the cell nests. He described the cell nests as aggregates of cells lying in the submucosa; some were deep in the submucosa, while others were positioned immediately beneath the epithelium. Serial sectioning revealed no connections between the aggregates of cells.

Radiosensitivity of the Urodele Amphibians

Determination of ionizing radiation $LD_{50/30}$ values for urodele amphibians is complicated by many factors including the mode of radiation death and environmental temperatures at the time of exposure. Death from exposure to very high radiation doses (6000-12000 R) was considered by Brunst (1958a, 1958b) to be a result of damage to the skin, gills, spleen, and peripheral portion of the liver. The skin and gill damage interferes with the respiratory processes of the organism. The spleen and peripheral portion of the liver were referred to as lymphatic organs by Brunst (1958a, 1958b) but were considered to be the principle hematopoietic organs of the newt in the absence of bone marrow (Jakowska, Nigrelli and Sparrow, 1958).

Brunst (1958a) exposed adult axolotls (Siredon mexicanum) to doses of 500, 1000, 3000, 6000, and 12000 R at temperatures of 22 to 23°C. He observed median survival times ranging from 11 days for the 12000 R exposure to 196 days for the 500 R exposure with approximately one-third of the latter group surviving over two years postexposure. An approximate $LD_{50/30}$ of 6000 R could be calculated from his data (Lappenbusch and Willis, 1970). Little radiation-induced gastrointestinal damage was observed in salamanders from all but the 12000 R exposure group. The damage observed in those animals was considered moderate and consisted of enlarged nuclei, membranous disruption,

and hypersecretion of the epithelial goblet cells (Brunst, 1958a, 1958b).

The $LD_{50/30}$ for the newt Diemictylus (Tritirus) viridescens was listed as 1468 R at 23°C by Jakowska, Nigrelli and Sparrow (1958). Death was considered the result of disruption of the osmoregulatory function of the skin, kidney, and lungs. They, as did Brunst (1958a), noted damage to the spleen and peripheral liver but essentially no damage to the intestinal epithelium.

Lappenbusch and Willis (1970) determined an $LD_{50/30}$ for Taricha granulosa of 30 kR with a postirradiation environmental temperature of 10°C. The same authors (Willis and Lappenbusch, 1976) indicated that the $LD_{50/30}$ was not a useful parameter for describing the response of amphibian species to ionizing radiation. They expressed a preference for the parameter, "minimal LD_{50}," proposed by Conger and Clinton (1973). That parameter is based on a postirradiation period long enough to include all the acute effects of the radiation exposure. Relating LD_{50} values to mean survival times, Willis and Lappenbusch (1976) concluded that, with a sufficiently long postirradiation period, an LD_{50} of less than 250 R was probable.

Willis and Lappenbusch (1976) exposed over 800 newts (Taricha granulosa) to X-rays and ^{60}Co gamma radiation with doses ranging from 0 to 80 kR. They reported a relationship between exposure dose and mean survival time (MST) which indicated that death of the animals resulted from hematopoietic, gastrointestinal, and central

nervous system syndromes. The authors did indicate, however, that deaths at the dose-independent plateau of the MST curve might have resulted from combined effects of the radiation on the hematopoietic system, intestine, and skin.

An extensive study relating radiosensitivity of urodele amphibians to cytological morphometric parameters was conducted by Sparrow et al. (1970). They learned that exposure to 1000 R ionizing radiation was lethal to nearly all the amphibians tested but that survival times of the different species were quite variable. Radiosensitivity parameters were related to the nuclear volume and interphase chromosome volume (nuclear volume divided by number of chromosomes) of hepatocytes from the animals. Results indicated that median survival times increased as the species' nuclear volume and/or interphase chromosome volume increased. The same relationship was shown for the latent period (time between irradiation and the beginning of the steep slope of a survival curve) and the nuclear parameters. Based on an $LD_{50/30}$-interphase chromosome volume relationship, an $LD_{50/30}$ of 2473 R could be predicted for Taricha granulosa. For extended periods of time, Sparrow et al. (1970) noted that the LD_{50} for a species could be as low as 175 R. That value is close to the lethal threshold dose of 100 to 200 R (Lappenbusch and Willis, 1970) and the minimal LD_{50} of 250 R (Willis and Lappenbusch, 1976) for the species.

Environmental temperature affects the radiosensitivity of the newt. Exposure of Diemictylus (Triturus) viridescens to 9000 R

X-rays with a postirradiation holding temperature of 5°C resulted in survival times of up to five months (Jakowska, Nigrelli and Sparrow, 1958). The effect of temperature on postirradiation survival time was investigated by Willis and Prince (1967). With exposure of Taricha granulosa to 200 R and 48 kR and postirradiation holding temperatures of 10, 15, 20, and 25°C, they showed that mean survival time was inversely related to holding temperature. Algard, Friedmann, and McCurdy (1974) reported survival times for Taricha granulosa between 40 and 75 days and between 80 and 107 days for "active" and "hibernating" newts, respectively. Those values resulted from radiation doses between 2700 and 3600 rads with a postexposure holding temperature of 15°C. At the similar dose levels (2.5 to 3.5 kR) and for the same species, Willis and Lappenbusch (1976) listed survival times between 120 and 144 days with a holding temperature of 10°C.

Seasonal differences in radiosensitivity of Taricha granulosa have been reported. Lappenbusch and Willis (1970) observed that postirradiation survival times were significantly longer for males than for females in the spring of the year but observed no difference in the summer. Algard, Friedmann and McCurdy (1974) also reported seasonal differences in the radiosensitivity of Taricha granulosa. Newts collected in winter, referred to as inactive or "hibernating," were less radiosensitive than were newts collected in spring or summer. In the latter investigation, no mention was made concerning sex differences.

Cellular Kinetics in Newts and Other Poikilothermic Vertebrates

Other than the studies on intestinal epithelium of the goldfish and killifish discussed on pages 48 to 52 and the data on diurnal rhythms in newts discussed on pages 66 and 67, there is a dearth of cellular kinetic data for poikilothermic vertebrates in scientific literature. Uptake of tritiated thymidine by intestinal cells of a larger species of fish, the Coho salmon (Onchorhynchus kisutch), was studied by Johnson, Nakatani and Conte (1970). Injecting salmon with tritiated thymidine after acclimating groups of the fish to water temperatures of 5, 10, and 18°C, they radiochemically analyzed the nuclear protein fraction of tissue homogenates. They determined peak tritiated thymidine uptake by the intestinal cells to be 2.0, 6.2, and 9.3 nCi/mg of nuclear protein in fish acclimated to 5, 10, and 18°C respectively, at 120 minutes postinjection. Epithelial turnover times of more than 35 days, 23 days, and 13 days were calculated for fish in the 5, 10, and 18°C waters respectively. The same investigators also found that X-irradiation with exposures from 0 to 4000 R would reduce tritiated thymidine uptake by an amount concomitant with the dose.

Brunst (1950) reviewed the effects of X-irradiation on limb regeneration in urodele amphibians. It was observed that mitotic

activity of the limb bud regenerating after amputation was interrupted by pre- and postamputation irradiation. Comparing the reactivation of urodele limbs and mouse limbs to X-rays, Brunst, Sheremtieva-Brunst and Figgee (1953) observed similar radiation-induced responses, but the latent periods were considerably longer in the urodele amphibians. The techniques of cell cycle analysis were in their infancy at that point in time so no cellular kinetic data were available for the limb regenerating system.

Tritiated thymidine injections were used by O'Steen and Walker (1960) to investigate the cell labeling characteristics of Diemictylus (Triturus) viridescens. They classified the tissues of the newt on the basis of autoradiographic evidence of tritiated thymidine uptake. Group I consisted of tissues exhibiting labeling indices between zero and four percent and included loose connective tissue, bone, cartilage, muscle, neurons, medulla of liver, pancreas, and gastric epithelium. Group II included kidney tubules, cornea of the eye, oral epithelium, esophageal epithelium, and tracheal epithelium; these tissues had labeling indices between five and ten percent. Tissues with labeling indices greater than ten percent (Group III) were the intestinal epithelium (cell nests), seminiferous tubular epithelium, epidermis, spleen, cortex of the liver, and rectal epithelium. O'Steen and Walker (1960) also noted the migration of intestinal epithelial cells from the cell nests as reported by Patten (1960).

DNA synthetic activity and mitotic activity of lens regenerative tissue in the newt (Triturus viridescens) were investigated by Eisenberg and Yamada (1966). DNA synthesis was not detectable in the normal iris of the newt eye but was initiated in that tissue approximately five days after lentectomy. Initiation of mitotic activity in that same tissue did not occur until seven days after surgery. A gradual decline in DNA synthetic activity was observed between five and 35 days. Mitotic activity increased from seven to 15 days and then declined between 15 and 35 days postlentectomy. The investigators concluded that the cells synthesized DNA as a step preliminary to beginning a new cell cycle. The fact that there is not a wave of mitotic activity following the wave of DNA synthetic activity suggested that not all cells synthesizing DNA divide immediately afterward.

Research concerning erythropoietic activity in the same animal revealed a sequence of events similar to erythropoiesis in mammals. DNA synthesis and mitotic division in newt red blood cell precursors occurs in all but the final stage. In the newt, the final transition was from reticulocyte to erythrocyte; the reticulocyte is analogous to the late normoblast of human erythropoiesis (Grasso and Woodard, 1967).

The cellular kinetics of intestinal epithelium from the Western fence lizard (Sceloporus occidentalis) were investigated by Johnson, Dornfeld and Conte (1967). Using a colchicine technique with an environmental temperature of 26°C, they calculated

a mitotic duration of 1.8 hours. An epithelial turnover time was calculated from that value and the mitotic indices to be 4.2 to 7.8 days for the adult of the species. Tracing tritiated thymidine labeled cells from intestines of animals kept at 20°C by autoradiography, a turnover time of seven to nine days was estimated. A percent labeled mitosis curve was presented and a cell generation time of 20 to 22 hours was derived from it.

MATERIALS AND METHODS

Collection and Maintenance of Newts

The newts used in these experiments were collected from a small man-made reservoir approximately 1.5 miles west of Hoskins, Oregon. Newts were present in sufficient numbers so that they could be collected in a small-mesh landing net from the shore or by wading into shallower portions of the reservoir. Only male newts were collected and they were transported to the laboratory at Richland, Washington in polyethylene-lined 20-gallon tanks containing water from the reservoir. Upon arrival at the laboratory, the animals were transferred to similar containers filled with artificial pond water.[1]

Water containing newts to be used in studies at room temperature (22-23°C) was allowed to warm to that temperature while water containing newts to be used at cold temperatures was placed in a walk-in cold room and allowed to cool to 4°C. Light from an incandescent source was electrically timed to simulate the day/night cycle at the time of collection. The animals were allowed to acclimate to their respective environments for at least ten days.

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1. Artificial pond water:

0.058 g/l	NaCl
0.009 g/l	KCl
0.070 g/l	$CaCl_2 \cdot 2H_2O$
0.020 g/l	$NaHCO_3$

General Methods

Techniques used throughout this study will be described individually in this section. Variations in the techniques, if any, will be included in the appropriate part of the experimental design section.

X-Irradiation of Newts

All radiation exposures of the experimental animals were performed with a General Electric Maxitron 250 unit operated at 30 mA. and a 250 kVp voltage gradient. A filter, consisting of 0.25 mm of copper and 1.0 mm of aluminum, was placed in the X-ray beam during exposures.

Newts were exposed to the X-rays in individual, ventilated, plastic vials placed on a rotating lucite table (1 rpm). The target-to-surface distance was 91 cm, and a dose rate of 51 R per minute was calibrated using a Victoreen R-meter. If numerous exposures were scheduled, the dose rate was determined periodically to ensure consistency of exposure.

Administration of Colcemid[2]

Colcemid was used in all experiments in which the mitotic index was a measured parameter. Colcemid was dissolved in distilled water

2. Grand Island Biologicals, Grand Island, New York.

at a concentration of 1 mg/ml and was administered by intraperitoneal injection. Little information was available concerning the use of colcemid on poikilothermic vertebrates, so the dose had to be determined empirically. A dose was selected for maximum inhibition with minimum induction of nuclear pyknosis in intestinal epithelial proliferative cells. Newts used in this investigation weighed approximately 12 gm each and were injected with a 0.15 mg dose of colcemid. It was determined that five hours between injection of the drug and sacrifice of the animals was an appropriate time interval and it was used consistently throughout the study.

Administration of Tritiated Thymidine

For nuclear labeling experiments, tritiated thymidine[3] was administered by intraperitoneal injection. The stock solution was ^{3}H-methyl thymidine in sterile aqueous solution at a concentration of 1 mCi/ml and a specific activity of 6 Ci/mmole. It was diluted with sterile saline so that each animal was injected with 20 µCi in a 0.1 ml injection dose. That dose was empirically determined on the basis of a reported 1 µCi/g body weight dose administered to urodele amphibians by Scheving and Chiakulas (1965). At least four hours were allowed to elapse between tritiated thymidine injection and sacrifice of the animals.

3. Schwarz/Mann, Orangeburg, New York.

Necropsy and Histotechnique

At the scheduled time, newts were killed by decapitation and the digestive tract was removed from stomach to cloaca. The tract was fixed in ten percent neutral buffered formalin (phosphate buffers) for at least 24 hours. A section of the intestine located between 1 and 2 cm distal to the pyloric sphincter was chosen as a representative sample. That site was consistently used for histologic and autoradiographic evaluation throughout the investigation.

Tissue samples were dehydrated with ethanol to xylene in an Autotechnicon[4] and embedded in Paraplast.[5] Blocks, each containing four to five gut samples, were sectioned at a thickness of approximately 7 μm on a rotary microtome. A portion of the sectioned ribbon was mounted on each microscope slide so that each slide contained eight to ten cross-sections from each gut embedded in the Paraplast block. Duplicate slides were made from each block and processed simultaneously. All slides were stained with Periodic acid-Schiff (PAS) and hematoxylin stains. For autoradiography, slides were stained with the PAS reagents prior to dipping in liquid photographic emulsion because it was determined that immersion of developed autoradiographs in the periodic acid of the PAS-staining procedure removed silver grains from the emulsion layer. The hematoxylin nuclear stain was applied after autoradiographic exposure and development (Appendix I).

4. The Technicon Company, Chauncy, New York.
5. Sherwood Medical Industries, St. Louis, Missouri.

Autoradiographic Technique

The microscope slides were dipped, after PAS-staining, into a 45°C mixture of distilled water and Ilford L-4 Nuclear Track Emulsion, gel form (2:1). After a short drying time at room temperature, the slides were packed in light-tight slide boxes with a vial of desiccant and stored at 4°C for a three-week exposure time, after which they were developed in Kodak D-19. Development of the autoradiographs for seven minutes was followed by a rinse in distilled water and 30 seconds in Kodak Rapidfix. The slides were then stained with Harris' hematoxylin, dehydrated, and coverslips applied.

Microscopic Examination of Sections

Each microscope slide contained at least eight cross-sections of intestines from each of the newts from a treatment group of three to five animals. Counts for the determination of the mitotic indices, synthetic indices, and percent labeled mitoses were made on sections 1, 3, 5, 7, etc. of each intestine. That method generally revealed over 1000 proliferative cells and up to 50 mitotic figures with minimal probability of a cell being counted more than once. The duplicate slides were examined if it became necessary to increase numbers for statistical purposes. PLM values were based on 50 or more mitotic figures whenever possible and included nuclei with five or more silver grains in the photographic emulsion above them. Background

determinations were made by examining 100 mature epithelial cells on each slide. Unless a wrinkle or break in the tissue section was present, background was generally less than two silver grains per nucleus.

Experimental Design

The experiments conducted are listed in Table 2 which includes a brief description of the environmental conditions under which the animals were kept before and during the experiments. Two kinds of experiments are listed. Experiments 2a-c were designed to provide information on the cell cycle phase durations and the remainder were designed to determine the effects of environmental factors on mitotic activity. The time span listed is the period of time from the beginning of the experiment until the last group of animals was killed.

Diurnal Rhythms in Mitotic and Labeling Indices

A preliminary experiment was designed to reveal diurnal rhythms in the mitotic and DNA synthetic indices of cells in the intestinal epithelial cell nests. The schedules of injection and sacrifice are presented in Figures 1 and 2. Colcemid injections were used with eight groups of three newts per group (Experiment 1a); the groups correspond to groups 2 through 9 in Figure 1. Mitotic indices were calculated by dividing the number of mitotic figures from a series of

TABLE 2. List of Experiments Conducted Using the Intestinal Epithelial Proliferative Cells of the Rough-Skinned Newt as a Model.

Experiment #	No. of Groups	No. of Animals/Group	Time Span	Parameter Measured	Environmental Condition
1a	8	3	0 - 26 hrs	MI	Room temp with light-dark cycle
1b	8	3	0 - 26 hrs	LI	
2a	9	5	0 - 31 hrs	MI + LI + PLM	Room temp with light-dark cycle
2b	20	4	0 - 58 hrs	MI + LI + PLM	
2c	10	4	40 - 76 hrs	MI + LI + PLM	
3	20	4	0 - 78 hrs	MI + PLM	Room temp with 1000 R whole-body irradiation
4	9	3	0 - 68 hrs	MI + PLM	4°C with continuous darkness
5	10	4	0 - 32 hrs	MI	Room temp with light-dark cycle
6	10	4	0 - 32 hrs	MI	Room temp with light-dark cycle + injection ^{3}H-TdR
7	10	4	0 - 32 hrs	MI	4°C with light-dark cycle
8	10	3	0 - 32 hrs	MI	4°C with continuous darkness
9	10	4	0 - 32 hrs	MI	Room temp after 2 months at 4°C in darkness
10	10	4	0 - 32 hrs	MI	Room temp with light-dark cycle after 100 R whole-body irradiation
11	19	4	0 - 56 hrs	MI	Room temp with light-dark cycle after 200 R whole-body irradiation
12	10	4	0 - 32 hrs	MI	Room temp with light-dark cycle after 500 R whole-body irradiation

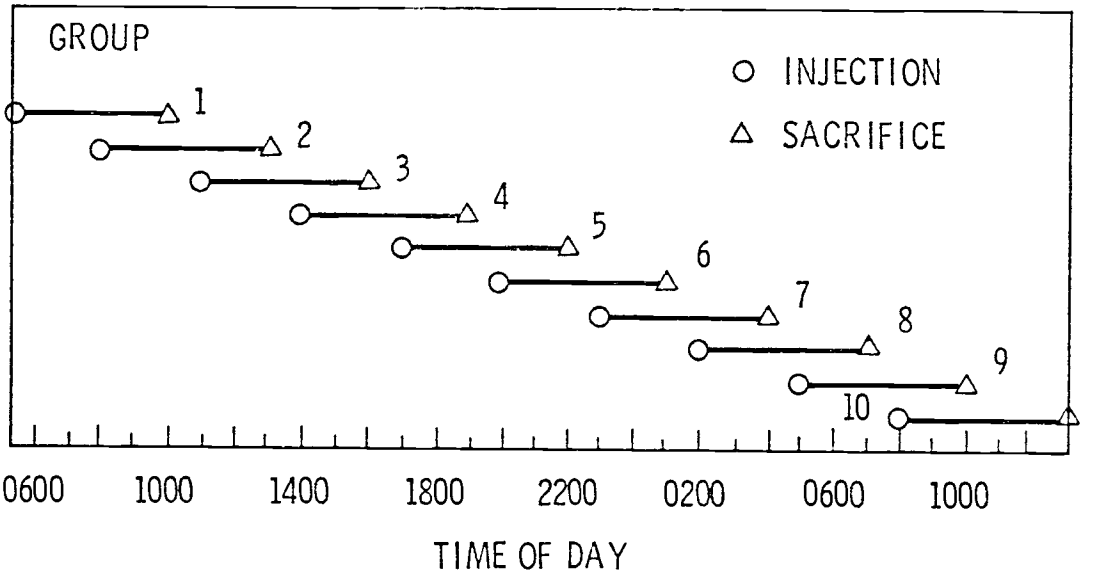


Figure 1. Colcemid injection and newt sacrifice schedule for determination of mitotic index fluctuations over a 24-hour period.

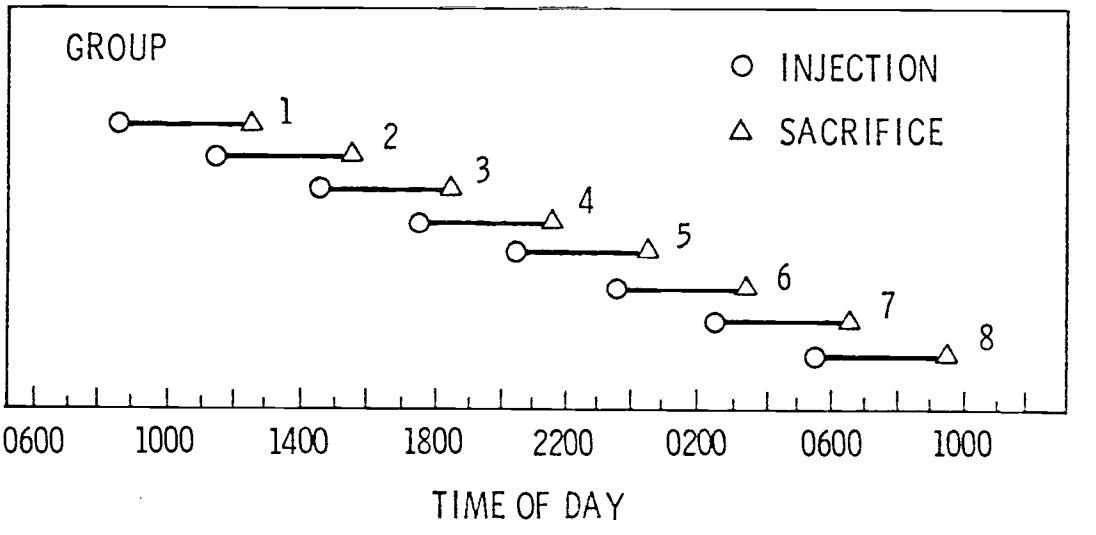


Figure 2. Tritiated thymidine injection and sacrifice schedule for determination of labeling index fluctuation over a 24-hour period.

sections by the total number of proliferative cells in those sections. Eight other groups with three newts in each group (Experiment 1b) were injected with tritiated thymidine according to the schedule shown in Figure 2. Labeling indices were calculated by dividing the number of labeled cells in a series of sections by the total number of proliferative cells in cell nests of those sections. Mitotic indices were calculated as described above. Before and during the two experiments, the newts were kept at room temperature (22-23°C) near a window for exposure to the natural light-dark cycle.

Cell Cycle Duration of the Epithelial Proliferative Cells

At Room Temperature. The objective of Experiment 2 was to determine the duration of the cell cycle phases in the intestinal epithelial proliferative cells at room temperature (22-23°C) by the percent labeled mitoses method. All newts in Experiment 2a were injected with ^{3}H-TdR at 0800 hours. Groups of five newts each were killed at three-hour intervals beginning at 1500 hours; the last group was killed 31 hours postinjection. Each newt was given an intraperitoneal injection of colcemid five hours before its scheduled sacrifice.

The number of mitotic figures in the cell nests fluctuated considerably during the experimental period and was relatively low at most of the sacrifice times. Experiments 2b and 2c were designed so

that the sacrifice times of all newts coincided with a time of peak mitotic activity (i.e., 1300 hours). In those experiments, the number of hours between injection of ^{3}H-TdR and sacrifice was regulated by injecting groups of animals at varying times prior to sacrifice. Each animal used in Experiments 2b and 2c was injected with colcemid at 0800 hours and sacrificed at 1300 hours. The twenty groups with four newts per group were injected with ^{3}H-TdR at times which resulted in the injection-to-sacrifice periods shown in Table 3. Experiment 2c was an overlapping extension of Experiment 2b. Ten groups, each containing four newts, were injected with ^{3}H-TdR with resultant injection-to-sacrifice periods also shown in Table 3.

After 1000 R X-Ray Exposures. The design of Experiment 3 was essentially the same as that used in Experiments 2b and 2c. Twenty groups with four newts per group were injected with ^{3}H-TdR with resultant injection-to-sacrifice periods shown in Table 3. Each of the groups was exposed to 1000 R whole-body X-irradiation, under the conditions previously described, prior to the ^{3}H-TdR injection. The newts were kept at room temperature (22-23°C) before, during, and after irradiation and injection. All newts in Experiment 3 were injected with colcemid at 0800 hours and sacrificed at 1300 hours.

At 4°C With Continuous Darkness. Experiment 4 was a preliminary investigation to study the effects of cold environmental temperature on the cell cycle of the intestinal epithelial proliferative cells.

TABLE 3. Time Between Tritiated Thymidine Injection and Sacrifice for Groups of Newts Used in Experiments 2b, 2c, 3 and 4.

Experiment #	No. of Groups	Animals/ Group	Treatment	Respective Times between Injection and Sacrifice (hours)
2b	20	4	Room temperature with light-dark cycle	5, 7, 9, 11, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 59, 52, 55, 58
2c	10	4	Room temperature with light-dark cycle	40, 44, 48, 52, 56, 60, 64, 68, 72, 76
3	20	4	Room temperature with light-dark cycle and 1000 R whole-body irradiation	5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 28, 33, 38, 43, 48, 54, 60, 66, 72, 78
4	9	3	4°C temperature and complete darkness	5, 12, 20, 28, 36, 44, 52, 60, 68

The newts were acclimated to cold (4°C) under unlighted conditions. Groups of three newts were injected with ^{3}H-TdR at times which resulted in injection-to-sacrifice times shown in Table 3. Colcemid was administered at 0800 hours and the animals were sacrificed at 1300 hours.

Influence of External Stimuli on the Daily Mitotic Index

Because of the difficulty in obtaining sufficient data for a complete PLM curve, a series of experiments was designed to determine the effects of various stimuli on the daily mitotic index of the newt intestinal epithelial proliferative cells.

MI at Room Temperature With a Light-Dark Cycle. Experiment 5 was a duplication of Experiment 1a but with more animals per group and two additional groups. Ten groups of four newts were injected with colcemid and sacrificed five hours later as shown in Figure 1. The animals were kept at room temperature (22-23°C) with a photoperiod of 14 hours of light and 10 hours of darkness (14L:10D) before and during the experiments.

MI After Tritiated Thymidine Injection. The effect of a single ^{3}H-TdR injection on the daily mitotic rhythm was investigated. Ten groups with four newts in each group were injected with ^{3}H-TdR at 0400 and, thereafter, treated as shown in Figure 1. The newts were acclimated to room temperature (22-23°C) with a 14L:10D cycle.

MI at 4°C With a Light-Dark Cycle. Newts used in Experiment 7 were acclimated to an environmental temperature of 4°C in the presence of a 14L:10D cycle. Ten groups with four newts per group were used in that experiment and the experimental design outlined in Figure 1 was utilized.

MI at 4°C With Continuous Darkness. Newts used in Experiment 8 were also acclimated to an environmental temperature of 4°C but were kept in continuous darkness. Ten groups, each containing three newts, were used in Experiment 8. They were injected with colcemid and sacrificed as shown in Figure 1.

MI at Room Temperature After Prolonged Cold and Darkness. Prolonged survival of amphibians in laboratories is routinely accomplished by storing them in a cold environment. During the course of this work, it was determined that nearly 100 percent of Taricha granulosa survived after three months in a 4°C environment. Experiment 9 was designed to determine the effect of prolonged cold storage with subsequent warming to room temperature on the mitotic index of the intestinal epithelial proliferative cells. Forty newts were kept in continuous darkness at 4°C for 60 days after which they were allowed to warm to room temperature over a 24-hour period two days before the experiment began. They were divided into ten groups of four, which were then treated with colcemid as outlined in Figure 1.

MI After Acute Exposure to X-Rays. The 1000 R whole-body X-ray exposure used in Experiment 3 caused a nearly complete cessation of

mitotic activity in the intestinal epithelial proliferative cells. Experiments 10, 11, and 12 were designed to determine the effects of 100 R, 200 R, and 500 R exposures, respectively, on the mitotic activity in those cells. The newts were irradiated between 0445 hours and 0500 hours and the colcemid injection-sacrifice schedule shown in Figure 1 was then initiated.

In Experiment 10, ten groups, each containing four newts, were used. Nineteen groups with four newts per group were used in Experiment 11. The first ten groups were injected and sacrificed as shown in Figure 1 and the remaining ten groups were continued on the same schedule for an extra day. Ten groups with four newts in each group were used in Experiment 12. They were injected with colcemid and sacrificed as shown in Figure 1.

Statistical Analysis of Data

Means and standard deviations listed in the tables of results were calculated with a Hewlett-Packard 45 pocket calculator.

An analysis of variance for many-sample comparison with one-way classification was used to determine whether or not any of the sample means from an experiment differed statistically from the others. A subsequent studentized range test was used to reveal which, if any, of the sample means was different from another or others.

An analysis of variance for a multiple observation, factorial experiment was used to elucidate differences between the sets of

sample means from animals which received different treatments. Thus, effects of two or more treatments can be compared, an effect of the time of day can be determined, and an interaction between the treatment and time of day can be identified.

Examples of those tests and the method for determining a 95 percent confidence interval are presented in Appendix III. Appendix III also contains the results of statistical analyses performed on data collected in this investigation.

RESULTS

Photomicrographs of newt (Taricha granulosa) intestinal cross-sections are shown in Figure 3. Included are low magnification photomicrographs for illustration of the general morphology and high magnification photomicrographs of mitotic figures and autoradiographically labeled cells.

Appendix II contains the detailed data from the epithelial cell kinetics experiments. Those data are summarized in Tables 4 through 14 within this section. All the indices are expressed as percents (x100) in the tables for ease of handling in statistical analyses.

Diurnal Rhythms in Mitotic and Labeling Indices

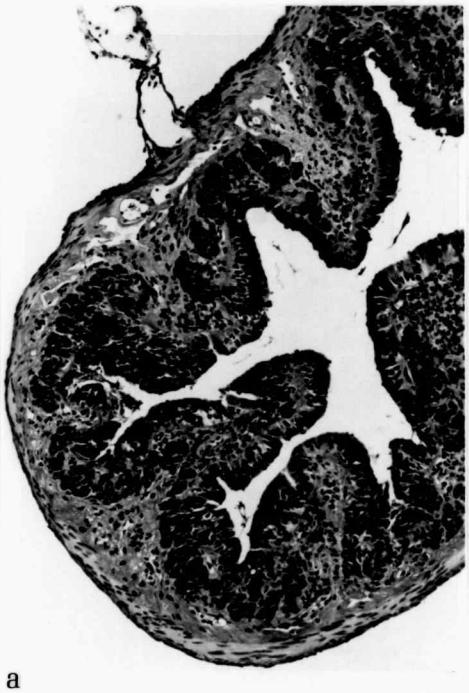
The results of a preliminary experiment (Experiment 1) are summarized in Tables 4 and 5 and are graphically illustrated in Figure 4. The overall mitotic index in the cell nests of colcemid-injected newts was 0.0068. Peak sample mitotic indices occurred at 1300 hours and at 0400 hours, and the lowest mean values occurred at 2200 and at 0100 hours. Peak sample mitotic indices occurred at 0630 and 2130 hours in the intestines of those animals injected with tritiated thymidine only. Low indices were observed between 0930 hours and 1530 hours and at 0300 hours. The overall mitotic index of

Figure 3a. Photomicrograph of an intestinal cross-section from a newt (Taricha granulosa) acclimated to room temperature (22-23°C). Periodic acid-Schiff's Reagent (PAS) and Hematoxylin stain (X60).

Figure 3b. Higher magnification photomicrograph of the same section as in Figure 3a. Cell nests are apparent in the submucosa. PAS and Hematoxylin (X150).

Figure 3c. Photomicrograph showing autoradiographically labeled cells in the cell nests. The newt was killed four hours after ^{3}H-TdR injection and the autoradiograph was exposed for 30 days before photographic development. Hematoxylin and Eosin (X300).

Figure 3d. Photomicrograph showing mitotic figures in the cell nests. The epithelial basement membrane is extended into the submucosa to surround the cell nests. PAS and Hematoxylin (X300).



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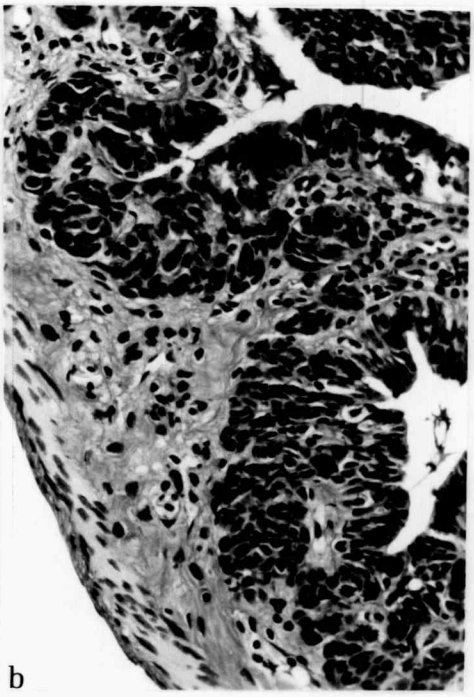

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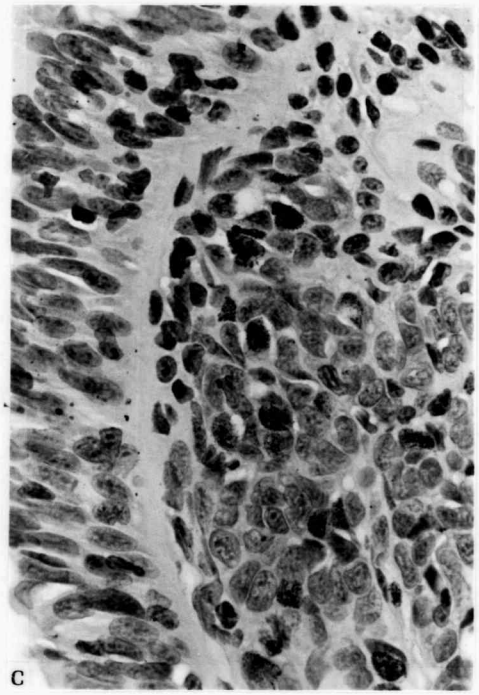

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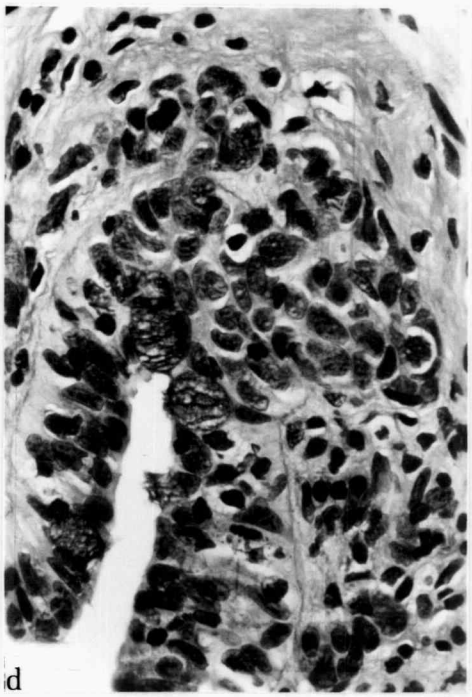


d

TABLE 4. Mean Mitotic Indices in the Intestinal Cell Nests of Newts (T. granulosa) at Three-Hour Intervals over a 21-hour Period. All newts were kept at a 22-23°C environmental temperature with a 14L:10D cycle and were injected with colcemid five hours before sacrifice (Experiment 1a).

Group #	Number of Animals	Sacrifice Time	MI + SD (X 100)
2	3	1300	1.10 ± 0.44
3	3	1600	0.52 ± 0.09
4	3	1900	0.71 ± 0.07
5	3	2200	0.45 ± 0.30
6	3	0100	0.39 ± 0.22
7	3	0400	1.04 ± 0.99
8	3	0700	0.64 ± 0.55
9	3	1000	0.59 ± 0.29
Overall Mean			0.68

TABLE 5. Mean Mitotic Indices in the Intestinal Cell Nest of Newts at Three-hour Intervals over a 21-hour Period. All newts were kept at a 22-23°C environmental temperature with a 14L:10D cycle and were injected with tritiated thymidine four hours before sacrifice (Experiment 1b).

Group #	No. of Animals	Sacrifice Time	MI + SD (X 100)	LI + SD (X 100)
2	3	1230	0.24 ± 0.06	25.2 ± 17.8
3	3	1530	0.20 ± 0.03	14.3 ± 2.4
4	3	1830	0.30 ± 0.05	7.5 ± 7.6
5	3	2130	0.46 ± 0.23	25.7 ± 5.1
6	3	0030	0.24 ± 0.10	10.2 ± 4.0
7	3	0330	0.17 ± 0.06	4.1 ± 4.8
8	3	0630	0.39 ± 0.04	13.9 ± 8.5
9	3	0930	0.21 ± 0.10	4.8 ± 5.3
Overall Mean			0.28	13.2

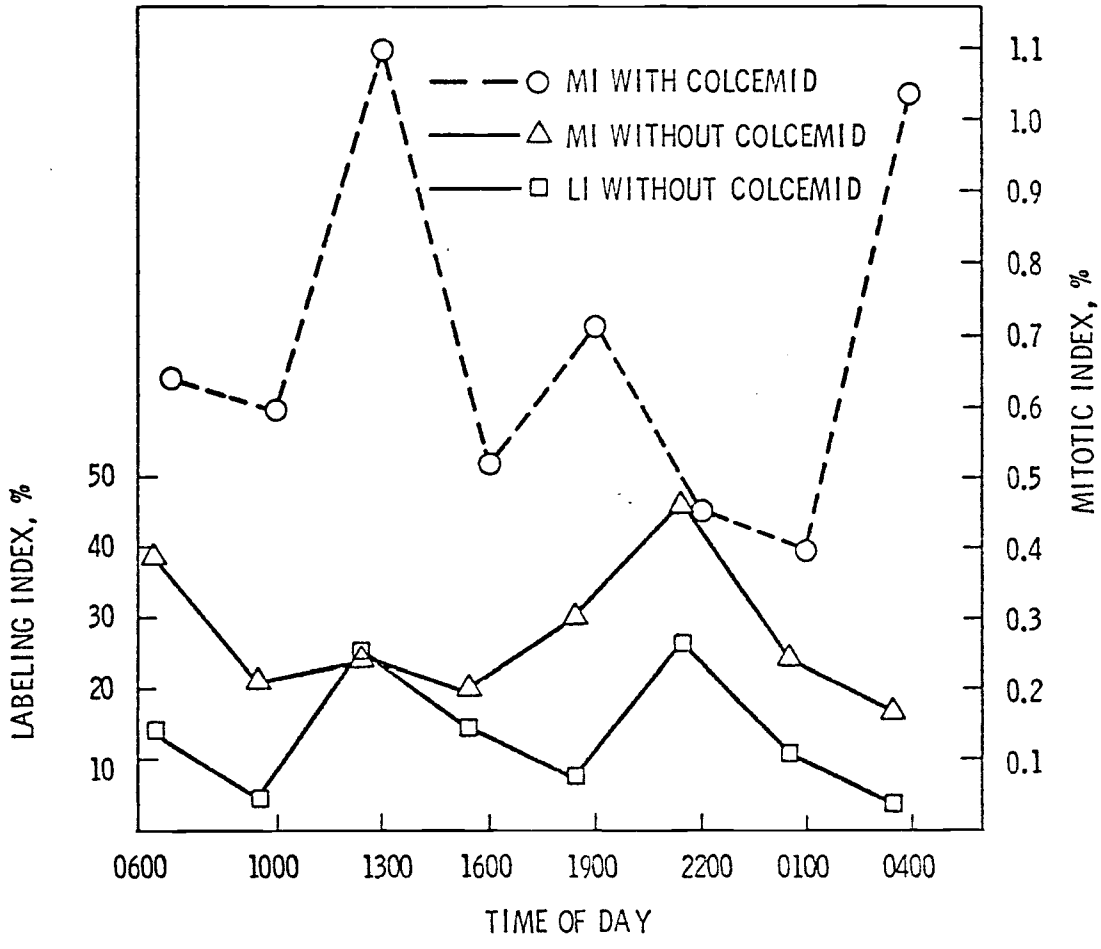


Figure 4. Sample mean mitotic indices in the intestinal cell nests of newts kept at an environmental temperature of 22-23°C with a 14L:10D cycle (Experiments 1a and 1b).

intestinal cell nests of those animals was 0.0028, nearly a factor of three less than that of the colcemid injected newts. Tritiated thymidine labeling index peaks occurred at 1230 and 2130 hours, and the lowest indices were at 0930, 1830, and 0330; the overall labeling index was 0.132.

Statistical analyses of data from Experiments 1a and 1b (Appendix III - D and E) revealed no significant differences between the sample mean mitotic indices within the 21-hour period whether or not colcemid was used. Comparison of the set of means from Experiment 1a with the set of means from Experiment 1b (Appendix III - F) showed that mitotic indices were significantly higher ($p < 0.01$) in the intestines of colcemid-treated newts and that there was a significant interaction ($p < 0.05$) between treatment and time of day. Colcemid was more or less effective at certain times of the 21-hour period even though the times could not be identified. An analysis of variance of sample mean labeling indices over the 21-hour period (Appendix III - G) indicated that a significant difference between two or more means existed but the subsequent studentized range test did not substantiate the difference.

Cell Cycle Duration in the Epithelial Proliferative Cells

Experiments 2a, 3, and 4 were designed to reveal the cell cycle phase times under three conditions: room temperature (2a); after 1000 R whole-body radiation (3); and at 4°C (4). The three experiments

were initially designed to provide preliminary data and, on the basis of that data, they were to be expanded to yield more complete information.

At Room Temperature

Newts were injected with ^{3}H-TdR at 0800 and groups of them were sacrificed periodically thereafter (Table 2) to elucidate the cell cycle at room temperature. The mean percent labeled mitoses (PLM), mean mitotic indices and mean labeling indices for each group are presented in Table 6; the PLM curve is illustrated in Figure 5. The first three points of the curve in Figure 5 (7 to 13 hours post ^{3}H-TdR injection) define the ascending leg and essentially 100 percent of the mitoses were labeled thereafter. The ascent of the curve is of approximately six hours duration and crosses the 50 percent line at approximately 9.5 hours postinjection.

No statistically significant peak or nadir sample means were present in the curve of mean mitotic indices (Appendix III - H). The last sample mean labeling index (1500 hours) was significantly greater than the lowest sample mean labeling index which occurred at 2400 hours (Appendix III - I). Because ^{3}H-TdR was administered to all newts of Experiment 2a at essentially the same time of day, there is no reasonable explanation for the significant difference in labeling means. The overall mitotic index was 0.0054 and the overall tritiated thymidine labeling index was 0.266.

TABLE 6. Mean Mitotic Indices, Labeling Indices, and Percent Labeled Mitoses in Intestinal Cell Nests of Newts Kept at an Environmental Temperature of 22-23°C with a 14L:10D Cycle. All animals were injected with tritiated thymidine at 0800 and were injected with colcemid five hours before sacrifice (Experiment 2a).

Group #	# of Animals	Sacrifice Time	Hours Post Injection	MI ± SD (X 100)	LI ± SD (X 100)	PLM ± SD
1	5	1500	7	0.64 ± 0.19	17.8 ± 6.9	18.1 ± 9.4
2	5	1800	10	0.55 ± 0.31	19.2 ± 8.8	61.7 ± 13.1
3	5	2100	13	0.79 ± 0.86	25.9 ± 7.7	92.0 ± 8.4
4	3	2400	16	0.22 ± 0.09	15.9 ± 7.5	96.3 ± 6.4
5	5	0300	19	0.35 ± 0.04	18.1 ± 10.6	94.6 ± 7.4
6	4	0600	22	0.41 ± 0.10	19.5 ± 4.1	96.7 ± 6.7
7	2	0900	25	0.21 ± 0.06	29.3 ± 9.0	100.0 ± 0
8	5	1200	28	0.21 ± 0.24	25.0 ± 5.6	100.0 ± 0
9	5	1500	31	0.56 ± 0.52	35.5 ± 7.8	96.6 ± 6.2
Overall Mean				0.54	26.6	

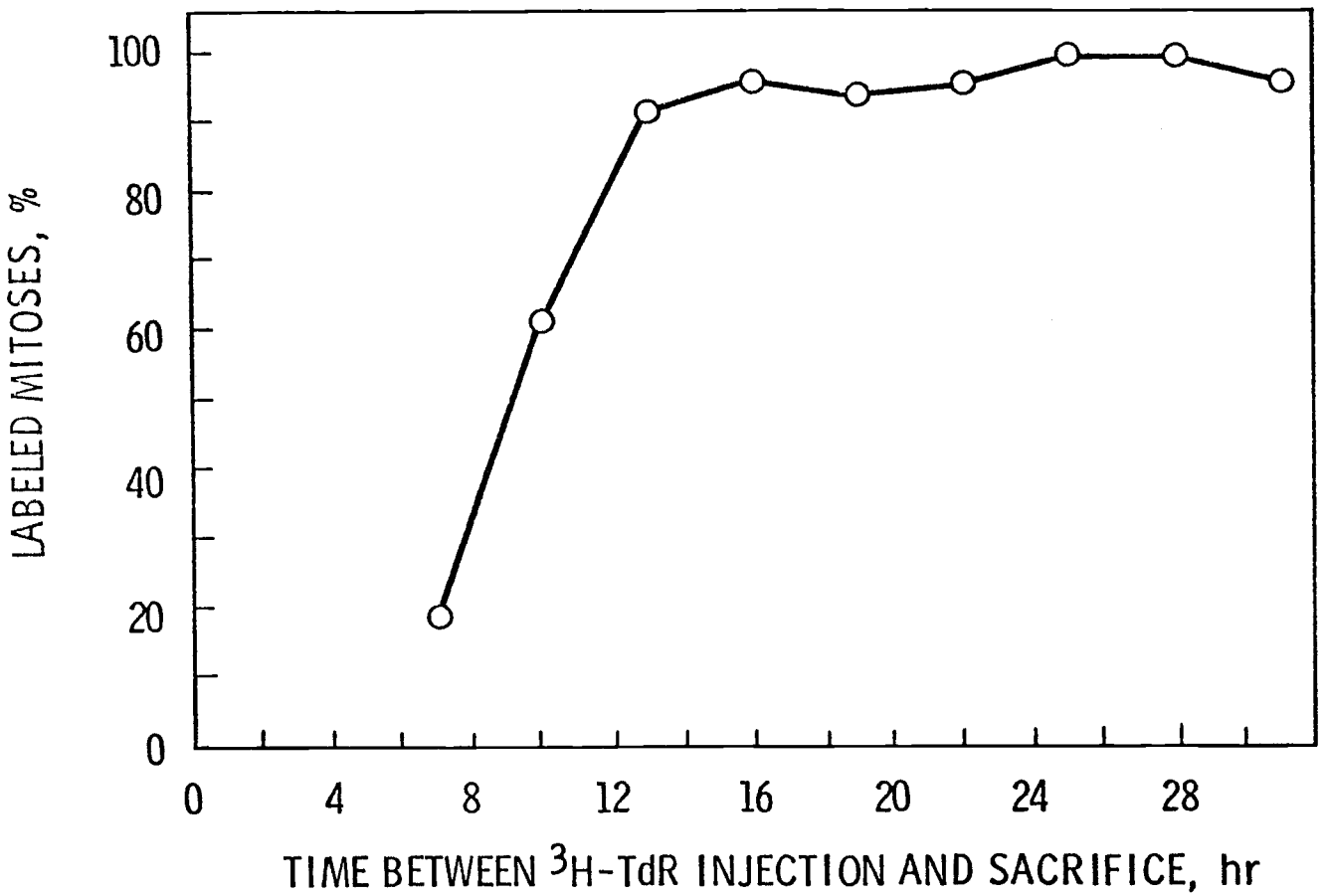


Figure 5. Percent labeled mitoses in the intestinal cell nests of newts between 7 and 31 hours after injection with tritiated thymidine (Experiment 2a).

Because of the low mitotic indices in some of the newts, particularly those at 0300 hours and 1200 hours, a decision was made to try another approach to the percent labeled mitoses method. Data from Experiment 1a (Table 4) indicated that a relatively high mitotic index could be obtained with an 0800-hour colcemid injection time followed by sacrifice at 1300 hours. Experiment 2b was, therefore, designed so that all the animals were sacrificed at that time; groups of newts were injected with ^{3}H-TdR at specified time intervals before that.

The mean mitotic indices, labeling indices, and percent labeled mitoses for each group of newts are presented in Table 7 and the percent labeled mitoses curve is illustrated in Figures 6 and 7. Figure 7 illustrates the similarity of data obtained by the two methods. Cell cycle phase time estimates can be derived from the PLM curves of Figures 6 and 7 using the method of Sherman and Quastler (1960). The sum, T_{G2} plus one half T_M, is the time period between the time ^{3}H-TdR was injected and the time at which 50 percent of the mitotic figures were labeled. T_S was estimated at 41 hours by including an artificial descending leg of the PLM curve having the same slope as the ascending leg. Because a second ascending portion of the curve could not be obtained, no generation time (T_C), could be estimated from the PLM curve of Figure 6.

Experiment 2c was designed to provide data which would allow an extension of the PLM curve through a second ascending portion. The

TABLE 7. Mean Mitotic Indices, Labeling Indices, and Percent Labeled Mitoses in the Intestinal Cell Nests of Newts Kept at an Environmental Temperature of 22-23°C with a 14L:10D Cycle. All newts were injected with colcemid between 0800 and 0830 hours and killed between 1300 and 1330 hours (Experiment 2b).

Group #	Animal #	Injection Time	Hours Post ^{3}H-TdR Injection	MI ± SD (X 100)	LI ± SD (X 100)	PLM ± SD
1	4	0800	5	2.32 ± 0.33	14.6 ± 5.2	1.1 ± 1.1
2	4	0600	7	2.68 ± 1.46	19.5 ± 11.2	21.8 ± 14.2
3	3	0400	9	1.33 ± 0.43	6.9 ± 2.4	43.3 ± 7.9
4	4	0200	11	1.52 ± 0.71	27.3 ± 11.4	77.9 ± 10.1
5	4	2400	13	2.31 ± 1.30	22.0 ± 9.4	94.8 ± 3.7
6	4	2100	16	1.76 ± 1.29	22.0 ± 8.8	97.8 ± 2.3
7	4	1800	19	1.91 ± 0.84	19.7 ± 9.9	98.9 ± 0.9
8	4	1500	22	1.62 ± 1.07	14.8 ± 6.2	99.5 ± 1.0
9	4	1200	25	1.05 ± 0.72	15.1 ± 8.5	100.0 ± 0
10	4	0900	28	1.42 ± 0.89	23.8 ± 13.4	100.0 ± 0
11	4	0600	31	0.87 ± 0.43	16.5 ± 8.3	100.0 ± 0
12	4	0300	34	0.45 ± 0.27	10.9 ± 4.5	95.4 ± 5.7
13	4	2400	37	0.70 ± 0.42	14.9 ± 3.5	95.9 ± 6.1
14	4	2100	40	0.81 ± 0.37	16.1 ± 7.7	96.8 ± 2.9
15	4	1800	43	0.58 ± 0.09	11.0 ± 3.0	96.4 ± 5.6
16	4	1500	46	0.60 ± 0.34	11.0 ± 4.0	93.4 ± 4.5
17	4	1200	49	0.90 ± 0.87	9.0 ± 2.0	73.5 ± 17.9
18	4	0900	52	1.26 ± 1.23	11.6 ± 7.4	73.6 ± 12.7
19	4	0600	55	1.50 ± 0.97	28.9 ± 17.5	63.5 ± 7.7
20	4	0300	58	1.16 ± 0.78	13.4 ± 3.4	67.3 ± 9.2
Overall Mean				1.33	16.5	

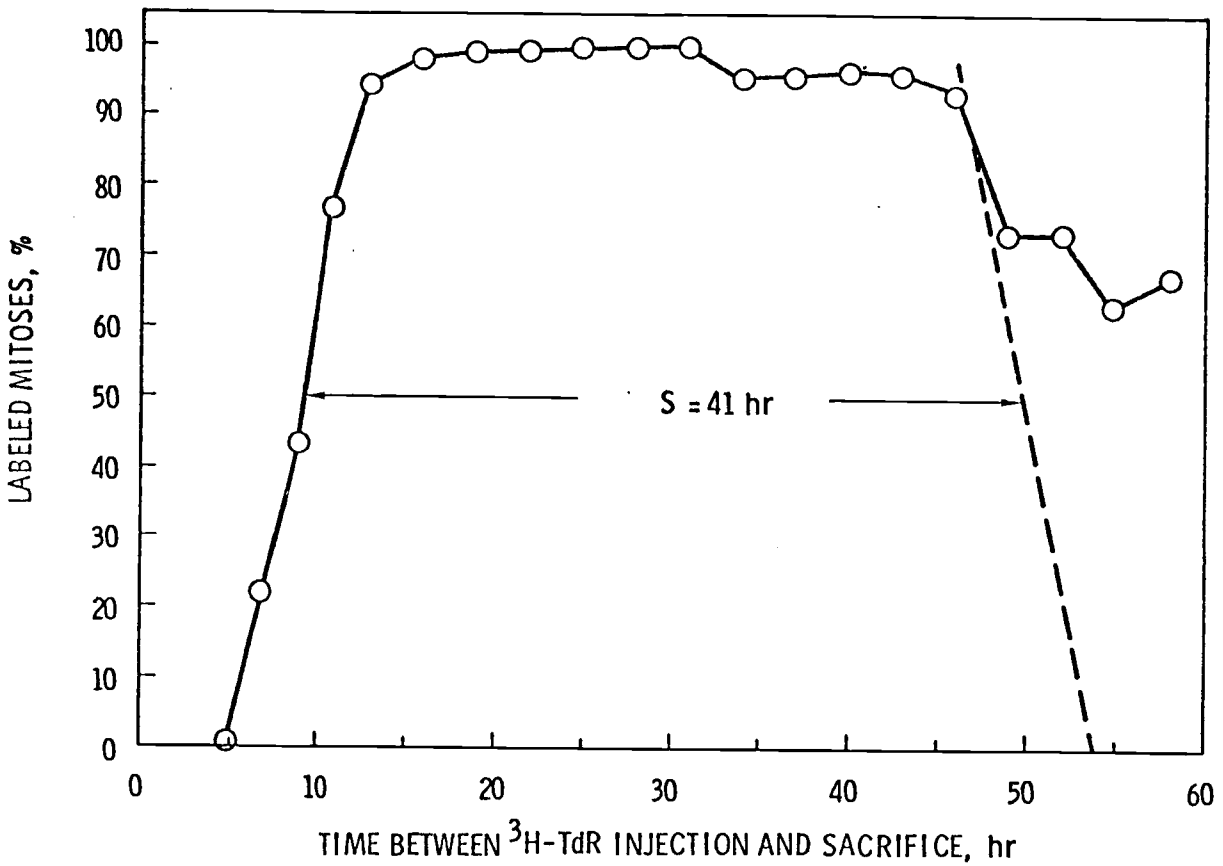


Figure 6. Percent labeled mitoses in the intestinal cell nests of newts between 5 and 58 hours after injection with tritiated thymidine (Experiment 2b).

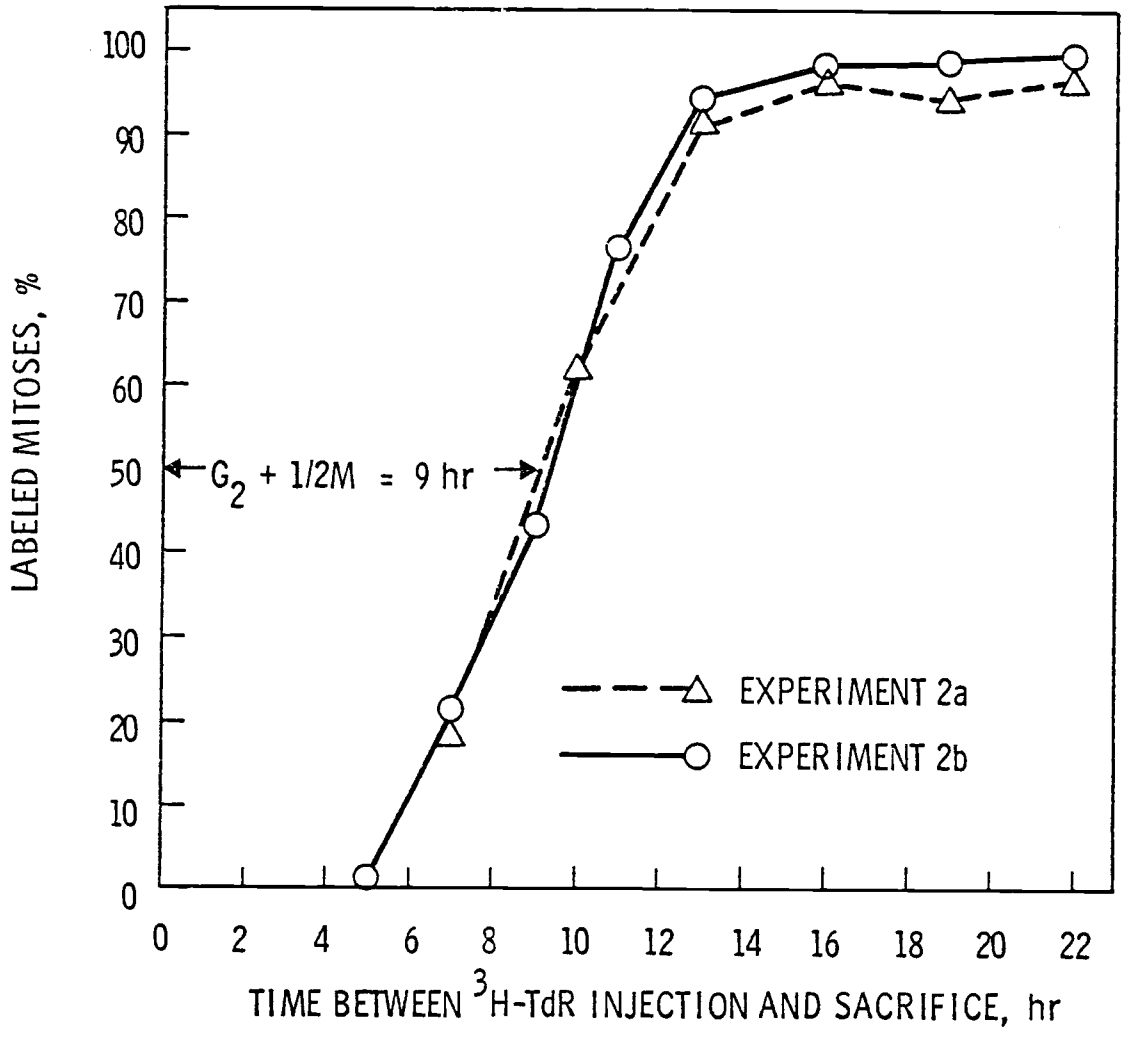


Figure 7. A comparison of the ascending legs of two percent labeled mitoses curves obtained by different methods (Experiments 2a and 2b).

mitotic indices from animals in that experiment were very low and counts of labeled mitoses fluctuated to such extremes that they could not be considered valid. Because that fluctuation was also evident in the final four points on the PLM curve from Experiment 2b data, no further attempts were made to extend the PLM curve.

Although all animals were killed at essentially the same time, there was a relatively large fluctuation of the sample mean mitotic indices in data from Experiment 2b. An analysis of variance was performed on the data (Appendix III - J) and it was shown that many significant differences ($p < 0.05$) between sample mean mitotic indices existed. Generally, mitotic indices were higher in intestines of animals injected with tritiated thymidine less than 24 hours before sacrifice than in those injected more than 24 hours before sacrifice (see Appendix III - J for details). There is no reasonable explanation for the differences in mitotic indices.

The labeling index fluctuated considerably over the 58-hour duration of Experiment 2a. An analysis of variance (Appendix III - K) showed that three of the values were significantly higher than the lowest value and six of the values were significantly lower than the highest value (see Appendix III - K for details). No diurnal cycle was apparent in the data except that the three significant peak values occurred in the early morning hours, and in each of the three consecutive mornings.

After 1000 R X-Ray Exposure

The cell renewal system in the intestinal epithelial cell nests of the newt is apparently quite radiosensitive. Exposure to 1000 R X-rays effectively stopped mitotic activity in those cells for the duration of Experiment 3 and no PLM curve could be obtained.

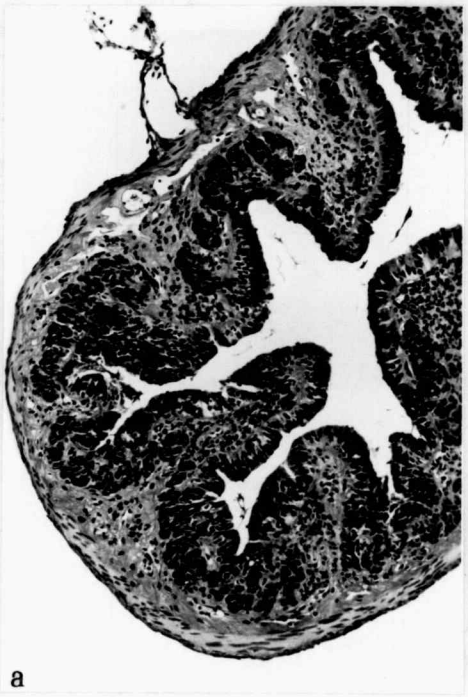
Histologically, a progression of lesions leading toward a depletion of the cell nests was observed. Large numbers of pyknotic nuclei were present in the proliferative areas of intestinal cross-sections within 24 hours postexposure. A progressive loss of cell nests was also noted during the first 24 hours, and the submucosa became vacuolar so that it had a "spongy" appearance. By 36 hours postexposure, the reduced number of cell nests and a vacuolar submucosa were characteristic histological features. Intestinal cross-sections from two of the three newts in the last group of animals (78 hours postexposure) were nearly devoid of cell nests. Throughout the progression of degenerative changes in the proliferative areas, only subtle changes were evident in the mucosal layer. The most prominent change was a decrease in numbers of cells present and, consequently, the mucosa became thinner. In three of the 75 animals, disorientation of cells in the epithelial layer was evident but was not severe and was limited to small foci. Photomicrographs of X-ray induced histological changes are presented in Figure 8.

Figure 8a. Photomicrograph of an intestinal cross-section from a nonirradiated newt (Taricha granulosa). Periodic acid-Shiff's Reagent (PAS) and Hematoxylin stain (X60).

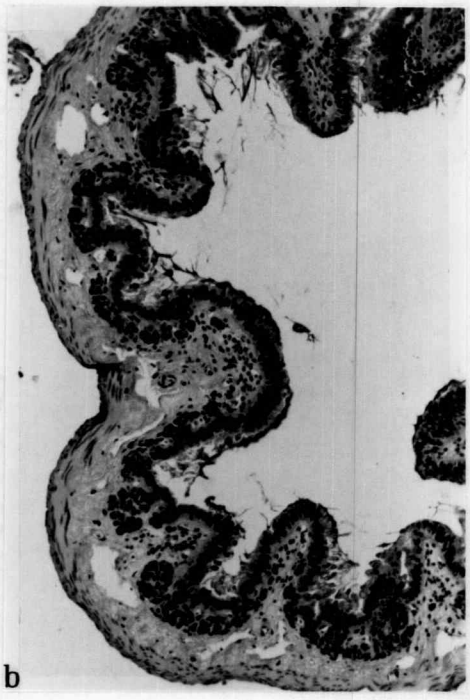
Figure 8b. Photomicrograph of an intestinal cross-section from a newt 31 hours after exposure to 500 R X-rays. There is a reduction in size and depletion in number of cell nests. PAS and Hematoxylin (X60).

Figure 8c. Photomicrograph of an intestinal cross-section from a newt 78 hours after exposure to 1000 R X-rays. The cell nests were severely reduced in number and in size. PAS and Hematoxylin (X60).

Figure 8d. Higher magnification photomicrograph of the same section as in Figure 8c. The mucosal depth was reduced and pyknotic nuclei were present in the mucosa. PAS and Hematoxylin (X150).



a



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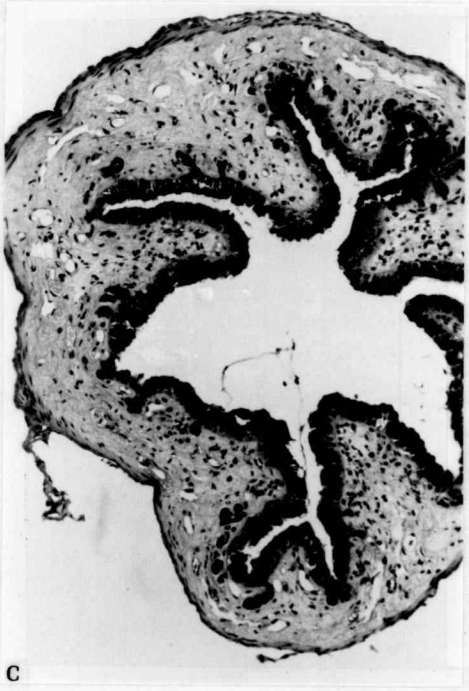

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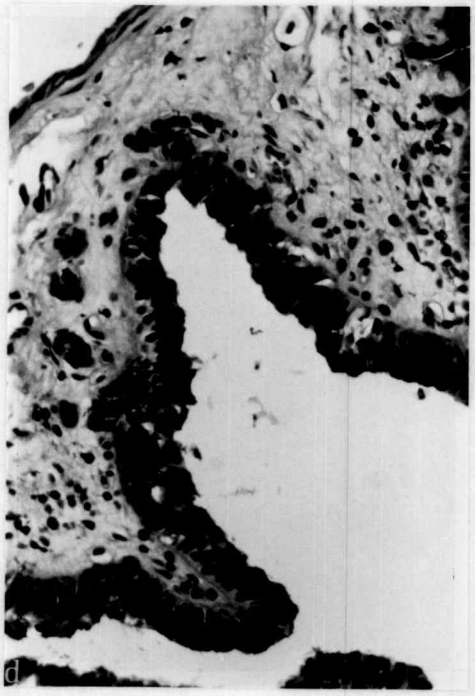

d

At 4°C With Continuous Darkness

Acclimation of the newts to 4°C and to darkness resulted in reduced mitotic activity in intestinal cell nests. Such environmental conditions were maintained before, during and after ^{3}H-TdR injection of newts in Experiment 4 until they were killed. So few mitotic figures could be found in intestinal cross-sections that no PLM data could be obtained. No evidence of histological damage was observed.

Influence of External Stimuli on the Daily Mitotic Index

Observations made in Experiments 1, 2, 3, and 4 concerning the mitotic index of the newt intestinal cell nests led to Experiments 5, 6, 7 and 8, which were conducted simultaneously, and to Experiments 9, 10, 11 and 12, which were conducted shortly thereafter.

MI at Room Temperature with a Light-Dark Cycle

Considerable time had elapsed since the preliminary investigation of the mitotic index fluctuation in the cell nests over a 21-hour period (Experiment 1a), so another experiment was conducted (Experiment 5) in which newts were kept at 22 to 23°C with a 14L:10D cycle. Mean mitotic indices of the newt intestinal cell nests from groups containing four newts each were determined at three-hour intervals spanning a 27-hour period. An injection of colcemid was administered five hours before sacrifice; the newts were injected and killed according to the schedule shown in Figure 1.

Peak and nadir sample mitotic indices were not as pronounced in Experiment 5 as they were in the preliminary investigation (Experiment 1), but they did occur at approximately the same times. Peak indices were at 1300 to 1600 hours and the minimum sample mitotic index occurred at 0400 hours; the overall mitotic index was 0.027. Data from Experiment 5 are presented in Table 8 and are illustrated in Figure 9. An analysis of variance (Appendix III - L) indicates that none of the sample mean mitotic indices was significantly different from another.

MI After a Tritiated Thymidine Injection

In Experiment 6, 40 newts were injected with tritiated thymidine at 0400 hours. Groups containing four newts each were injected with colcemid and killed at times corresponding to the groups of Experiment 5 (Figure 1). Data from Experiment 6 are presented in Table 9 and are compared with those from Experiment 5 in Figure 9. The single injection of tritiated thymidine had an apparent initial disruptive effect on mitotic activity. It was followed by a longer period of relatively low activity and then by a gradual rise in the mitotic index to values approximating those for animals without the ^{3}H-TdR injection.

The overall mitotic index was 0.0218, only slightly lower than the 0.0274 determined in Experiment 5 above. Analyses of variance

Table 8. Mean Mitotic Indices in the Intestinal Cell Nests of Newts at Three-hour Intervals over a 27-hour Period. All newts were kept at a 22-23°C environmental temperature with a 14L:10D cycle and were injected with colcemid five hours before sacrifice (Experiment 5).

Group #	Number of Animals	Sacrifice Time	MI + SD (X 100)
1	4	1000	3.19 ± 1.09
2	4	1300	3.44 ± 0.91
3	4	1600	3.41 ± 1.51
4	4	1900	3.08 ± 1.03
5	4	2200	2.27 ± 0.75
6	4	0100	3.22 ± 1.82
7	4	0400	1.67 ± 0.67
8	4	0700	2.28 ± 1.10
9	4	1000	2.02 ± 0.60
10	4	1300	2.81 ± 0.64
Overall Mean			2.74

TABLE 9. Mean Mitotic Indices in the Intestinal Cell Nests of Newts at Three-hour Intervals over a 27-hour Period. All newts were kept at an environmental temperature of 22-23°C with a 14L:10D cycle, injected with tritiated thymidine at 0400 hours and injected with colcemid five hours before sacrifice (Experiment 6).

Group #	Number of Animals	Sacrifice Time	MI + SD (X 100)
1	4	1000	1.78 ± 0.83
2	4	1300	2.57 ± 1.24
3	3	1600	1.95 ± 0.73
4	4	1900	2.87 ± 1.00
5	4	2200	1.72 ± 1.02
6	4	0100	1.58 ± 1.02
7	4	0400	1.57 ± 0.43
8	4	0700	2.04 ± 0.66
9	4	1000	2.54 ± 3.05
10	4	1300	3.20 ± 3.76
Overall Mean			2.18

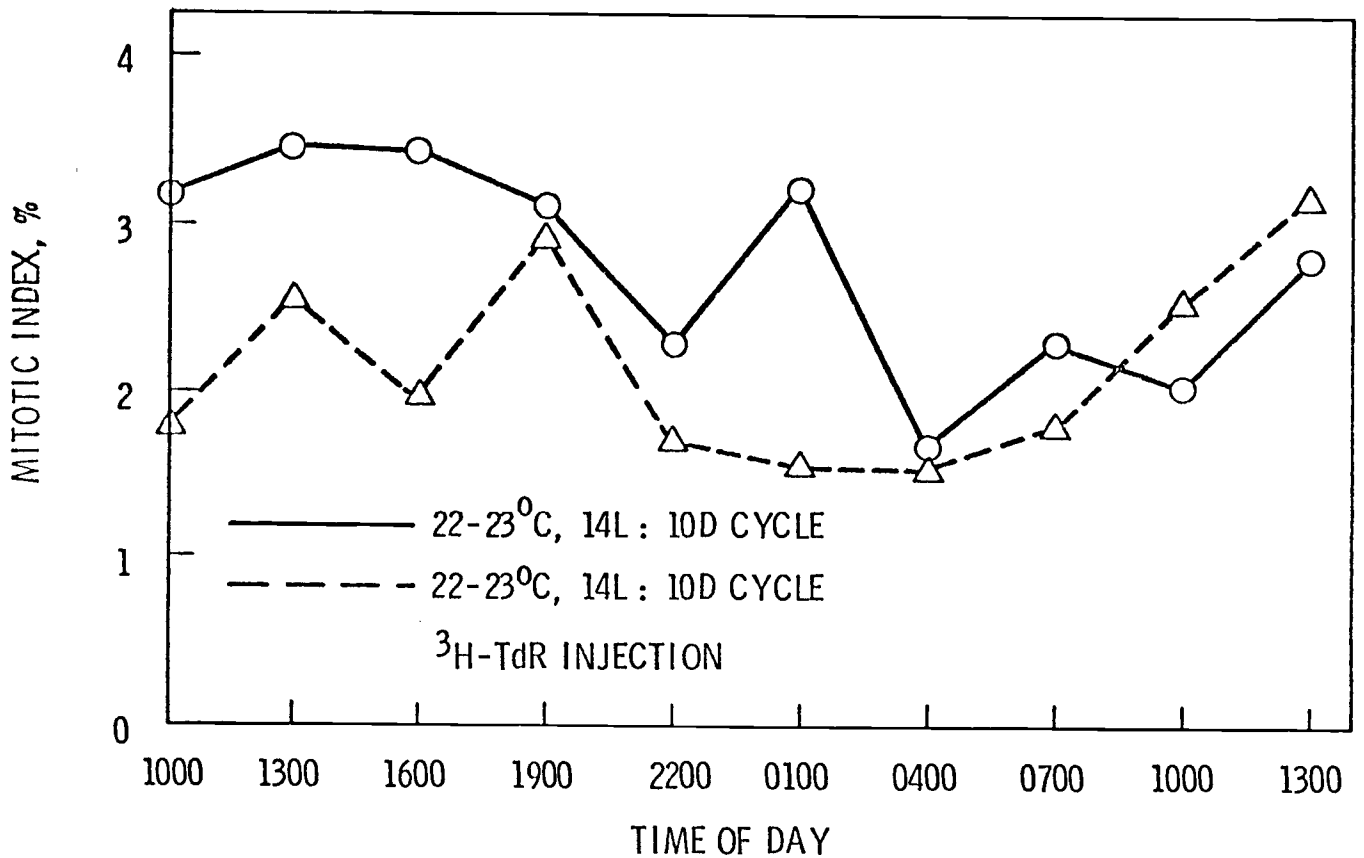

Figure 9. A comparison of sample mean mitotic indices from newts which were injected with tritiated thymidine and newts which were not injected with tritiated thymidine (Experiments 5 and 6).

(Appendices III-M and N) indicate that there was no significant difference between sample mean mitotic indices in Experiment 6 and that the ^{3}H-TdR injection produced no significant differences between the two sets of mitotic indices from animals of Experiments 5 and 6.

MI at 4°C With a Light-Dark Cycle

Lowering the environmental temperature significantly decreased the mitotic activity in the intestinal cell nests of the newt. All animals in Experiment 7 were kept at 4°C with a 14L:10D cycle. Ten groups with four newts in each group were injected with colcemid and subsequently killed at times corresponding with the groups of Experiment 5 (Figure 1). The results of Experiment 7 are listed in Table 10 and are compared to those from Experiment 5 in Figure 10.

The overall mean mitotic index in Experiment 7 was 0.012. Peak and nadir values of the sample mean mitotic indices were not pronounced. However, the lowest value occurred at 0400, the same time as in Experiments 5 and 6. An analysis of variance with the sample means from Experiment 7 indicated that none was significantly different from another (Appendix III - O).

MI at 4°C With Continuous Darkness

The mitotic index of newt intestinal cell nests was significantly lower in darkness than with a 14L:10D cycle at the same 4°C

TABLE 10. Mean Mitotic Indices in Intestinal Cell Nests of Newts at Three-hour Intervals over a 27-hour Period. All newts were kept at an environmental temperature of 4°C with a 14L:10D cycle and were injected with colcemid five hours before sacrifice (Experiment 7).

Group #	Number of Animals	Sacrifice Time	MI + SD (X 100)
1	4	1000	1.07 ± 0.54
2	4	1300	1.12 ± 0.66
3	4	1600	1.58 ± 0.76
4	4	1900	1.23 ± 0.31
5	4	2200	1.54 ± 1.77
6	4	0100	1.15 ± 0.62
7	4	0400	0.79 ± 0.19
8	4	0700	0.90 ± 0.92
9	4	1000	1.42 ± 0.42
10	4	1300	1.21 ± 0.72
Overall Mean			1.20

environmental temperature (Experiment 8). Groups of three newts each were injected with colcemid and killed at times corresponding to those of the previous three experiments (Figure 1). The overall mean mitotic index was 0.0031 and none of the sample means from Experiment 8 differed significantly from another (Appendix III - P). Data from Experiment 3 are summarized in Table 11 and are graphically compared to those of Experiments 5 and 7 in Figure 10.

The three sets of means from Experiments 5, 7 and 8, which are graphically presented in Figure 10, were statistically compared using an analysis of variance for a multiple observation, factorial experiment (Appendix III - Q). Mitotic indices in the intestinal cell nests of newts acclimated to a 4°C environmental temperature were significantly lower ($P < 0.01$) than those of newts maintained at room temperature (22-23°C); both groups were exposed to the 14L:10D cycle. Mitotic indices in the intestinal cell nests of newts maintained in complete darkness were significantly lower ($P < 0.01$) than those of newts which were exposed to the 14L:10D cycle; both groups were acclimated to a 4°C environmental temperature.

MI After Prolonged Cold and Darkness

There was a compensatory increase in mitotic activity in the intestinal cell nests of newts when the newts were kept for two months in darkness at 4°C and subsequently warmed to room temperature with

TABLE 11. Mean Mitotic Indices in the Intestinal Cell Nests of Newts at Three-hour Intervals over a 27-hour Period. All newts were kept at an environmental temperature of 4°C in complete darkness and were injected with colcemid five hours before sacrifice (Experiment 8).

Group #	Number of Animals	Sacrifice Time	MI + SD (X 100)
1	3	1000	0.30 ± 0.48
2	3	1300	0.23 ± 0.10
3	3	1600	0.41 ± 0.11
4	3	1900	0.41 ± 0.24
5	3	2200	0.48 ± 0.13
6	3	0100	0.28 ± 0.21
7	3	0400	0.31 ± 0.16
8	3	0700	0.24 ± 0.13
9	3	1000	0.19 ± 0.06
10	3	1300	0.21 ± 0.14
Overall Mean			0.31

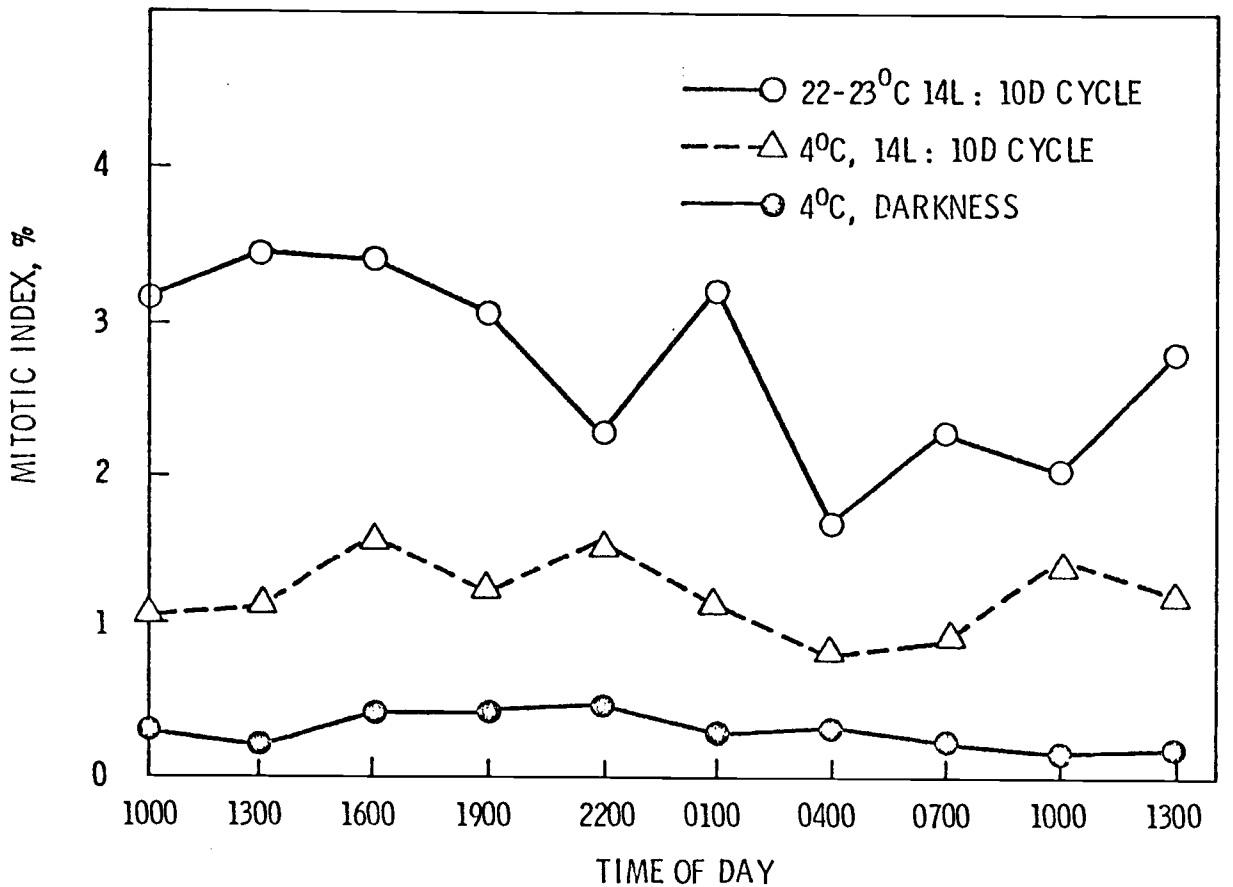

Figure 10. A comparison of sample mean mitotic indices from newts kept at different environmental temperatures with or without a light-dark cycle.

a 14L:10D cycle (Experiment 9). Ten groups containing four newts each were injected with colcemid and killed at times corresponding to those of Experiment 5 above (Figure 1). Data from Experiment 9 are summarized in Table 12 and are compared to those of Experiment 5 in Figure 11.

The overall mitotic index was 0.0375 and the peak sample mean mitotic index, at 1600 hours, was significantly higher ($P < 0.05$) than the sample means at 1900 hours and 0700 hours and the sample means 1000 hours and 1300 hours of the second day of the experiment (Appendix III - R). The sample mean mitotic index at that time of day in Experiment 5 was also relatively high. Statistical comparison of the sets of means from Experiments 5 and 9 (Appendix III - S) indicated that the mean mitotic indices were significantly greater ($P < 0.05$) in Experiment 9 and was attributed to a compensatory increase in mitotic activity after a period of relatively low activity such as that in animals of Experiment 8.

MI After Exposure to X-Irradiation

The overall mitotic index of the intestinal cell nests in newts exposed to 100 R X-rays was 0.0023 between five and 29 hours post-exposure. The last sample mean (32 hours postirradiation) was higher than all the previous sample means of the experiment, although it was not statistically higher (Appendix III - T). The high sample

TABLE 12. Mean Mitotic Indices in the Intestinal Cell Nests of Newts at Three-hour Intervals over a 27-hour Period. All newts were kept at an environmental temperature in complete darkness for 60 days and were warmed to 22-23°C with a 14L:10D cycle two days prior to the beginning of the experiment. All animals were injected with colcemid five hours before sacrifice (Experiment 9).

Group #	Number of Animals	Sacrifice Time	MI ± SD (X 100)
1	4	1000	3.38 ± 0.41
2	4	1300	3.31 ± 1.26
3	4	1600	6.82 ± 1.92
4	4	1900	2.79 ± 0.88
5	4	2200	5.28 ± 1.96
6	4	0100	3.80 ± 2.26
7	4	0400	4.07 ± 1.71
8	4	0700	2.45 ± 0.72
9	4	1000	2.68 ± 2.04
10	4	1300	2.87 ± 2.36
Overall Mean			3.75

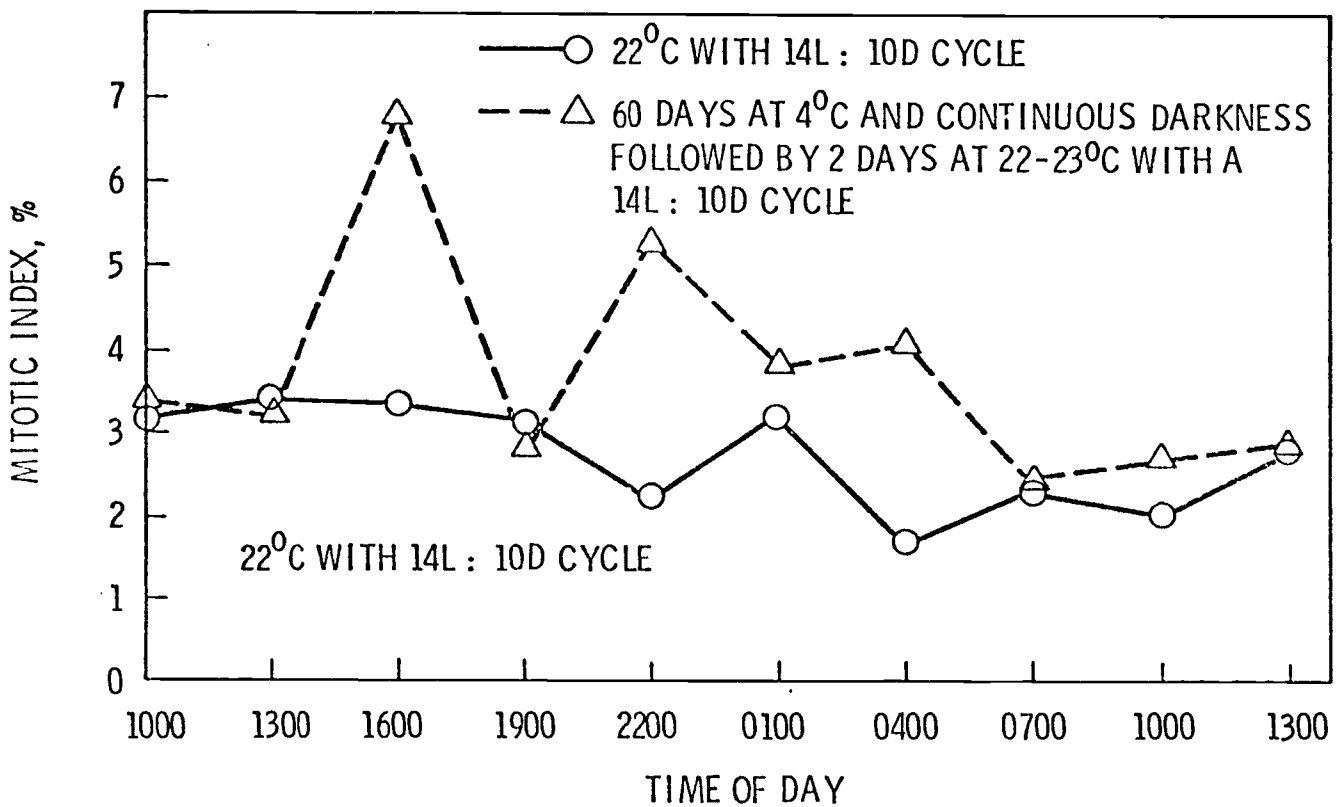

Figure 11. A comparison of sample mean mitotic indices in the intestinal cell nests of newts after a prolonged cold and dark environment with those of newts acclimated to a 22-23°C environment with a 14L:10D cycle (Experiments 5 and 9).

mean was possibly indicative of recovery from the radiation-induced cell cycle block. Data from that experiment (Experiment 10) are presented in Table 13 and graphically illustrated in Figure 12.

Whole-body exposures of newts to 200 R X-rays (Experiment 11) depressed the mitotic index of the cell nests for a longer time than did exposure to 100 R. The sample means remained low (overall mean = 0.0021) for the first 53 hours postexposure and only the final sample mean, 56 hours postexposure, was considerably higher than the overall mean (Table 14 and Figure 12).

That final sample mean from Experiment 11 was significantly higher ($P = 0.05$) than eight of the sample means from earlier times in the experiment and indicated recovery from the radiation-induced cell cycle block.

Experiment 12, in which the newts were exposed to 500 R X-rays, was done after Experiment 10 but before Experiment 11. The intent was to compare depression of mitotic activity from a 100 R exposure and a 500 R exposure. Exposure to 500 R, however, depressed the mitotic activity to essentially zero for the first 32 hours, so it was decided to expose more groups of newts to 200 R and sacrifice the groups over a longer period of time (Experiment 11).

X-ray-induced histological lesions in newts exposed to 100 R (Experiment 10) were confined to small numbers of pyknotic nuclei in the cell nests appearing within 24 hours postexposure. The duration of the experiment was too short to allow a further progression of

TABLE 13. Mean Mitotic Indices in the Intestinal Cell Nests of Newts at Three-hour Intervals over a 27-hour Period. All animals were exposed to 100 R X-rays between 0445 and 0500 hours, kept at room temperature (22-23°C) with a 14L:10D cycle, and injected with colcemid five hours before sacrifice (Experiment 10).

Group #	Number of Animals	Sacrifice Time	MI + SD (X 100)
1	4	1000	0.11 ± 0.07
2	4	1300	0.03 ± 0.03
3	4	1600	0.07 ± 0.05
4	4	1900	0.06 ± 0.04
5	4	2200	0.05 ± 0.04
6	4	0100	0.23 ± 0.20
7	4	0400	0.87 ± 0.81
8	4	0700	0.23 ± 0.06
9	4	1000	0.66 ± 0.77
10	4	1300	1.74 ± 2.30

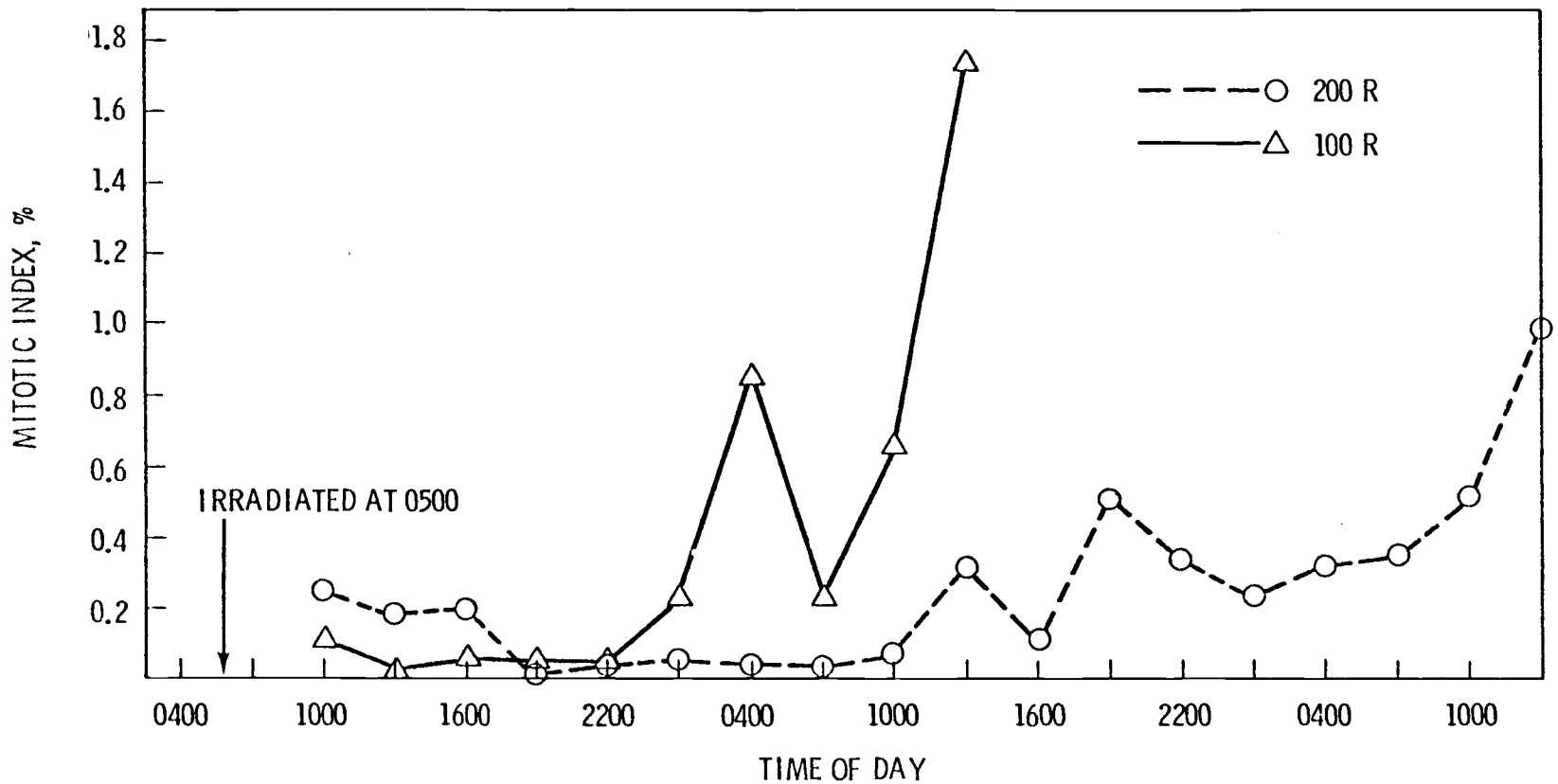

Figure 12. Dose-response curves of the sample mean mitotic indices in the intestinal cell nests of newts exposed to 100 R and 200 R X-rays (Experiments 10 and 11).

TABLE 14. Mean Mitotic Indices in the Intestinal Cell Nest of Newts at Three-hour Intervals over a 27-hour Period. All animals were exposed to 200 R X-rays between 0445 and 0500 hours, kept at room temperature (22-23°C) with a 14L:10D cycle, and injected with colcemid five hours before sacrifice (Experiment 11).

Group #	Number of Animals	Sacrifice Time	MI + SD (X 100)
1	4	1000	0.25 ± 0.14
2	4	1300	0.19 ± 0.08
3	4	1600	0.20 ± 0.10
4	4	1900	0.01 ± 0.01
5	4	2200	0.03 ± 0.02
6	4	0100	0.05 ± 0.03
7	4	0400	0.04 ± 0.05
8	4	0700	0.03 ± 0.02
9	4	1000	0.04 ± 0.04
10	4	1000	0.07 ± 0.06
11	4	1300	0.31 ± 0.46
12	4	1600	0.11 ± 0.12
13	4	1900	0.51 ± 0.75
14	4	2200	0.44 ± 0.24
15	4	0100	0.23 ± 0.07
16	4	0400	0.32 ± 0.14
17	4	0700	0.36 ± 0.22
18	4	1000	0.52 ± 0.46
19	4	1300	1.00 ± 1.17

changes. Lesions similar to those described for newts exposed to 1000 R, although considerably less severe, did occur after 200 R X-ray exposure (Experiment 11). Nuclear pyknosis was present within 24 hours and loss of cell nests from the proliferative areas was also apparent by that time. Diminished intestinal mucosal depth was evident at 36 hours postexposure but was not as pronounced as it was in newts exposed to 1000 R. Between 44 and 56 hours postexposure, a gradual return to normal microscopic structure began so that intestines of the last group of newts (56 hours postexposure) appeared nearly normal.

No discernable histopathologic differences were noted between intestines of newts exposed to 500 R (Experiment 12) and those exposed to 200 R. The same early changes were observed to approximately the same degree of severity but no further progression of changes was observed due to the short duration of the experiment.

DISCUSSION

Counting Error and Within-Sample Variation

All cell counts were accomplished visually with a light microscope and are subject to errors which would contribute to within-sample variation. A maximum counting error of five percent was assigned to estimates of intestinal epithelial proliferative cells, a value based on repeated estimates from slides occasionally as long as two years between estimates. Estimates were determined by counting, at 200X magnification, the proliferative cells in an intestinal cross-section by tens; the cells were too numerous to count individually. The procedure was subject to constant checking by counting cells individually in a portion of the section and the method was determined to be reliable.

Counts of mitotic figures and autoradiographically labeled cells could be made with considerably more accuracy with 400X magnification. An estimated maximum counting error of two percent was assigned to those values and to the labeled mitoses values.

Calculating a maximum error for the fraction of labeled cells or the fraction of cells in mitosis resulted in an estimate of 5.4 percent. That was done using the formula,

$$V_R = \sqrt{V_P^2 + V_M^2},$$

where V_R was the error estimate of the fraction, V_P was the estimated error for counting of proliferative cells (five percent), and V_M was the estimated error for counting of mitotic or labeled cells (two percent). The maximum error for the percent labeled mitosis was 2.8 percent, calculated with the same formula but with two percent estimated counting errors for mitotic cells and for labeled mitotic cells.

Within most samples (groups of newts) high variation of individual observations and consequent high standard deviations of the means were characteristic. The average standard deviation ranged from 30 percent of its mean in Experiment 1b to 84 percent of its mean in Experiment 11. The fact that the last sample mean in Experiment 11 did not prove to be significantly different from the 18 previous sample means in that experiment could be attributed to the high sample variance. The high sample variance contributed to the failure of the studentized range test to identify significantly different sample means after the analysis of variance indicated that some existed. That occurred in Experiments 1b (labeling indices), 7 and 9 (mitotic indices).

The relatively high standard deviations listed in Tables 4 through 14 were a cause of considerable concern until it was noted that other authors reporting cell kinetics data rarely listed variance estimates. None were included, for example, in reports by Scheving and Chiakulas (1965), Grasso and Woodard (1967), and Grosset and

Odartchenko (1975a, 1975b); all presented mitotic and/or labeling index data from urodele amphibians. Because no variance estimates were listed, three possible conclusions could be drawn: (1) the estimates were not calculated, (2) within-sample variation was so small that it was considered negligible, or (3) estimates were purposely omitted because they detracted from the objectives of the investigation.

Standard errors, standard deviations, or ranges of cell kinetic data were relatively high in reports of authors who included them. For example, Chiakulas and Scheving (1966) listed standard errors from nine percent to more than 50 percent for tritiated thymidine labeling indices in the epidermis of larval newts. Eisenberg and Yamada (1966) presented graphs in which a mean labeling index of 19 percent was shown with a range from ten to 28 percent and a mean mitotic index of ten percent had a range from two to 18 percent. Those were approximate values read from graphs in which cellular kinetics data from lens regeneration in newts were presented. Garcia and Johnson (1972) listed some labeling indices from the intestines of goldfish with standard errors which were approximately 75-90 percent of the means. The overall mean mitotic and synthetic indices from Experiments 1, 2, and 5 through 11 are presented in Table 15. Also listed in that table is the average coefficient of variation calculated from sample means of each experiment.

TABLE 15. Overall Mean Mitotic and Labeling Indices from Experiments 1, 2 and 5 through 11.

Experiment Number	Overall Mean Mitotic Index (X 100)	Average* Coefficient of Variation	Overall Mean Labeling Index (X 100)	Average* (SD/Mean)
1a	0.68	52.5		
1b	0.28	30.3	13.2	67.0
2a	0.54	55.0	26.6	35.0
2b	1.33	57.0	16.5	40.0
5	2.74	37.0		
6	2.18	59.0		
7	1.20	45.0		
8	0.31	60.0		
9	3.75	44.0		
10	0.23	84.0		
11	0.21	74.0		

* From Tables 4 through 14, the coefficient of variation was calculated for each sample mean. The listed value is the arithmetic average of the coefficients of variation from the experiment.

The high sample variance was due, at least in part, to the relatively small numbers of animals in each group. Other possible reasons might have included: (1) individual differences in the effect of colcemid on the animals, (2) individual differences in uptake of tritiated thymidine by the animals, and (3) natural individual variation in cell proliferative activity in the intestine. Each of those and other possibilities are discussed subsequently.

Effect of Colcemid on the Mitotic Index

The use of colcemid as a mitotic arresting agent was considered necessary to obtain data from which to construct a percent labeled mitosis curve for the intestinal cell nests of the newt. Results of Experiment 1b (Table 5) indicated that numbers of mitotic figures would have been too low to provide valid percent labeled mitoses data. In two experiments conducted simultaneously, the overall mean mitotic index for 21-hour periods was 0.0028 without colcemid (Table 5) and 0.0068 with colcemid injections five hours before sacrifice (Table 4). Overall mean mitotic indices of 0.0054 from Experiment 2a (Table 6) and 0.0133 from Experiment 2b (Table 7) substantiated the mitotic arresting properties of colcemid in the intestinal cell nests of newts. All newts in Experiment 2b were killed at a time of day when mitotic activity would be at a peak; that was the most apparent reason for the two-fold increase in the mitotic index over that from Experiment 2a.

Some of the high sample variances could have been a result of the colcemid injections. The average standard deviation was 30 percent of its mean in Experiment 1b (no colcemid injection) contrasted with coefficients of variations of 52.5, 55, and 57 percent in data from Experiments 1a, 2a, and 2b, respectively, in which colcemid was used (Table 15). Differences in the effects of the drug on individual animals could account for the higher variation in sample means when colcemid was used.

Héroux (1960) questioned the effectiveness of colchicine at low temperatures, relating its failure in his experiments to the marked decrease in mitotic activity in the ears of cold-acclimated rats. Data from Experiments 7 and 8 of this investigation did not indicate a failure of colcemid at low temperatures in the poikilothermic newt. The overall mean mitotic index in Experiment 7 (4°C) was lower by a factor of three than that in Experiment 5 (22-23°C). The difference could have been construed as an effect of lower temperatures on the action of colcemid except that the overall mean mitotic index in Experiment 8, also at 4°C, was about four-fold lower than that in Experiment 7. Environmental temperature, although a possible contributor, was not solely responsible for the difference in mitotic indices. The other difference between environmental conditions in Experiments 7 and 8 was the absence of a 14L:10D cycle in Experiment 8. No empirical evidence in scientific literature on the subject indicates an effect of light on the mitotic-arresting properties of colcemid.

An effective dose of colcemid for use with poikilothermic vertebrates was not easily obtainable from existing literature. Leblond (1959) cited a dose of 1 mg colchicine per 1 kg body weight for general use with small mammals. Assuming a 30 gram mouse, a total dose of 0.03 mg of colchicine would be administered. Hampton (1966) empirically determined doses of 0.004 and 0.008 mg per mouse to be inefficient for long-term mitotic arrest and used 0.04 mg per mouse which, assuming a 30 gram mouse, would be 1.33 mg per kilogram body weight. Johnson, Dornfeld and Conte (1967) used a 0.004 mg dose of colchicine per 10 gram fence lizards and calculated mitotic duration and cellular turnover time in the intestinal epithelium of those animals. It was interesting to note, however, that mitotic indices which they listed were no higher in colchicine treated animals than in control animals.

Preliminary experiments indicated that the colcemid dose for newts had to be considerably higher than any of those doses. An effective mitotic inhibiting dose per newt was determined to be 0.15 mg or approximately 12.5 mg per kilogram body weight. The most effective time period between injection of colcemid and sacrifice of the animal was simultaneously determined to be five hours. That dose and time period were then routinely used without further investigation.

Tritiated Thymidine Incorporation and Autoradiography

A suitable dose of ^{3}H-TdR for use with newts was quite easily deduced from existing literature; numerous authors have listed doses for poikilothermic vertebrates. Hyodo (1965b) reported using doses of 5, 10, and 20 µCi (specific activity (s.a.): 8.6 Ci/mM) per animal in goldfish weighing five to seven grams. Hyodo-Taguchi (1970) injected six- to ten-gram goldfish with 15 µCi each (s.a.: 5.5 Ci/mM). Johnson, Dornfeld and Conte (1967) used 0.5 µCi per gram body weight in western fence lizards (no specific activity reported) and Johnson, Nakatani and Conte (1970) used a dose of 1.5 µCi per gram body weight (s.a.: 12.8 Ci/mM) in juvenile Coho salmon.

Doses of tritiated thymidine for use in urodele amphibians were reported by Grasso and Woodard (1967) who used 75 to 80 µCi per animal (s.a.: 1.5 Ci/mM) to study erythropoiesis in Triturus. O'Steen and Walker (1960) used 20 µCi doses to study cellular dynamics in various tissues of the same animal and Scheving and Chiakulas (1965) used 1 µCi per gram to study epidermal cell labeling in Amblystoma larvae.

Generally, those investigators using the highest doses of ^{3}H-TdR also reported the shortest exposure time necessary for autoradiography. Grasso and Woodard (1967) who used 75 to 80 µCi per animal reported an exposure period of one to two weeks while O'Steen and Walker (1960) exposed autoradiographs for three to four weeks or more after

using a 20 μCi dose in the same species. Brugal (1973) used a dose of 0.02 μCi in larval newts and reported a 50-day autoradiographic exposure period.

The time of availability of ^{3}H-TdR for incorporation into tissues after its administration to poikilothermic animals is controversial. Hyodo (1965b) estimated an incorporation time of two hours in the goldfish and Johnson, Nakatani and Conte (1970) reported that maximum incorporation had occurred in the intestinal epithelium of the Coho salmon within two hours at any of the environmental temperatures used in their experiments. Scheving and Chiakulas (1965) estimated a two-hour ^{3}H-TdR incorporation time in the epidermis of larval newts and Grasso and Woodard (1967) stated that ^{3}H-TdR was incorporated into newt blood cells between zero and two hours post-injection. Results of other investigations (Grosset and Odartchenko, 1975a) indicated that ^{3}H-TdR injected intraperitoneally was effective for approximately two hours in the newt, Triturus cristatus, but the authors did not consider that a "flash" label. They chose to use intracardiac injections to get more rapid incorporation of ^{3}H-TdR into the erythropoietic tissues of the animal.

Labeling indices obtained for intestinal epithelium of poikilothermic invertebrates vary considerably in the data of different investigators. Hyodo (1965b) and Hyodo-Taguchi (1970) reported approximately 100 labeled cells per intestinal cross-section of goldfish three hours after ^{3}H-TdR injection. Other data from that

investigation placed the number of epithelial cells per section at 3000 or more which resulted in a labeling index of 0.033 or less. Garcia and Johnson (1972) also reported a similar labeling index in the intestines of goldfish at 10°C but discovered a rapid decline in the labeling index to less than 0.01 at 30°C.

An intestinal epithelial ^{3}H-TdR labeling index of 0.23 was listed by O'Steen and Walker (1960). That value was divided into an index for the surface layer, 0.02, and an index for the cell nests, 0.42, one day postinjection. Brugal and Pelmont (1975) calculated an intestinal epithelial labeling index of approximately 0.42 in embryonic newts but did not specify the precise cell population from which it was derived.

Potten, Hagemann and Reiland (1972) reported a failure of some individual mice to incorporate ^{3}H-TdR into DNA even though sampling indicated its distribution throughout the body of the animal. That phenomenon involved two to six percent of mice they used and was either specific to a few organs or general for all organs. A similar condition in newts could have accounted for the exceedingly low labeling indices in the intestines of six animals in Experiments 2a and 2b (Appendices II-D and E).

A small-scale preliminary experiment was conducted and it was determined that a 15 µCi (s.a.: 6 Ci/mM) per newt intraperitoneal dose was sufficient to produce a maximum number of labeled nuclei in the intestinal cell nests within two hours postinjection. No improvement in label incorporation was noted if doses of 20 or 25 µCi per

animal were used. Autoradiographs from those animals were considered sufficiently exposed in approximately three weeks. Longer exposure times resulted in such dense labeling of mitotic figures that they were not readily recognizable as such. The above described methods resulted in mean labeling indices of approximately 0.20 (Tables 6 and 7).

Intestinal Cell Proliferative Activity

Observations made during this investigation substantiated the role of the cell nests as intestinal epithelial cell proliferative zones in Taricha granulosa. Very few labeled cells were observed in the surface epithelium during the first 24 hours after ^{3}H-TdR administration and mitotic activity was confined to the cell nests with the exception of an occasional mitotic figure in the basal zone of the epithelial layer. The role of the cell nests in the intestines of urodele amphibians was first proposed by Patten and Andrew (1954) and confirmed with cell labeling techniques by Patten (1960) and O'Steen and Walker (1960).

Cell Cycle Phase Durations

Cellular kinetics data from the intestinal epithelial cell nests of Taricha granulosa was subject to diverse interpretations because

the relationships between phase duration, mitotic indices, and tritiated thymidine labeling indices cited for mammalian tissues are not necessarily valid in that poikilothermic system. Attempts to interpret the data from that system were made more difficult because of the lack of agreement between researchers investigating similar systems. In the following discussion, several interpretive methods are discussed in attempts to apply them to data resulting from this investigation; problems associated with each are noted.

The percent labeled mitoses curves illustrated in Figures 4, 5, and 6 are very similar in appearance to those obtained by Grosset and Odartchenko (1975a) for erythroblasts of the newt, Triturus. Although the curves of Figures 4 and 5 were obtained by slightly different methods (Experiments 2a and 2b), their similarity, shown in Figure 6, was remarkable. Because colcemid was used to effect a five-hour period of mitotic arrest before the animals were killed, each point in the curve should be located two to three hours earlier than it is shown; the points shown were assigned to the sacrifice time. That shift would affect only the G_2 + 1/2M duration, however, and other inferences drawn from the curve would remain the same.

Using the technique proposed by Quastler and Sherman (1959), a DNA synthesis time of 41 hours was derived from the PLM curve of Figure 6. The descending leg of the curve was not well-defined so a line with the negative slope of the ascending leg was drawn in at the point where the PLM fell decisively below the peak value. That

method was one used by Grosset and Odartchenko (1975a) with data from erythroblasts of Triturus and the T_S, so derived, was verified by them with a double labeling technique. They attributed the poor definition of a descending leg to individual cell variation in the progression through the cell cycle.

Mitotic duration, also estimated by the method of Quastler and Sherman (1959), was approximately 8 hours. That method, however, made no allowance for individual cell variation in the length of the G_2 phase. The time required for the PLM curve to ascend from zero to its peak value is a reflection of T_{G2} and T_M variability as well as the length of the labeling "pulse" (Quastler and Sherman, 1959; Lipkin, 1971). Odartchenko et al. (1964) considered the duration of the premitotic gap (G_2) to be more variable than T_M in the erythroblasts of dogs and that was substantiated for erythroblasts of newts, frogs, lizards, and chickens by Grosset and Odartchenko (1975b). Two useful parameters were obtained from the ascending leg of the PLM curve. They were the minimum T_{G2}, represented by the time from injection of the DNA precursor until the first labeled mitotic figure occurred (Quastler and Sherman, 1959), and T_{G2} + 1/2 T_M, the period of time between injection and the time when the PLM curve reached 50 percent (Lesher, Fry and Kohn, 1961; Lesher, Fry and Sacher, 1961). Allowing approximately one hour for incorporation of the ^{3}H-TdR label and subtracting approximately two hours because of the five-hour colcemid treatment, a minimum T_{G2} of two hours and a T_{G2} + 1/2 T_M duration of approximately six hours was graphically derived.

Independent determination of T_M would allow T_{G2} to be calculated by simple subtraction (Sisken, 1964; Thrasher, 1966).

Other methods of determining T_M exist; for example, the colchicine technique of Storey and Leblond (1951) which depends on a determination of a daily mitotic rate. In their report, the daily mitotic rate consisted of a sum of the mitotic indices, expressed as percents, from four consecutive six-hour periods of mitotic arrest by colchicine. Data were not collected from four six-hour periods in this investigation but the data from Experiment 5 was used to yield an estimate of the daily mitotic rate. There were ten sample means at three-hour intervals over a 27-hour period which were grouped into four separate sets of means from nonoverlapping five-hour time periods. One of those sets, for example, included the sample means from groups of animals which were killed at 1000, 1600, 2200, and 0400 hours; they represent four five-hour periods of colcemid-induced mitotic arrest with an hour between each period. A sum of each set of four sample means was calculated and the four sums were averaged. The resultant average, 10.58 percent, was multiplied by 1.2 to adjust it from a 20-hour period to a 24-hour period. The result indicated that 13.02 percent of the intestinal proliferative cells underwent mitosis per day and 100 percent divided by that figure yielded a 7.68 day turnover time for the intestinal epithelium. Turnover time is equal to the mitotic duration divided by the mitotic index (Leblond and Walker, 1956) so, algebraically, a T_M in the newt intestinal cell nests was calculated to be 0.52 hours using the mitotic index of

0.0028 from Experiment 1b. Another method of calculating turnover time and T_M was suggested by Leblond and Walker (1956). With it, the time period of mitotic arrest by colcemid was divided by the mitotic index at the end of that time. Dividing five hours by the average mitotic index from Experiment 5, 0.0274, resulted in a turnover time of 7.60 days which would result in a T_M of 0.51 hours. Both methods resulted in nearly identical estimates of the turnover time. The above relationships were later condensed into the equation, $M = pt/q$, by Leblond (1959); those relationships were discussed on pages 20 and 21 of this report.

If 0.51 was a reasonable estimate of T_M, the individual cell variability of T_{G2}, as reflected in the ascending leg of the PLM curve, would be greater than seven hours. A minimum T_{G2} of two to three hours was graphically obtained from the PLM curve and, with a T_M of 0.5 hours, a mean T_{G2} of five to six hours would result. Maximum T_{G2} would be approximately 10 to 11 hours. If a T_M of 3.45 hours, as reported by Grosset and Odartchenko (1975b) for erythroblasts of Triturus, is more reasonable, durations of G_2 between two and eight hours with a mean T_{G2} of four hours would result. With the longer mitotic time, however, the probability becomes greater that variation in T_M accounted for some of the slope of the ascending PLM curve and, if that occurred, the T_{G2} variability was proportionately smaller.

At least two probable errors are inherent in the colcemid technique. One error involves the effective time of action on the cells by colcemid; Leblond (1959) and Bertalanffy (1964) noted a lag time of up to 30 minutes after administration for mammalian systems. Another error could result if the administered colcemid dose were too low or if the sensitivity of the cells was less than expected. Not all cells entering mitosis during the time between administration and sacrifice would be subject to mitotic arrest. If the effective time of colcemid in newts was less than the time period between injection and sacrifice, the calculated T_M would be longer than the actual T_M and, if numerous cells escaped the mitotic block, the calculated T_M would be shorter than the actual T_M. No empirical evidence was collected from the intestinal cell nests of Taricha to indicate whether either of those conditions existed.

The method for estimating mitotic duration used by Odartchenko et al. (1964) and Grosset and Odartchenko (1975b) was not clearly described by those authors. It required knowledge of the durations of prophase and telophase for solution of T_M and knowledge of the T_M for calculation of the duration of prophase and telophase. Their data, however, did indicate that T_M in Triturus was at least three hours. At any rate, the method was not compatible with the use of colcemid because, with colcemid, essentially all mitotic figures observed were in metaphase.

A number of methods are available for determining the generation times (T_C) of proliferative cells. Quastler and Sherman (1959)

advocated measurement of time between two corresponding points on the first and second waves of the PLM curve. Unfortunately, no second wave of the PLM curve was obtained in this investigation so indirect methods of calculating T_C had to be used.

One of the simplest means of calculating a generation time is by using the relationship, $N_S/N = T_S/T$, in which N_S is labeling index (cells in S phase), N is the number of proliferative cells, T_S is the duration of S phase, and T is the generation time (Quastler and Sherman, 1959). A shortened version of the equation, $T_C = T_S/LI$, was used by Bertalanffy (1964) for a mammalian system and by Grosset and Odartchenko (1975a) for erythroblasts of the newt. That formula, applied to data from Taricha granulosa, resulted in a T_C of 41/0.266 or 154.1 hours. The mean mitotic index from Experiment 2a was used on the denominator rather than that from Experiment 2b. The indices from which the former was derived were from groups of animals killed over a 24-hour period while all animals in Experiment 2b were killed at essentially the same time of day. Generation times, calculated from the equation, $T_C = T_S/LI$, would be affected by errors in the determination of the labeling index. If the cell labeling process or detection of labeled cells were inefficient, the LI estimate would have been low and the generation time estimate would have been too high. If the population contained cells which were not subject to subsequent division, the LI would have been estimated too high, and consequently the T_C estimate would have been low (Quastler and Sherman, 1959).

A similar method of estimating T_C was used by Brown and Oliver (1968) for estimating the generation time of basal cells in hamster cheek pouch epithelium. The relationship, $T_C = 0.693\ (T_S)/LI$, was used and three assumptions were made concerning the cell population: (1) all cells were proliferating, (2) the cells were exponentially distributed throughout the cell cycle, and (3) all labeled cells would have eventually divided. Data from this investigation used in that formula resulted in a T_C estimate of 106.8 hours.

Fry, Lesher and Kohn (1961a) proposed a relationship for use with a labeled cohort of cells; it was $T = G\ \overline{I}$ and, in it, T was the duration of mitosis, G was the generation time, and $\overline{I}$ was the mitotic index of the labeled cells of the population under consideration. The mean labeled mitotic index calculated from data of Experiment 2a of this investigation was 0.018 and using that value and the 0.51 hour T_M derived by the colcemid technique, a generation time of 27.7 hours resulted. The duration of DNA synthesis in the intestinal epithelial proliferative cells of Taricha was at least 40 hours so a T_G calculated that way was meaningless. However, if a T_M of three hours (Grosset and Odartchenko, 1975b) was used, a T_G of 163 hours resulted. An attractive aspect of that method is that inefficiency of labeling in the intestinal cell nests by tritiated thymidine should have a proportionate effect on the number of labeled cells and on the number of labeled mitoses in the cell population.

Another method, a second-order approximation, used by Leith et al. (1975) involves the relationship:

$$T_C = \frac{T_S + [T_S^2 + 2(2T_2\ T_S + T_S^2)\ (LI)]^{1/2}}{2\ (LI)/\ln 2}$$

where T_2 is the time period between injection and 50 percent labeled mitoses. Applied to data from this investigation, a T_C of 158.6 hours resulted. A relationship between T_S and T_C for tumors and several normal epithelia was postulated by Mendelsohn (1975); in it, $T_S = 4.0 + 0.3\ T_C$. Using the estimated T_S of 41 hours for the intestinal cell nests of Taricha in that relationship and solving for T_C resulted in a generation time estimate of 123.3 hours.

Whichever interpretive method was used, the resultant generation time was over 100 hours in length. With an estimated T_S of 41 hours and $T_{G2} + 1/2\ T_M$ of six hours, T_{G1} was estimated to be 50 hours or more. Data were not available which would allow definition of a G_1 or a possible G_o phase.

A G_o phase of the cell cycle is usually considered as a factor responsible for the failure of a second wave of the PLM curve to materialize (Wolfsberg, 1964; Epifanova and Terskikh, 1969; Burns and Tannock, 1970; Burns, 1975). The difference between T_C and the sum of T_{G2}, T_M, and T_S, usually considered T_{G1}, was divided into T_{G1} and

T_{Go} by Burns and Tannock (1970) and Burns (1975) on the basis of proposed mathematical relationships between cell cycle parameters. Applying those relationships to the data from Experiments 2a and 2b produced some interesting, though speculative, results. The rate of cell production from the mitotic cycle, Kp, was defined as LI/T_S; that rate was 0.65 percent/hour in the intestines of the newts from Experiments 2a and 2b. If the true generation time of those cells was 80 hours (not including a possible T_{Go}), the relationship, $2-e^{KpT}C$, would indicate that approximately 32 percent of the cell population was in the G_o phase. True generation times of 60 hours and 100 hours yielded approximate proportions of the cell populations in G_o phase of 52 percent and 8.5 percent, respectively.

The plateau of the PLM curve after the initial wave of the PLM curve was considered important by Burns (1975) in a theoretical model. From it, the true generation time was calculated using the equation, $P = T_S/T_C$; P was the level of the plateau. Subsequently, the growth fraction of the cell population was determined using the relationship GF = 2LI/P. A growth fraction of 1.0 would result in the intestinal cell nests of the newt if the true generation time was 80 hours and the PLM curve stabilized at a plateau about the 50 percent level. A longer true generation time could only result if the plateau were stabilized at a lower level. Unfortunately, no conclusion could be reached regarding the plateau in Experiment 2b unless the experimental period could have been extended for several more hours.

A formula for determination of the growth fraction of a cell population, T_C (LI)/T_S, was presented by Kisieleski, Baserga and Lisco (1961). For an 80-hour T_C, a 41 hour T_S and a 26.6 percent labeling index, a growth fraction of 52 percent of the cells in the newt intestine resulted from use of their formula. If the growth fraction in the intestinal epithelial cells was 1.0, the maximum T_C would have to have been less than 154 hours in that formula; used that way, it was a reversion back to the LI/T_S relationship used to calculate generation time by Quastler and Sherman (1959). A growth fraction of 1.0 was one of the assumptions prerequisite for that relationship.

Data from Experiments 1, 2, and 5, although inconclusive, were interpreted to produce the following estimates: a mean T_{G2} of five to six hours, a T_M between one-half and three hours, a mean T_S of approximately 41 hours, and a T_C of well over 100 hours; it may approach 150 hours. Those numbers would result in a 55- to 95-hour period for T_{G1} and T_{Go}, if there is a T_{Go} involved. A cell turnover time of approximately 7.6 days was also calculated. Results of comparable investigations with poikilothermic vertebrates are not plentiful and those reported were generally subject to many of the same interpretive errors as the results from this investigation. Cellular kinetics parameters measured in cell populations of seven poikilothermic species are listed in Table 16. Because the parameters were not all measured the same way for all species, they are not directly comparable. The results of Grosset and Odartchenko (1975a;

TABLE 16. Cellular Kinetics of Cell Populations From Poikilothermic Vertebrates

Species	Common Name	Cell Population	Environmental Temperature (°C)	Phase Durations (hours) T_M	T_{G2}	T_S	T_{G1}	T_C	Turnover Time (Days)	Reference
Taricha granulosa	rough-skinned newt	intestinal cell nests	22-23	2-3	5-6	41	>50	>100	7.6	This report
Triturus cristatus	crested newt	erythro-blasts	18-20	3.45	0.9	41		60-80		Grosset and Odartchenko (1975a and 1975b)
Lacerta viridis	green lizard	erythro-blast	18-20	1.7	0.9	15		28		"
Pleurodeles waltlii	newt (larva)	intestinal cell nests	20	2	1	24	2	29		Brugal and Pelmont (1975)
Sceloporus occidentalis	western fence	intestinal epithelium	26	1.8				20-22	7-9	Johnson, Dornfield and Conte (1967)
Carassius auratus	goldfish	intestinal epithelium	10			62.4		288		Garcia and Johnson (1972)
			20			24.0		166		"
			37			4.8		127		"
Oryzias latipes	killifish	intestinal epithelium	4-6	36	44	very long				Hyodo-Taguchi and Egami (1969)
			15	4	4	24				"
			23-25	2	1-2	9	5	18		"

1975b) are, perhaps, the most directly comparable to the results from Taricha granulosa because both studies were done with adult animals and the PLM curves drawn from both sets of data were very similar. Their data, however, was obtained from erythroblasts of animals in which anemia had been induced with an injection of phenylhydrazine. There was no discussion concerning the effect of the drug, itself, on the cell cycle.

Data from the intestines of larval newts obtained by Brugal and Pelmont (1975) were not directly comparable with data from Taricha granulosa. Their results showed a marked decrease in mitotic activity and in the growth fraction of the intestinal epithelial proliferative cells from one larval stage to the next. Changes in those parameters which would occur between the larval stages and adulthood were not characterized.

The generation time determined for the intestinal epithelial cells of the goldfish at 20°C by Garcia and Johnson (1972) was similar to that calculated for Taricha granulosa in this investigation. It was calculated the same way, $T_C = T_S/LI$, which means that the LI must have been proportionately higher in the goldfish to compensate for the shorter T_S.

The estimate of T_C in the intestinal epithelium of the fence lizard made by Johnson, Dornfeld and Conte (1967) may have been considerably shorter than the actual T_C. If their PLM curve for the cell population was directly translated, T_S would be approximately

18 hours, T_{G2} would be at least three hours, and T_C would be between 25 and 30 hours, values that would be similar to those obtained by Grosset and Odartchenko (1975b) for the intestinal epithelium of the green lizard.

Cell cycles reported for poikilothermic cell populations are consistently longer than those for most mammalian systems. Generation times of 130 or 135 hours for the basal cells of hamster cheek pouch epithelium (Brown and Oliver, 1968) and 260 hours for epithelium in the forestomach of a mouse (Wolfsberg, 1964) are among the longest reported for mammalian tissues. The T_S time for those two cell populations were estimated to be 8.6 hours and 13.5 hours, respectively, so the authors considered the G_1 and/or G_o periods responsible for the long generation times. Cell cycle phase durations reported by Quastler and Sherman (1959) for intestinal epithelium of the mouse are fairly representative for that mammalian cell population. They were approximately one hour for T_{G2}, 30 minutes for T_M, 7.5 hours for T_S, and 9.5 hours for T_{G1} with a T_C of 18.75 hours. All of those phase durations were considerably shorter than the corresponding phase durations in the poikilothermic species listed in Table 16. One exception was the cell cycle phase durations in the intestine of the killifish at an environmental temperature of 23 to 25°C which were reported by Hyodo-Taguchi and Egami (1969). They were comparable to many mammalian cell cycles.

Diurnal Rhythms

Diurnal rhythms in the tritiated thymidine labeling index and the mitotic index exist in cells of a variety of mammalian species (Table 1) and in cells of urodele amphibians (pages 64 and 65). The diurnal rhythm in the mitotic indices for the intestinal cell nests of Taricha granulosa were intuitively apparent in the data from Experiments 1, 5, 6, and 7 even though the peaks and nadirs were not statistically demonstrable except in Experiment 9.

An attempt was made to define the rhythm of mitotic activity more precisely and the results of the attempt are presented in Table 17 and Figure 11. For each time of day in each experiment, the overall mean was subtracted from the sample mean and the difference was divided by the overall mean; the quotients are listed as percent deviation from the overall mean in Table 17. The sums of the quotients from Experiments 1a and 5 through 9 for each time of day were calculated and are graphically illustrated in Figure 13. Corresponding sums which included quotients from Experiments 5, 7, and 8, only, were calculated separately because animals in those experiments were treated similarly except for the photoperiod and environmental temperatures to which they were exposed. Both curves in Figure 11 define a diurnal rhythm in the mitotic activity of proliferative cells in the intestinal epithelium of newts. Peak activity occurred in the afternoon and evening while the lowest activity occurred in the early morning hours.

TABLE 17. Percent Deviation of Each Sample Mean in an Experiment From the Overall Mean of That Experiment

Time of Day	Experiment Number						Sums Experiments	
	1a	5	6	7	8	9	5, 7, & 8	All
1000		+16.4	-18.3	-10.8	-3.2	-10.3	+2.4	-26.2
1300	+61.8	+25.5	+17.9	-6.7	-25.8	-12.2	-7.0	+60.5
1600	+23.5	+24.5	-10.6	+31.7	+32.2	+80.9	+88.4	+135.2
1900	+4.4	+12.4	+31.7	+2.5	+32.2	-26.0	+47.1	+57.2
2200	-33.8	-17.2	-21.1	+28.3	+54.8	+54.4	+65.9	+65.4
0100	-42.6	+17.5	-27.5	-4.2	-9.7	-6.1	+3.6	-72.6
0400	+52.9	-39.1	-28.0	-34.2	0.0	+8.0	-73.3	-40.4
0700	-5.9	-16.8	-6.4	-25.0	-22.6	-35.0	-64.4	-111.7
1000	-13.2	-26.2	+16.5	+18.3	-38.7	-28.9	-46.7	-72.3
1300		+2.2	+46.8	0.8	-32.3	-23.9	-29.3	-6.4

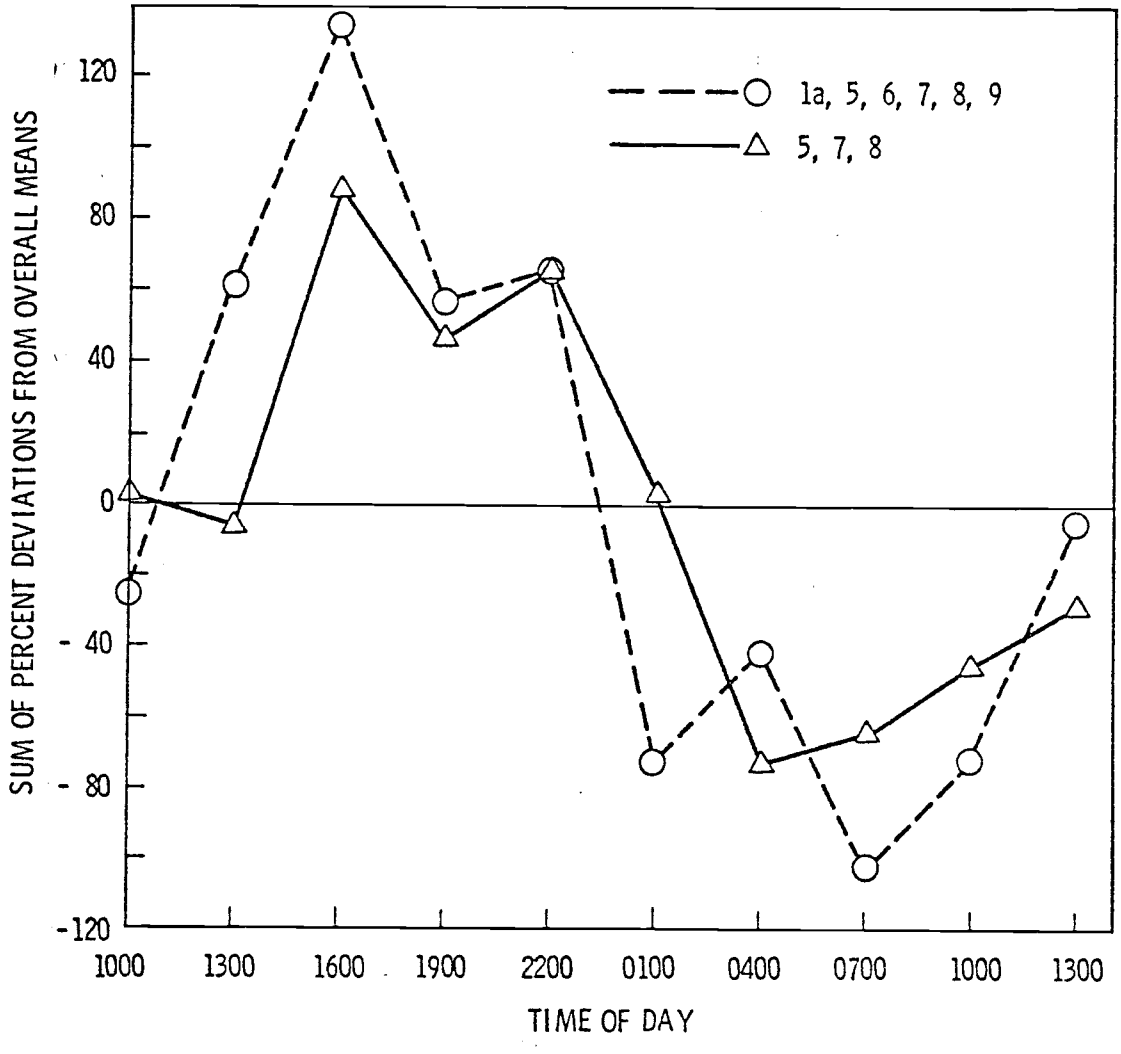

Figure 13. Combined deviations of the sample means of Experiments 1, 5, 6, 7, 8 and 9 from the overall means for those experiments.

If the peak and nadir values were adjusted back in time two to three hours because of the five-hour period of mitotic arrest by colcemid, they corresponded quite well with the results that Scheving and Chiakulas (1965) and Chiakulas and Scheving (1966) obtained from the epidermis of urodele larvae. Some differences existed, however, between their results and the results from Taricha granulosa. The second peak in mitotic activity (0230) reported by those authors was not reflected in Figure 11 even though it did show up in the graph of data from Experiment 1a. No diurnal rhythm could be demonstrated in the LI of intestinal cell nests in Taricha granulosa. The only similarities between the LI curve from Experiment 1b and that of Scheving and Chiakulas (1965) and Chiakulas and Scheving (1966) were the low labeling indices which occurred between 0600 and 1000 hours and at 1900 hours.

Diurnal fluctuation in the mitotic or DNA synthetic indices would have a pronounced effect on calculated values for T_M, T_C, and turnover times. For example, if the formula of Leblond (1959) were applied to data from Experiments 1a and 1b, values for T_M, in hours, ranged between 0.82 at 0400 hours and 5.11 at 2200 hours. If, however, overall daily mitotic and synthetic indices were used in the calculation, the effect of diurnal variation would be negligible.

Effect of Environmental Temperature

Lowered environmental temperature and, therefore, body temperature of Taricha granulosa from room temperature to 4°C resulted in an

overall mitotic index nearly half that at room temperature. Garcia and Johnson (1972) demonstrated a temperature dependence (Q_{10} = 2.6) for DNA synthesis in intestinal epithelial cells of a goldfish but considered the remaining phases of the cell cycle relatively insensitive to temperature changes. They attributed the decreased LI to the temperature dependence of enzyme-mediated reactions within the system.

Data from other studies with poikilothermic vertebrates (Table 16) support the conclusion of Garcia and Johnson (1972) that T_S is lengthened at lower temperatures. Hyodo-Taguchi and Egami (1969) drew a similar conclusion from observations in the intestiinal epithelium of the killifish in which they found a considerable temperature dependence for T_{G2} and T_M. Intestinal epithelial renewal rates based on ^{3}H-TdR incorporation by cells in the Coho salmon, Onchorhynchus kisutch, were also shown to be temperature dependent (Johnson, Nakatani and Conte, 1970).

Some proportion of the decrease in number of colcemid arrested mitotic figures at lowered temperatures could probably be attributed to the slower action of colcemid at the lower temperature. As discussed on page 133, however, the mitotic index of the same cell system was considerably lower in the absence of a light-dark cycle even though the environmental temperature remained the same (Experiment 8). Thus, reduction in the mitotic index could not reasonably be attributed to slowing of the action of colcemid. The lower

mitotic index at the lower temperature should probably be attributed to cells entering mitosis at such a reduced rate that colcemid failed to demonstrate an accumulation of mitotic cells, as suggested by Héroux (1960). Logically, an increased duration of any phase or phases of the cell cycle would reduce the rate of cells entering mitosis unless the duration of another phase was correspondingly decreased.

Effect of the Light-Dark Cycle

The mitotic index in the intestinal cell nests of Taricha granulosa was demonstrated to vary with perturbations of the daily light-dark cycle. Overall mitotic indices were nearly five-fold lower in animals kept in complete darkness than in animals kept in the presence of a 14L:10D cycle with the same 4°C environmental temperature (Experiments 7 and 8). That overall index was approximately 11 percent of the overall index from Experiment 5 in which the newts were kept at room temperature with a 14L:10D cycle.

When newts were kept at 4°C in complete darkness for two months with subsequent warming to room temperature (Experiment 9), a compensatory increase in intestinal mitotic activity was noted over the next 24 hours. The overall mitotic index from those animals was nearly 40 percent higher in animals acclimated to room temperature with a 14L:10D cycle. Despite all that time in darkness, the pattern of diurnal rhythm of mitotic activity remained in the intestinal cells of those animals. The peak activity at 1600 hours was

significantly higher than four of the lowest sample mitotic indices; that was the only experiment in which a statistically significant peak mitotic index was demonstrated.

A reversal of diurnal rhythms in mitotic activity in mouse intestinal epithelium was accomplished through reversal of the daily light-dark cycle by Sigdestad, Lesher and Scott (1975); no such phenomenon has yet been reported in poikilothermic vertebrates. No attempt was made to reverse the diurnal rhythm in Taricha granulosa but the results from Experiments 7, 8, and 9 clearly demonstrated the influence of environmental temperature and a photoperiod on mitotic activity in the intestinal cell nests of the newt.

Comparison of diurnal rhythms in cell proliferative activity from mammals and other vertebrate species (Table 1) revealed an interesting pattern. In rodent species, peak mitotic activity generally occurred between 2400 and 1200 hours and the lowest mitotic activity was noted in the daylight hours. The diurnal rhythm of intestinal cell nest mitotic activity in Taricha granulosa was highest near noon or early afternoon while the lowest activity was noted in animals killed in the early morning hours. That difference might reflect the fact that rodents are primarily nocturnal in nature while newts are diurnal creatures.

One of the activities of animals generally related to a day-night cycle is feeding; numerous investigators, including Sigdestad, Lesher and Scott (1975), considered the feeding cycle responsible for increased mitotic activity in the gut. Passage of food through the

gut would result in abrasion of the epithelial lining, thereby creating a need for cell replacement. The newts used in this investigation were not fed and their intestinal tracts were found to be essentially empty when they were dissected. That was especially true of the newts kept in a cold and dark environment for two months before they were killed. The higher than normal mitotic activity in the intestinal cell nests of those animals after their long confinement was not a result of feeding activity.

Effect of Ionizing Radiation

A slight depressive effect of tritiated thymidine on the mitotic index on the intestinal cell nests was present in the data from Experiment 6 as graphically illustrated in Figure 9. There was an initial slight depression of the sample mean mitotic indices and, after a series of low means, a slight compensatory increase occurred. Differences between the two curves were not statistically significant but that could have been complicated by the inherent diurnal rhythm and the increased mitotic index toward the end of the experiment.

Similar perturbations of the daily mitotic rhythm were shown by Møller, Larsen, and Faber (1974) in hamster cheek pouch epithelium. They observed an initial increase in mitotic activity followed by depression and subsequent compensatory increase. They also established the time of day of ^{3}H-TdR injection as a factor influencing the overall effect. Payne and Shaw (1971) compared the effect of orally

administered ^{3}H-TdR in mice to a 25 R X-ray exposure. If the effects of 100 and 200 R X-ray exposure (Experiments 10 and 11) were extrapolated to 25 R, the effect of ^{3}H-TdR on mitotic activity in the intestine of Taricha granulosa didn't appear to have been severe.

Relatively low whole-body X-ray exposure doses cause a cessation of mitotic activity in the intestinal cell nests of Taricha granulosa (Experiments 10 and 11). The length of time of mitotic arrest by X-rays was demonstrated to be dose dependent for the two different doses used in those experiments. Evidence of individual differences in radiosensitivity was also present in the data from Experiments 10 and 11. Data from the last sacrifice times (Appendices II-K and II-L) contain large differences in the mitotic indices of individual animals which were not as pronounced earlier in the experiments. Within-sample variation, as indicated by the standard deviations of the sample means, was also very high in Experiments 10 and 11 (Table 15). The individual mitotic indices and the high standard deviations reflected either differences in sensitivity to the exposure dose, differences in recovery from the effects of exposure, or both.

The phase of the cell cycle at which the X-ray-induced block occurred could not be ascertained from the data collected from Experiments 10 and 11. The almost immediate drop of the sample mitotic indices (within five hours postirradiation) indicated that the "G_2 block", well documented in mammalian systems, was, at least, partially responsible. Little correlation could be drawn between the

radiosensitivity data for Taricha granulosa presented by Lappenbusch (1970) and the diurnal rhythm of mitotic activity in the intestinal cell nests observed in this investigation. The time of highest radiosensitivity reported by Lappenbusch (1970) was 2100 hours. That corresponded to a time shortly after the peak mitotic activity. Lowest radiosensitivity was reported to be 2400 hours and that corresponded to a period of relatively low mitotic activity in the intestinal cell nests of the newt. Perhaps, with more complete knowledge of the cell cycle in those cells, the radiosensitivity of the organism could have been more closely related to proliferative activity of the cells. Lappenbusch (1970) gave no indication of what the cause of death might have been although the mean survival time of 73.9 days after 1000 R X-ray exposure could be construed to indicate a hematopoietic syndrome, as suggested by Brunst (1958a), rather than a gastrointestinal syndrome.

The effect of a 1000 R X-ray exposure on the epithelial cells of newt intestine was very subtle, histologically. Gastrointestinal injury is usually not considered a cause of radiation-induced death in urodele amphibians (Brunst, 1958a). The intestinal mucosal layer was determined, in this investigation (Experiment 3), to be quite intact up to 78 hours postexposure and the most conspicuous change was the decreased numbers of cell nests at that time. Lappenbusch and Willis (1970) reported survival times for Taricha granulosa greater than 100 days after 5000 R exposure and the 73.9-day survival time reported by Lappenbusch (1970) indicated that some degree of

recovery might have occurred in the intestine of Taricha granulosa after 78 hours. Photomicrographs of the small intestine of Necturus maculosis (mud puppy) taken by Prasad (1968) 11 days after 150 kR exposure showed minimal damage to the epithelium.

Seasonal Variations

Changes in metabolic parameters in amphibians have been associated with the season of the year. Fromm and Johnson (1955) measured maximum oxygen consumption and carbon dioxide production by frogs in the spring. Lappenbusch and Willis (1970) reported a pronounced seasonal influence on survival times of nonirradiated Taricha granulosa; newts collected in summer survived 2.5 times longer than those collected in spring. Female newts collected in spring had slightly shorter survival times after 3000 R radiation exposure than did summer-collected females but no differences were noted between radiosensitivities of males collected at both times. Algard, Friedmann and McCurdy (1974) reported winter-collected Taricha granulosa to be considerably less radiosensitive than newts collected in spring and summer.

Newts used in this investigation were collected at two different times of the year and, what could be considered a seasonal difference in intestinal mitotic activity, was observed. Newts collected in summer were those of Experiments 1, 2, 3, and 4. The newts of

Experiments 5 through 12 were collected in early spring. Experiments 1a and 5 were conducted under identical conditions except for the time of year yet the overall mean mitotic indices differ considerably. The overall mean mitotic index of Experiment 1a was 0.0068 and that for Experiment 5 was 0.0274, a four-fold difference. Furthermore, the overall mean mitotic index in Experiment 2a was 0.0054 and those animals were collected in the same season as the newts used for Experiment 1. No other evidence is available to substantiate a seasonal variation but the differences cited above were otherwise inexplicable.

Conclusions and Summary

The cell cycle in intestinal epithelial proliferative cells of the rough-skinned newt, Taricha granulosa, is of very long duration. An attempt to determine the duration of the individual phases of that cell cycle, in vivo and at an environmental temperature of 22 to 23°C, was only partially successful. Techniques combining mitotic arrest by colcemid, tritiated thymidine autoradiography, and light microscopy were used to collect data from which the cell cycle phase durations in that cell population were calculated or deduced. The G_2 duration and DNA synthesis time were graphically determined to be five to six hours and 41 hours, respectively from a percent labeled mitosis curve. Data from the PLM curve and the colcemid technique, as well as data from other investigations, were taken into consideration and the duration of mitosis was estimated to be two to three hours. The generation time was calculated by several methods and was considered to be longer than 100 hours. Extremely low mitotic indices in the intestinal cell nests precluded attempts to analyze the cell cycle in that cell population at low environmental temperatures and after 1000 R X-ray exposure. The influence of artificial and natural factors on mitotic activity in the intestinal epithelial proliferative cells (cell nests) of newts was investigated as those factors affected the mitotic indices of animals over a 27-hour period. Mitotic activity in the intestinal

cell nests of newts kept at room temperature (22-23°C) with a 14L:10D cycle was subject to a diurnal periodicity. Peak mitotic indices generally occurred in the early afternoon hours and the lowest mitotic indices were recorded during early morning hours. That diurnal rhythm persisted, though slight in some instances, as the mitotic activity was perturbed by manipulating the environmental conditions to which the newts were acclimated.

An environmental temperature of 4°C with a 14L:10D cycle resulted in diminished mitotic activity in the intestinal cell nests. There was more than a two-fold difference in overall mitotic indices at the two temperatures. With an environmental temperature of 4°C and complete darkness, the overall mitotic index was nearly four-fold less than it was at the same temperature with a 14L:10D cycle. The differences between mitotic indices under the above three environmental conditions were statistically significant ($P < 0.01$).

Newts subjected to a dark and cold (4°C) environment for 2 months were warmed to room temperature over a two-day period and the mitotic indices in their intestinal cell nests were determined over a 27-hour period thereafter. A diurnal periodicity was evident with a statistically significant ($P < 0.05$) peak mitotic index at 1600 hours. Intestinal cell nest mitotic activity was significantly greater ($P < 0.05$) in those newts than in newts acclimated to room temperature for a longer time.

Ionizing radiation had a disruptive effect on mitotic activity in the intestinal cell nests of newts. An intraperitoneal injection of tritiated thymidine produced an initial, slightly depressive effect on mitotic activity in the cell population but the mitotic index returned to normal or slightly higher levels within the 27-hour period.

Whole-body exposure of newts to 100 R X-irradiation essentially stopped mitotic activity in the intestinal cell newts for 32 hours. An X-ray exposure dose of 200 R essentially stopped mitotic activity in the cell nests for 56 hours. Recovery of mitotic activity in the cell nests was shown by data from both groups of animals after both those time periods. Whole-body exposure of newts to 1000 R X-irradiation severely depressed mitotic activity in the intestinal cell nests for at least 78 hours and resulted in diminished numbers of the cell nests although the mature cells of the intestinal epithelium appeared to be relatively radioresistant.

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APPENDICES

APPENDIX I - HISTO-TECHNICAL PROCESSING

Xylene 1	5 minutes	
Xylene 2	3 minutes	
Absolute ethanol	3 minutes	
95% ethanol	3 minutes	
70% ethanol	3 minutes	
50% ethanol	3 minutes	
distilled water	3 minutes	
periodic acid solution	5 minutes	
distilled water	rinse	
Schiff's reagent	15 minutes	
running tap water	10 minutes	
photographic emulsion	dip	for autoradiography
exposure	21 days	
developer (Kodak D-19)	7 minutes	
distilled water	rinse	
fixer (Kodak Rapidfix)	1 minute	
running tap water	10 minutes	
Erlich's Hematoxylin	5 minutes	
running tap water	3 minutes	
dilute acid-alcohol solution	rinse	
running tap water	1 minute	
dilute ammonium hydroxide solution	rinse	
running tap water	1 minute	

APPENDIX I (cont.)

50% ethanol	3 minutes
70% ethanol	3 minutes
95% ethanol	3 minutes
absolute ethanol	3 minutes
xylene 1	3 minutes
xylene 2	5 minutes
coverslip mounting	

APPENDIX II

Experimental Data In Detail

II-A. Results of Experiment 1a

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
2	1	1	2400	28	1.17		
	2	1	1270	19	1.50		
	3	1	1270	8	0.63	1300	1.10 ± 0.44
3	4	1	2160	9	0.42		
	5	1	1170	7	0.60		
	6	1	1270	7	0.55	1600	0.52 ± 0.09
4	7	1	1410	11	0.78		
	8	2	1150	8	0.70		
	9	1	2360	15	0.64	1900	0.71 ± 0.07
5	10	1	1640	2	0.12		
	11	1	1590	11	0.69		
	12	1	1270	7	0.55	2200	0.45 ± 0.30
6	13	2	1640	2	0.24		
	14	1	1360	4	0.29		
	15	1	1100	7	0.64	0100	0.39 ± 0.22
7	16	1	1320	7	0.53		
	17	1	1190	26	2.18		
	18	1	1910	8	0.42	0400	1.04 ± 0.99
8	19	1	1380	0	0.00		
	20	1	1540	15	0.98		
	21	1	1060	10	0.94	0700	0.64 ± 0.55
9	22	1	2130	9	0.42		
	23	1	1190	11	0.92		
	24	1	1660	7	0.42	1000	0.59 ± 0.29

II-B. Results of Experiment 1b (Mitotic indices)

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
2	1	4	5320	11	0.21		
	2	4	5760	18	0.31		
	3	4	5280	10	0.19	1230	0.24 ± 0.06
3	4	4	2880	6	0.21		
	5	4	4960	9	0.16		
	6	4	5560	12	0.22	1530	0.20 ± 0.03
4	7	3	3840	14	0.36		
	8	3	2760	8	0.29		
	9	3	4260	11	0.26	1830	0.30 ± 0.05
5	10	4	4920	14	0.29		
	11	4	4000	29	0.73		
	12	4	2440	9	0.37	2130	0.46 ± 0.23
6	13	3	2400	7	0.29		
	14	3	2670	9	0.30		
	15	3	2340	3	0.13	0030	0.24 ± 0.10
7	16	3	3120	7	0.22		
	17	3	4650	5	0.11		
	18	3	2160	4	0.19	0330	0.17 ± 0.06
8	19	4	3920	15	0.38		
	20	4	4880	26	0.53		
	21	4	2400	6	0.25	0630	0.39 ± 0.14
9	22	4	1720	1	0.06		
	23	3	3360	4	0.12		
	24	3	2250	10	0.44	0930	0.21 ± 0.20

II-C. Results of Experiment 1b (Labeling Indices)

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total Labeled Cells	LI (X100)	Group Sacrifice Time	Group LI ± SD (X100)
1	1	2	2860	144	5.0		
	2	1	1490	576	38.7		
	3	1	1420	454	32.0	1230	25.2 ± 17.8
2	4	2	1440	222	15.4		
	5	1	1240	142	11.5		
	6	1	1390	221	15.9	1530	14.3 ± 2.4
3	7	1	1280	33	2.6		
	8	2	1840	68	3.7		
	9	1	1420	230	16.2	1830	7.5 ± 7.6
4	10	1	1230	297	24.1		
	11	1	1000	314	31.4		
	12	2	1220	265	21.7	2130	25.7 ± 5.1
5	13	2	1600	126	7.9		
	14	2	1780	266	14.9		
	15	2	1560	123	7.9	0030	10.2 ± 4.0
6	16	1	1040	12	1.2		
	17	1	1550	24	1.5		
	18	2	1440	139	9.7	0030	4.1 ± 4.8
7	19	1	980	139	14.2		
	20	1	1220	271	22.2		
	21	2	1200	64	5.3	0630	13.9 ± 8.5
8	22	3	1290	16	1.2		
	23	1	1120	25	2.2		
	24	2	1500	163	10.9	0930	4.8 ± 5.3

II-D. Results of Experiment 2a.

Group No.	Animal No.	No. of Sections	Total Proliferative Cells	Total # of Mitoses	Total # of Labeled Cells	Individual MI (X100)	Individual LI (X100)	Individual PLM	Sacrifice Time
1	1	4	2920	23	644	0.79	22.1	13.4	
	2	4	3960	25	576	0.63	14.5	32.0	
	3	4	4160	19	456	0.45	11.0	21.1	
	4	4	5440	48	755	0.88	13.9	17.4	
	5	4	3280	15	908	0.46	27.7	6.7	1500
2	6	4	3280	11	743	0.34	22.7	63.6	
	7	3	2580	24	579	0.93	22.4	66.7	
	8	3	2490	5	332	0.20	13.3	60.0	
	9	3	2070	17	155	0.82	7.5	41.2	
	10	3	2850	13	856	0.46	30.0	76.9	1800
3	11	4	4000	90	1428	2.25	35.7	90.0	
	12	4	1520	13	408	0.86	26.8	100.0	
	13	3	1800	1	544	0.06	30.2	100.0	
	14	3	2010	10	333	0.50	16.6	90.0	
	15	3	1740	5	351	0.29	20.2	80.0	2100
4	16	4	2960	9	697	0.30	23.5	89.0	
	17	4	3400	4	289	0.12	8.5	100.0	
	18	4	2040	5	320	0.25	15.7	100.0	2400
	19	Died							
	20	Died							
5	21	4	2360	7	585	0.30	24.7	85.7	
	22	4	2040	8	328	0.39	16.1	87.5	
	23	3	2190	7	123	0.32	5.6	100.0	
	24	3	2490	10	804	0.40	32.3	100.0	
	25	3	2040	7	237	0.34	11.6	100.0	0300
6	26	4	3040	15	464	0.49	15.3	86.7	
	27	4	2040	10	365	0.49	17.9	100.0	
	28	3	2370	7	590	0.30	24.9	100.0	
	29	3	2040	7	406	0.34	19.9	100.0	0600
	30	Died							

II-D. (Continued)

Group No.	Animal No.	No. of Sections	Total Proliferative Cells	Total # of Mitoses	Total # of Labeled Cells	Individual MI (X100)	Individual LI (X100)	Individual PLM	Sacrifice Time
7	31	5	3000	5	687	0.17	22.9	100.0	
	32	4	3160	8	1124	0.25	35.6	100.0	
	33								
	34								
	35								0900
8	36	4	2840	3	592	0.11	20.8	100.0	
	37	4	2880	1	976	0.03	33.9	100.0	
	38	3	2850	17	757	0.60	26.5	100.0	
	39	3	1890	1	378	0.05	20.0	100.0	
	40	3	1200	3	285	0.25	23.8	100.0	1200
9	41	4	2160	7	1357	0.22	42.9	85.7	
	42	4	1760	8	464	0.45	26.4	100.0	
	43	3	2700	40	1185	1.48	43.9	97.5	
	44	3	2550	9	882	0.35	34.6	100.0	
	45	3	2190	7	649	0.32	29.6	100.0	1500

II-E. Results of Experiment 2b

Group No.	Animal No.	No. of Sections	Total Proliferative Cells	Total # of Mitoses	Total # of Labeled Cells	Individual MI (X100)	Individual LI (X100)	Individual PLM	Injection Time
1	1	5	5350	114	434	2.13	8.1	0.0	
	2	3	3570	100	579	2.80	16.2	1.0	
	3	6	5280	110	1090	2.08	20.6	0.9	
	4	6	4920	111	665	2.26	13.5	2.6	0800
2	5	6	5520	46	641	0.83	11.6	34.8	
	6	4	3040	126	1076	4.14	35.4	31.7	
	7	5	2650	93	506	3.50	19.1	4.3	
	8	4	3560	80	415	2.25	11.7	16.3	0600
3	9	5	2850	51	273	1.79	9.6	37.3	
	10	6	7080	67	361	0.95	5.1	52.2	
	11	5	5000	62	300	1.24	6.0	40.3	0400
	12	Died							
4	13	5	4550	100	811	2.20	17.8	81.0	
	14	5	4140	31	1494	0.75	36.1	80.7	
	15	5	5250	107	2002	2.04	38.1	84.1	
	16	6	4620	50	785	1.08	17.0	62.0	0200
5	17	5	2600	62	285	2.38	11.0	92.0	
	18	5	4300	60	1210	1.40	28.1	94.0	
	19	3	2790	115	488	4.12	17.5	92.2	
	20	5	3300	44	1028	1.33	31.2	100.0	2400
6	21	5	3300	19	649	0.58	19.7	100.0	
	22	5	4900	68	835	1.38	17.0	97.1	
	23	5	6700	98	1092	1.46	16.3	94.9	
	24	4	2920	105	1024	3.60	35.1	99.2	2100
7	25	6	4800	45	1560	0.94	32.5	100.0	
	26	4	4080	109	887	2.67	21.7	99.1	
	27	3	4110	105	617	2.55	15.0	98.2	
	28	4	7000	103	668	1.47	9.5	98.1	1800

II-E (Continued)

Group No.	Animal No.	No. of Sections	Total Proliferative Cells	Total # of Mitoses	Total # of Labeled Cells	Individual MI (X100)	Individual LI (X100)	Individual PLM	Injection Time
8	29	6	5280	70	755	1.33	14.3	100.0	
	30	4	5160	102	760	1.98	14.7	100.0	
	31	6	3780	12	288	0.32	7.6	100.0	
	32	4	3400	97	771	2.85	22.7	98.0	1500
9	33	5	4050	32	464	0.79	11.5	100.0	
	34	5	6900	11	546	0.16	7.9	100.0	
	35	5	3650	58	999	1.59	27.4	100.0	
	36	5	3650	61	495	1.67	13.6	100.0	1200
10	37	6	4020	53	822	1.31	20.4	100.0	
	38	6	4800	93	1163	1.94	24.2	100.0	
	39	5	4050	90	1676	2.22	41.4	100.0	
	40	6	6900	15	630	0.22	9.1	100.0	0900
11	41	6	3960	13	383	0.33	9.7	100.0	
	42	6	3480	48	312	1.37	9.0	100.0	
	43	6	6780	57	1613	0.84	23.8	100.0	
	44	6	7560	71	1780	0.94	23.5	100.0	0600
12	45	4	2960	10	251	0.33	8.5	100.0	
	46	5	4100	30	640	0.73	15.6	93.3	
	47	4	2800	4	191	0.14	6.8	100.0	
	48	4	2840	17	357	0.60	12.6	88.2	0300
13	49	4	5120	28	1025	0.55	20.0	96.4	
	50	4	4440	8	588	0.18	13.2	100.0	
	51	4	3200	32	455	1.00	14.2	100.0	
	52	4	2880	31	348	1.08	12.1	87.1	2400
14	53	4	3640	15	273	0.41	7.5	93.3	
	54	4	2080	24	304	1.15	14.6	95.8	
	55	4	5240	57	1340	1.09	25.6	98.2	
	56	4	2400	14	402	0.58	16.8	100.0	2100

II-E (Continued)

Group No.	Animal No.	No. of Sections	Total Proliferative Cells	Total # of Mitoses	Total # of Labeled Cells	Individual MI (X100)	Individual LI (X100)	Individual PLM	Injection Time
15	57	5	5650	39	634	0.69	11.2	97.4	
	58	5	5550	32	839	0.58	15.1	100.0	
	59	5	3200	19	285	0.59	8.9	100.0	
	60	4	3600	17	311	0.47	8.6	88.2	1800
16	61	4	3840	20	530	0.52	13.8	90.0	
	62	4	1600	3	183	0.19	11.4	100.0	
	63	4	1920	13	99	0.68	5.2	92.3	
	64	4	2280	23	312	1.01	13.7	91.3	1500
17	65	4	2640	8	304	0.30	11.5	100.0	
	66	4	3440	24	312	0.70	9.1	66.7	
	67	4	4520	99	375	2.19	8.3	60.6	
	68	4	2880	12	203	0.42	7.0	66.7	1200
18	69	4	2080	59	272	2.84	13.1	83.1	
	70	4	2840	7	230	0.24	8.1	85.7	
	71	4	3000	10	121	0.33	4.0	60.0	
	72	4	3960	64	845	1.62	21.3	65.5	0900
19	73	5	3450	80	915	2.32	26.5	63.8	
	74	4	4400	89	1057	2.02	24.0	56.2	
	75	5	3040	4	359	0.13	11.8	75.0	
	76	5	4900	74	2614	1.51	53.3	66.2	0600
20	77	4	3480	80	613	2.30	17.6	66.3	
	78	5	3650	36	490	0.99	13.4	69.4	
	79	5	3100	18	284	0.58	9.2	77.8	
	80	5	3450	27	457	0.78	13.2	55.6	0300

II-F. Results of Experiment 5

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
1	1	2	3420	127	3.71		
	2	2	2740	116	4.23		
	3	3	4140	129	3.12		
	4	4	6520	111	1.70	1000	3.19 ± 1.09
2	5	2	5160	213	4.13		
	6	2	3880	129	3.32		
	7	4	4520	99	2.19		
	8	3	2820	116	4.11	1300	3.44 ± 0.91
3	9	2	4440	186	4.19		
	10	3	2670	114	4.27		
	11	4	2960	34	1.15		
	12	2	2800	113	4.04	1600	3.41 ± 1.51
4	13	2	5280	124	2.35		
	14	2	3000	102	3.40		
	15	2	2460	108	4.39		
	16	4	4960	108	2.18	1900	3.08 ± 1.03
5	17	5	7300	92	1.26		
	18	3	4230	109	2.58		
	19	4	3800	84	2.21		
	20	2	4460	135	3.03	2200	2.27 ± 0.75
6	21	2	5340	119	2.23		
	22	2	3280	152	4.63		
	23	2	2840	138	4.86		
	24	5	5000	58	1.16	0100	3.22 ± 2.89
7	25	3	3840	101	2.63		
	26	4	5560	90	1.62		
	27	4	2440	33	1.35		
	28	4	4480	49	1.09	0400	1.67 ± 0.67
8	29	2	4000	124	3.10		
	30	5	7600	60	0.79		
	31	5	4250	89	2.09		
	32	3	2940	91	3.12	0700	2.28 ± 1.10
9	33	4	6800	107	1.57		
	34	4	4720	71	1.50		
	35	4	3000	67	2.23		
	36	4	3720	103	2.77	1000	2.02 ± 0.60
10	37	3	4680	102	2.18		
	38	4	3880	105	2.71		
	39	2	3740	138	3.69		
	40	4	2200	58	2.64	1300	2.81 ± 0.64

II-G Results of Experiment 6

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
1	1	3	5760	127	2.20		
	2	5	4100	60	1.46		
	3	5	4050	32	0.79		
	4	5	3250	87	2.68	1000	1.78 ± 0.83
2	5	3	4020	99	2.46		
	6	2	3600	148	4.11		
	7	5	4100	44	1.07		
	8	4	3640	96	2.64	1300	2.57 ± 1.24
3	9	4	4440	97	2.18		
	10	4	4680	53	1.13		
	11	4	3680	93	2.53	1600	1.95 ± 0.73
	12	Died					
4	13	2	2860	94	3.29		
	14	2	2500	91	3.64		
	15	5	4300	60	1.40		
	16	4	3200	101	3.16	1900	2.87 ± 1.00
5	17	4	3560	57	1.60		
	18	3	3000	92	3.07		
	19	4	3000	49	1.63		
	20	4	2720	16	0.59	2200	1.72 ± 1.02
6	21	4	4200	17	0.40		
	22	4	5040	109	2.16		
	23	4	4040	108	2.67		
	24	4	2000	22	1.10	0100	1.58 ± 1.02
7	25	5	4800	90	1.88		
	26	4	9480	113	1.19		
	27	4	2840	57	2.01		
	28	4	5200	63	1.21	0400	1.57 ± 0.43
8	29	4	4560	105	2.30		
	30	4	6320	71	1.12		
	31	4	3080	82	2.66		
	32	4	4320	89	2.06	0700	2.04 ± 0.66
9	33	2	2900	153	5.28		
	34	4	4960	48	0.97		
	35	4	2320	56	2.41		
	36	4	2400	36	1.50	1000	2.54 ± 3.05
10	37	4	5120	110	2.15		
	38	2	1580	138	8.73		
	39	4	3920	15	0.38		
	40	4	3160	49	1.55	1300	3.20 ± 3.76

II-H. Results from Experiment 7

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
1	1	4	6240	44	0.71		
	2	4	3360	37	1.10		
	3	4	1480	27	1.82		
	4	4	1960	13	0.66	1000	1.07 ± 0.54
2	5	4	3200	15	0.47		
	6	4	3000	60	2.00		
	7	4	2560	21	0.82		
	8	4	2400	28	1.17	1300	1.12 ± 0.66
3	9	4	2840	75	2.64		
	10	5	1250	14	1.12		
	11	4	1320	21	1.59		
	12	4	1560	15	0.96	1600	1.58 ± 0.76
4	13	4	2520	29	1.15		
	14	4	3520	57	1.62		
	15	4	2400	21	0.88		
	16	4	2280	29	1.27	1900	1.23 ± 0.31
5	17	4	2160	9	0.42		
	18	4	3240	99	3.06		
	19	4	1760	20	1.14		
	20	4	2520	39	1.55	2200	1.54 ± 1.77
6	21	4	2480	24	0.54		
	22	4	4440	74	1.67		
	23	4	1160	8	0.69		
	24	4	2400	41	1.71	0100	1.15 ± 0.62
7	25	4	4200	42	1.00		
	26	4	2200	17	0.77		
	27	4	2040	11	0.54		
	28	4	2240	19	0.85	0400	0.79 ± 0.19
8	29	4	1840	13	0.71		
	30	4	2560	45	1.76		
	31	4	2120	13	0.61		
	32	4	3080	16	0.52	0700	0.90 ± 0.92
9	33	4	1480	29	1.96		
	34	4	4400	50	1.14		
	35	4	2920	30	1.03		
	36	4	2560	40	1.56	1000	1.42 ± 0.42
10	37	4	2200	21	0.95		
	38	4	3080	70	2.27		
	39	4	3120	31	0.99		
	40	4	1600	10	0.63	1300	1.21 ± 0.72

II-I. Results of Experiment 8

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
1	1	3	2100	2	0.10		
	2	3	2970	9	0.30		
	3	3	3060	15	0.49	1000	0.30 + 0.48
2	4	4	2000	5	0.25		
	5	4	1960	6	0.31		
	6	4	2480	3	0.12	1300	0.23 ± 0.10
3	7	4	2160	10	0.46		
	8	4	2520	12	0.48		
	9	4	1760	5	0.28	1600	0.41 ± 0.11
4	10	4	5400	37	0.69		
	11	4	1520	4	0.26		
	12	4	2840	8	0.28	1900	0.48 ± 0.13
5	13	4	1840	8	0.43		
	14	4	2120	8	0.38		
	15	4	2240	14	0.63	2200	0.48 ± 0.13
6	16	4	1840	5	0.27		
	17	4	1320	1	0.08		
	18	4	2000	10	0.50	0100	0.28 ± 0.21
7	19	4	1880	9	0.48		
	20	4	2080	6	0.29		
	21	4	1280	2	0.16	0400	0.31 ± 0.16
8	22	4	2120	8	0.38		
	23	4	2440	3	0.12		
	24	4	2400	5	0.21	0700	0.24 ± 0.13
9	25	4	2240	4	0.18		
	26	4	2080	3	0.14		
	27	4	2040	5	0.25	1000	0.19 ± 0.06
10	28	4	1720	2	0.12		
	29	4	2160	3	0.14		
	30	4	1840	7	0.38	1300	0.21 ± 0.14

II-J. Results of Experiment 9

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
1	1	2	3340	109	3.26		
	2	3	3360	131	3.90		
	3	5	3950	115	2.91		
	4	4	3360	116	3.45	1000	3.38 ± 0.41
2	5	2	3320	171	5.15		
	6	4	2600	72	2.77		
	7	4	4040	93	2.30		
	8	3	3120	94	3.01	1300	3.31 ± 1.26
3	9	2	1660	108	6.51		
	10	3	1860	127	6.83		
	11	2	2180	203	9.31		
	12	4	2200	102	4.64	1600	6.82 ± 1.92
4	13	3	2250	92	4.09		
	14	5	4000	87	2.18		
	15	4	3760	90	2.39		
	16	3	4200	104	2.48	1900	2.79 ± 0.88
5	17	3	2280	166	7.28		
	18	3	3390	133	3.92		
	19	4	2040	135	6.62		
	20	5	3700	122	3.30	2200	5.82 ± 1.96
6	21	4	3080	90	2.92		
	22	2	2880	206	7.15		
	23	4	2440	54	2.21		
	24	4	1720	50	2.91	0100	3.80 ± 2.26
7	25	3	1860	116	6.24		
	26	5	3500	108	3.09		
	27	4	3320	79	2.38		
	28	2	2040	93	4.56	0400	4.07 ± 1.71
8	29	4	3360	113	3.36		
	30	5	3100	53	1.71		
	31	5	3500	73	2.09		
	32	5	3400	90	2.65	0700	2.45 ± 0.72
9	33	5	4400	134	3.05		
	34	4	4920	103	2.09		
	35	4	2360	8	0.34		
	36	2	2860	150	5.24	1000	2.68 ± 2.04
10	37	4	8080	24	0.30		
	38	5	3150	73	2.32		
	39	4	2120	127	5.99		
	40	4	3600	103	2.86	1300	2.87 ± 2.36

II-K. Results of Experiment 10

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
1	1	4	3760	6	0.16		
	2	4	3000	2	0.07		
	3	4	3600	1	0.03		
	4	4	3200	5	0.16	0100	0.11 ± 0.07
2	5	4	1880	1	0.05		
	6	4	7280	1	0.01		
	7	4	2760	0	0.00		
	8	4	2880	2	0.07	0400	0.03 ± 0.03
3	9	4	3200	1	0.03		
	10	4	2280	3	0.13		
	11	4	2760	2	0.07		
	12	4	2640	1	0.04	0700	0.07 ± 0.05
4	13	4	3000	2	0.07		
	14	4	2040	1	0.05		
	15	4	2000	2	0.10		
	16	4	1640	0	0.00	1000	0.06 ± 0.04
5	17	4	3120	1	0.03		
	18	4	1880	0	0.00		
	19	4	2480	2	0.08		
	20	4	3760	3	0.08	1300	0.05 ± 0.06
6	21	4	3040	2	0.07		
	22	4	2480	1	0.04		
	23	4	3120	11	0.35		
	24	4	2040	9	0.44	1600	0.23 ± 0.20
7	25	4	1800	9	0.50		
	26	4	3680	74	2.01		
	27	4	2160	3	0.14		
	28	4	2960	25	0.84	1900	0.87 ± 0.81
8	29	4	3200	8	0.25		
	30	4	1800	3	0.17		
	31	4	1880	4	0.21		
	32	4	2320	7	0.30	2200	0.23 ± 0.06
9	33	4	2080	8	0.38		
	34	4	2160	39	1.80		
	35	4	2880	11	0.38		
	36	4	2280	2	0.09	0100	0.66 ± 0.77
10	37	4	2160	5	0.23		
	38	4	2720	32	1.18		
	39	4	2000	8	0.40		
	40	4	2160	111	5.13	0400	1.74 ± 2.30

II-L. Results of Experiment 11

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
1	1	5	1750	4	0.23		
	2	5	2450	4	0.16		
	3	5	4000	6	0.15		
	4	5	5350	24	0.45	1000	0.25 ± 0.14
2	5	5	5450	11	0.20		
	6	5	4250	3	0.07		
	7	5	3700	9	0.24		
	8	5	2850	7	0.25	1300	0.19 ± 0.08
3	9	5	2850	9	0.32		
	10	5	3050	7	0.23		
	11	5	5150	7	0.14		
	12	5	2100	2	0.10	1600	0.20 ± 0.10
4	13	5	3600	1	0.03		
	14	5	7750	1	0.01		
	15	4	3480	0	0.00		
	16	4	2320	0	0.00	1900	0.01 ± 0.01
5	17	5	3800	1	0.03		
	18	5	3600	1	0.03		
	19	5	3800	2	0.05		
	20	5	4150	0	0.00	2200	0.03 ± 0.02
6	21	5	4100	3	0.07		
	22	5	4150	0	0.00		
	23	5	4250	2	0.05		
	24	4	3560	2	0.06	0100	0.05 ± 0.03
7	25	5	3650	1	0.03		
	26	5	2950	1	0.03		
	27	5	2950	0	0.00		
	28	5	7300	8	0.11	0400	0.04 ± 0.05
8	29	5	9800	5	0.05		
	30	4	6760	2	0.03		
	31	4	2160	0	0.00		
	32	4	4280	2	0.05	0700	0.03 ± 0.02
9	33	5	3400	1	0.03		
	34	5	3000	1	0.03		
	35	5	2400	0	0.00		
	36	5	7050	6	0.09	1000	0.04 ± 0.04

II-L. (Continued)

Group No.	Animal No.	No. Sections	Total Proliferative Cells	Total # Mitoses	MI (X100)	Group Sacrifice Time	Group MI ± SD (X100)
10	37	5	6150	4	0.07		
	38	5	5100	7	0.14		
	39	5	4650	3	0.06		
	40	5	6750	0	0.00	1000	0.07 ± 0.06
11	41	4	5080	6	0.12		
	42	4	4280	5	0.12		
	43	4	2640	0	0.00		
	44	4	1400	14	1.00	1300	0.31 ± 0.74
12	45	4	3480	7	0.20		
	46	4	4120	0	0.00		
	47	4	3360	0	0.00		
	48	4	2600	6	0.23	1600	0.11 ± 0.12
13	49	4	2080	34	1.63		
	50	4	5720	3	0.05		
	51	4	2720	2	0.07		
	52	4	2640	8	0.30	1900	0.51 ± 0.75
14	53	4	2680	13	0.49		
	54	4	2720	12	0.44		
	55	4	1280	9	0.70		
	56	4	4760	5	0.11	2200	0.44 ± 0.24
15	57	4	2400	4	0.17		
	58	4	2840	9	0.32		
	59	4	3080	6	0.19		
	60	4	3680	8	0.22	0100	0.23 ± 0.07
16	61	4	2080	5	0.24		
	62	4	4000	21	0.53		
	63	4	5080	13	0.27		
	64	4	3440	9	0.26	0400	0.32 ± 0.14
17	65	4	4240	11	0.26		
	66	4	3120	6	0.19		
	67	4	4520	31	0.69		
	68	4	2680	8	0.30	0700	0.36 ± 0.22
18	69	3	3120	37	1.19		
	70	4	1840	3	0.16		
	71	4	2680	8	0.30		
	72	4	2600	11	0.42	1000	0.52 ± 0.46
19	73	4	2680	63	2.35		
	74	4	1240	5	0.40		
	75	4	2520	6	0.24		
	76	Died				1300	1.00 ± 1.17

APPENDIX III

Statistical Methods

III-A. Short Method for Determining the 95% Confidence Interval of a Mean

Observations:	X_1	$(X_1)^2$
	X_2	$(X_2)^2$
	X_3	$(X_3)^2$
	X_4	$(X_4)^2$
	X_5	$(X_5)^2$
	ΣX	ΣX^2

Mean: $\frac{\Sigma X}{N}$

Sum of Squares (SS) = $\Sigma X^2 - \frac{(\Sigma X)^2}{N}$

Estimate of variance = $\sqrt{SS/(N-1)/N} = s_{\bar{x}}$

Critical value of "t" from table (P = 0.05 + 4 d.f.) = 2.78

95% Confidence Interval = $s_{\bar{x}}$ (2.78)

III-B. An Example of an Analysis of Variance: One-Way Classification, Many-Sample Comparison

	TIME OF DAY				
	0400	0700	1000	1300	1600
Observations (X)	2	1	2	4	2
	4	2	4	6	2
	6	1	4	6	4
Sums	12	4	10	16	8
Sums^2	144	16	100	356	64

$$\Sigma X^2 = 210$$

$$T = 12 + 4 + 10 + 16 + 8 = 50$$

$$T^2 = 2500$$

$$\Sigma \text{Sums}^2 = 580$$

Preliminary Calculations

Type of Total	Total of Squares	Number of Items Squared	Number of Observations Per Squared Item	Total of Squares Per Observation
Grand	2500	1	15	166.67
Times of Day	580	5	3	193.33
Observations	210	15	1	210.00

III-B. (Continued)

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Times of Day	193.33 - 166.67 = 26.66	4	6.67
Error	210.00 - 193.33 = 16.67	10	1.67

$F = 3.99\ (4,10)$

Critical "F" values

at: $P = 0.05 = 3.48\ (4,10)$

at: $P = 0.01 = 5.99\ (4,10)$

Studentized Range Test

$$K = K^* \sqrt{\frac{VE}{Nm}}$$

K*- from table of factors for studentized range.

Ve = error-variance estimate

Nm = number of observations per sample

$$K = 4.65 \sqrt{\frac{1.67}{3}} = 3.47$$

Ranked Means =	1.33	2.66	3.33	4.00	5.13
Time of Day =	0700	1600	1000	0400	1300

CONCLUSION:

The means determined for 0700 and 1300 hours are significantly different $P < 0.05$.

III-C. An Example of an Analysis of Variance: Multiple Observation, Factorial Experiment

Time of Day	Treatment 1 Observations (X)	X^2	Treatment 2 Observations (X)	X^2
0400	2	4	.2	.04
	4	16	.4	.16
	6	36	.6	.36
	12	56	1.2	.56
0700	1	1	.1	.01
	2	4	.2	.04
	1	1	.1	.01
	4	6	0.4	.06
1000	2	4	.2	.04
	4	16	.4	.16
	4	16	.4	.16
	10	36	1.0	.36
1300	4	16	.4	.16
	6	36	.6	.36
	6	36	.6	.36
	16	88	1.6	.88
1600	2	4	.2	.04
	2	4	.2	.04
	4	16	.4	.16
	8	24	0.8	.24

III-C. (Continued)

$$T = 12 + 4 + 10 + 16 + 8 + 1.2 + 0.4 + 1.0 + 1.6 + 0.8 = 55.0$$

$$T^2 = 3025.0$$

$$T_c^2 = (12 + 4 + 10 + 16 + 8)^2 + (1.2 + 0.4 + 1.0 + 1.6 + 0.8)^2$$

$$T_r^2 = (12 + 1.2)^2 + (4 + 0.4)^2 + (10 + 1.0)^2 + (16 + 1.6)^2 + (8 + 0.8)^2 = 670.29$$

$$T^2_{comb.} = (12)^2 + (4)^2 + (10)^2 + (16)^2 + (8)^2 + (1.2)^2 + (0.4)^2 + (1.0)^2 + (1.6)^2 + (0.8)^2 = 585.8$$

$$\Sigma X^2 + 56 + 6 + 36 + 88 + 24 + .56 + .06 + .36 + .88 + .24 = 212.10$$

Preliminary Calculations

Type of Total	Total of Squares	Number of Items Squared	Number of Observations Per Squared Item	Total of Squares Per Observation
Grand	3025	1	30	100.83
Treatment	2525	2	1	168.33
Times of Day	670.29	5	6	111.72
Combinations	585.8	10	3	195.27
Observations	212.10	30	1	212.10

III-C. (Continued)

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Treatment	168.33 - 100.83 = 67.50	1	67.50
Times of Day	111.72 - 100.83 = 10.89	4	2.72
TmT X Times (Interaction)	(195.27 - 100.83) - 67.50 - 2.72 = 24.22	4	6.06
Error	212.10 - 195.27 + 16.83	20	0.84

F ratios =

$$\frac{\text{TmTs}}{\text{error}} = \frac{67.5}{.84} = 80.36 \ (1,20 \text{ DF}) \ P < 0.01$$

$$\frac{\text{Times}}{\text{error}} = \frac{2.72}{.84} = 3.23 \ (4,20 \text{ DF}) \ P < 0.05$$

$$\frac{\text{interaction}}{\text{error}} = \frac{6.06}{.84} = 7.21 \ (4,20 \text{ DF}) \ P < 0.01$$

III-D. Analysis of Variance for Experiment 1a (Mitotic Index)

$T = 14.33$

$T^2 = 205.35$

$\Sigma sums^2 = 29.13$

$\Sigma X^2 = 11.24$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	9.71 - 8.56 = 1.15	7	0.16
Error	11.24 - 9.71 = 1.53	16	0.09

$$F = \frac{0.06}{0.09} = 1.77 \ (7,16) \ \text{n.s.}$$

CONCLUSION:

None of the sample mean mitotic indices was significantly different from any other.

III-E. Analysis of Variance of Experiment 1b (Mitotic Index)

$T = 6.62$

$T^2 = 43.82$

$\Sigma sums^2 = 6.13$

$\Sigma X^2 = 2.32$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	2.04 - 1.83 = 0.21	7	0.03
Error	2.32 - 2.04 = 0.28	16	0.018

$$F = \frac{0.03}{0.018} = 1.67 \ (7,16) \text{ n.s.}$$

CONCLUSION:

None of the sample mean mitotic indices was significantly different from any other.

III-F. Analysis of Variance To Compare the Mean Mitotic Indices from Experiments 1a and 1b

$T = 20.95$

$T^2 = 438.9$

$T_r^2 = 58.93$

$T^2_{comb.} = 35.26$

$\Sigma X^2 = 13.55$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Treatment	10.38 - 9.14 = 1.24	1	1.24
Time of Day	9.82 - 9.14 = 0.68	7	0.097
Tmt X Time (Interaction)	(11.75 - 9.14) - 1.24 - 0.097 = 1.27	7	0.18
Error	13.55 - 11.75 = 1.80	32	0.056

F Ratios

$$\frac{\text{Treatment}}{\text{Error}} = \frac{1.24}{0.056} = 22.14 \ (1,32) \quad P < 0.01$$

$$\frac{\text{Time}}{\text{Error}} = \frac{0.097}{0.056} = 1.73 \ (7,32) \quad \text{n.s.}$$

$$\frac{\text{Interaction}}{\text{Error}} = \frac{0.18}{0.056} = 3.21 \ (7,32) \quad P < 0.05$$

CONCLUSION:

The set of sample mean mitotic indices from Experiment 1a (with colcemid) was significantly higher than the set of sample mean mitotic indices from Experiment 1b (without colcemid). The effect of colcemid was greater or lesser at certain times of the day; the specific time was not identifiable.

III-G. Analysis of Variance for Experiment 1b (Labeling Index)

$T = 3.17$

$T^2 = 10.07$

$\Sigma sums^2 = 1.71$

$\Sigma X^2 = 0.68$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	0.57 - 0.42 = 0.15	7	0.021
Error	0.68 - 0.57 = 0.11	16	0.0068

$$F = \frac{0.021}{0.0068} = 3.09 \ (7,16) \quad P < 0.05$$

Studentized Range Test

$$k = 4.9 \sqrt{\frac{0.0068}{3}} = 0.23$$

Group:	4	1	2	7	5	3	8	6
Sacrifice Time:	2130	1230	1530	0630	0030	1830	0930	0330
MI(%):	25.7	25.2	14.3	13.9	10.2	7.5	4.8	4.1

CONCLUSION:

No two sample mean labeling indices were significantly different from each other.

III-H. Analysis of Variance for Experiment 2a (Mitotic Index)

$T = 18.67$

$T^2 = 348.57$

$\Sigma sum^2 = 50.98$

$\Sigma X^2 = 18.67$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	10.20 - 8.71 = 1.49	7	0.21
Error	15.09 - 10.20 = 4.89	32	0.15

$$F = \frac{0.21}{0.15} = 1.4 \ (7,32) \quad n.s.$$

CONCLUSION:

None of the sample mean mitotic indices was significantly different from any other.

III-I. Analysis of Variance for Experiment 2a (Labeling Index)

$T = 1{,}030.6$

$T^2 = 1{,}062{,}136.3$

$\Sigma Sums^2 = 126{,}403.74$

$\Sigma X^2 = 27{,}074.44$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	25,280.75 - 23,603.03 = 1,677.72	8	209.72
Error	27,074.44 - 25,280.75 = 1,793.69	36	49.82

$$F = \frac{209.72}{49.82} = 4.21 \ (8,36) \quad P < 0.01$$

Studentized Range Test

$$k = 4.68 \sqrt{\frac{49.82}{5}} = 14.77$$

Group:	9	7	3	8	6	2	5	1	4
Sacrifice Time:	1500	0900	2100	1200	0600	1800	0300	1500	2400
LI(%):	35.5	29.3	25.9	25.0	19.5	19.2	18.1	17.8	15.9

CONCLUSION:

The sample mean labeling index which occurred at 1500 hours was significantly higher than that which occurred at 2400 hours.

III-J. Analysis of Variance for Experiment 2b (Mitotic Index)

$T = 106.95$

$T^2 = 11{,}438.30$

$\Sigma sums^2 = 691.25$

$\Sigma X^2 = 214.47$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	172.81 - 142.98 = 29.12	19	1.53
Error	214.47 - 172.81 = 41.66	60	0.69

$$F = \frac{1.53}{0.69} = 2.22 \ (19,60) \quad P < 0.05$$

Studentized Range Test

$$k = 3.74 \sqrt{\frac{0.69}{4}} = 1.55$$

Group:	2	1	5	7	6	8	4	19	10	3
Injection Time:	0600_1	0800_1	2400_1	1800_1	2100_1	1500_1	0200_1	0600_3	0900_2	0400_1
MI(%):	2.68	2.32	2.31	1.91	1.76	1.62	1.52	1.50	1.42	1.33

Group:	18	20	9	17	11	14	13	16	15	12
Injection Time:	0900_3	0300_3	1200_2	1200_3	0600_2	2100_2	2400_2	1500_2	1800_2	0300_2
MI(%):	1.26	1.16	1.05	0.90	0.87	0.81	0.70	0.60	0.58	0.45

Note: Injection times with a subscript of 1 indicate these groups of newts which were injected within 24 hours of sacrifice. A subscript of 2 indicates injection between 24 and 48 hours before sacrifice and a subscript of 3 indicates injection more than 48 hours before sacrifice.

CONCLUSIONS:

The sample mean mitotic index for group 2 was significantly higher than those for groups 9, 11, 12, 13, 14, 15, 16, and 17.

The sample means from groups 1 and 5 were significantly higher than those for groups 12, 13, 15, and 16.

III-K. Analysis of Variance for Experiment 2b (Labeling Index)

$T = 1,315.0$

$T^2 = 1,729,225.0$

$\Sigma Sums^2 = 97,456.94$

$\Sigma X^2 = 28,548.46$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	24,364.24 - 21,615.31 = 2,748.93	19	144.68
Error	28,548.46 - 24,364.24 = 4,184.22	60	69.74

$$F = \frac{144.68}{69.74} = 2.07 \ (19,16) \quad P < 0.05$$

Studentized Range Test

$$k = 3.74 \sqrt{\frac{69.74}{4}} = 15.62$$

Group:	19	4	10	5	6	7	2	11	14	9
Injection Time:	0600_3	0200_1	0900_2	2400_1	2100_1	1800_1	0600_1	0600_2	2100_2	1200_2
LI(%):	28.9	27.3	23.8	22.0	22.0	19.7	19.5	16.5	16.1	15.1

Group:	13	8	1	20	18	15	16	12	17	3
Injection Time:	2400_2	1500_1	0800_1	0300_3	0900_3	1800_2	1500_2	0300_2	1200_3	0400_1
LI(%):	14.9	14.8	14.6	13.4	11.6	11.0	11.0	10.9	9.0	6.9

Note: Injection times with a subscript of 1 indicate those groups of newts which were injected within 24 hours of sacrifice. A subscript of 2 indicates injection between 24 and 48 hours before sacrifice and a subscript of 3 indicates injection more than 48 hours before sacrifice.

CONCLUSION:

The sample mean labeling indices for groups 4 and 19 were significantly higher than those for groups 3, 12, 15, 16, 17 and 18.

The sample mean for group 10 was significantly higher than that for group 3.

III-L. Analysis of Variance for Experiment 5

$T = 109.52$

$T^2 = 11{,}994.63$

$\Sigma sums^2 = 1{,}256.90$

$\Sigma X^2 = 349.20$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	314.23 - 299.87 = 14.36	9	1.60
Error	349.20 - 314.23 = 34.97	30	1.17

$$F = \frac{1.60}{1.17} = 1.37 \ (9,30) \quad \text{n.s.}$$

CONCLUSION:

None of the sample mean mitotic indices was significantly different from any other.

III-M. Analysis of Variance for Experiment 6

$T = 87.31$

$T^2 = 7,623.04$

$\Sigma sums^2 = 809.91$

$\Sigma X^2 = 274.82$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	202.48 - 190.58 = 11.90	9	1.32
Error	274.82 - 202.48 = 72.34	30	2.41

$$F = \frac{1.32}{2.41} = 0.55 \ (9,30) \quad n.s.$$

CONCLUSION:

None of the sample mean mitotic indices was significantly different from any other.

III-N. Analysis of Variance to Compare the Mean Mitotic Indices From Experiments 5 and 6

$T = 199.01$

$T^2 = 39,604.98$

$Tc^2 = 20,003.09$

$Tr^2 = 4,097.40$

$T^2_{comb.} = 2,105.53$

$\Sigma X^2 = 621.90$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Treatment	500.08 - 495.06 = 5.02	1	5.02
Time of Day	512.18 - 495.06 = 17.12	9	1.90
TmT X Time (Interaction)	(526.38 - 495.06) - 5.02 - 1.90 = 24.40	9	2.71
Error	621.90 - 526.38 = 95.52	60	1.59

F Ratio

$$\frac{\text{Treatment}}{\text{Error}} = \frac{5.02}{1.90} = 2.64 \ (1,60) \quad \text{n.s.}$$

$$\frac{\text{Time}}{\text{Error}} = \frac{1.90}{1.59} = 1.19 \ (9,60) \quad \text{n.s.}$$

$$\frac{\text{Interaction}}{\text{Error}} = \frac{2.71}{1.59} = 1.70 \ (9,60) \quad \text{n.s.}$$

CONCLUSION:

There was no significant effect of the ^{3}H-TdR injection on the mitotic index on intestinal cell nests of the newt.

III-O. Analysis of Variance for Experiment 7

$T = 48.05$

$T^2 = 2,308.80$

$\Sigma sums^2 = 240.4$

$\Sigma X^2 = 72.37$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	65.96 - 57.72 = 8.24	9	0.92
Error	72.37 - 65.96 = 6.41	30	0.21

$$F = \frac{0.92}{0.21} = 4.38 \ (9,30) \quad P < 0.01$$

Studentized Range Test

$$k = 4.82 \sqrt{\frac{0.21}{4}} = 1.10$$

CONCLUSION:

No sample mean mitotic index was significantly different from any other.

III-P. Analysis of Variance for Experiment 8

$T = 9.16$

$T^2 = 83.91$

$\Sigma sums^2 = 9.13$

$\Sigma X^2 = 3.45$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	3.04 - 2.80 = 0.24	9	0.0267
Error	3.45 - 3.04 = 0.41	20	0.0205

$$F = \frac{0.0267}{0.0205} = 1.30 \ (9,20) \text{ n.s.}$$

CONCLUSION:

No sample mean mitotic index was significantly different from any other.

III-Q. Analysis of Variance to Compare the Mean Mitotic Indices from Experiments 5, 7 and 8

$T = 169.79$

$T^2 = 28,828.64$

$Tc^2 = 14,452.76$

$Tr^2 = 2,968.46$

$T^2_{comb.} = 1,513.59$

$\Sigma X^2 = 426.14$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Treatment	361.32 - 240.24 = 121.08	2	60.54
Time of Day	247.37 - 240.24 = 7.13	9	0.79
TmT X Time (Interaction)	(378.40 - 240.24) - 60.54 - 0.79 = 76.83	18	4.27
Error	426.14 - 378.40 = 47.74	90	0.53

F Ratios

$$\frac{\text{Treatment}}{\text{Error}} = \frac{60.54}{0.53} = 114.23\ (2,90)\quad P < 0.01$$

$$\frac{\text{Time}}{\text{Error}} = \frac{0.79}{0.53} = 1.49\ (9,90)\quad \text{n.s.}$$

$$\frac{\text{Interaction}}{\text{Error}} = \frac{4.27}{0.53} = 8.06\ (18,90)\quad P < 0.01$$

CONCLUSIONS:

The mitotic indices from Experiment 7 were significantly lower than those from Experiment 5.

The mitotic indices from Experiment 8 were significantly lower than those from Experiment 7.

The environmental conditions under which the animals were maintained had a greater or lesser effect on the intestinal mitotic indices at unidentified times of the day.

III-R. Analysis of Variance for Experiment 9

$T = 148.71$

$T^2 = 22,114.66$

$\Sigma Sums^2 = 2,480.09$

$\Sigma X^2 = 698.82$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	620.03 - 552.87 = 67.16	9	7.46
Error	698.82 - 620.03 = 78.79	30	2.63

$$F = \frac{7.46}{2.63} = 2.84 \ (9,30) \quad P < 0.05$$

Studentized Range Test

$$k = 4.82 \sqrt{\frac{2.63}{4}} = 3.91$$

Group:	3	5	7	6	1	2	10	4	9	8
Sacrifice Time:	1600	2200	0400	0100	1000	1300	1300_2	1900	1000_2	0700
MI(%)	6.82	5.82	4.07	3.80	3.38	3.31	2.87	2.79	2.68	2.45

Note: A subscript of 2 on the sacrifice time indicates the second day of the experiment.

CONCLUSION:

The sample mean mitotic index at 1600 hours was significantly higher than those at 1900 hours and 0700 hours as well as those at 1000 hours and 1300 hours of the second day.

III-S. Analysis of Variance to Compare the Mean Mitotic Indices from Experiments 5 and 9

$T = 258.23$

$T^2 = 66,682.73$

$Tc^2 = 34,109.29$

$Tr^2 = 7,039.78$

$T^2_{comb.} = 37.40$

$\Sigma X^2 = 1,048.01$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Treatment	852.73 - 833.53 = 19.20	1	19.20
Time of Day	879.97 - 833.53 = 46.44	9	5.16
Tmt X Time (Interaction)	(935.00 - 833.53) - 19.2 - 5.16 = 77.27	9	8.58
Error	1,048.01 - 833.53 = 214.48	60	3.57

F Ratio

$$\frac{\text{Treatment}}{\text{Error}} = \frac{19.20}{3.57} = 5.38 \ (1,60) \quad P < 0.05$$

$$\frac{\text{Time}}{\text{Error}} = \frac{5.16}{3.57} = 1.41 \ (9,60) \quad \text{n.s.}$$

$$\frac{\text{Interaction}}{\text{Error}} = \frac{8.58}{3.57} = 2.40 \ (9,60) \quad P < 0.05$$

CONCLUSIONS:

The mitotic indices of Experiment 9 were significantly higher than those of Experiment 5.

The effect of the experimental conditions was significantly greater or lesser at a time or at times of the day which are not identified.

III-T. Analysis of Variance for Experiment 10

$T = 16.14$

$T^2 = 260.50$

$\Sigma sums^2 = 69.39$

$\Sigma X^2 = 37.14$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	17.34 - 6.51 = 10.83	9	1.20
Error	37.14 - 17.34 = 19.8	30	0.66

$$F = \frac{1.20}{0.66} = 1.82 \ (9,30) \quad \text{n.s.}$$

CONCLUSION:

None of the sample mean mitotic indices were significantly different from any other.

III-U. Analysis of Variance for Experiment 11

$T = 18.75$

$T^2 = 351.56$

$\Sigma sums^2 = 36.08$

$\Sigma X^2 = 15.35$

Analysis of Variance (ANOVA)

Source	Sum of Squares	Degrees of Freedom	Variance Estimation (Mean Square)
Time	9.02 - 4.63 = 4.39	18	0.24
Error	15.35 - 9.02 = 6.33	57	0.11

$$F = \frac{0.24}{0.11} = 2.18 \ (18,57) \quad P < 0.05$$

Studentized Range Test

$$k = 5.22 \sqrt{\frac{0.11}{4}} = 0.87$$

Group:	19	18	13	14	17	16	11	1	15	3
Sacrifice Time:	1300_3	1000_3	1900_2	2200_2	0700_2	0400_2	1300_2	1000_1	0100_2	1600_1
MI(%):	1.00	0.52	0.51	0.44	0.36	0.32	0.31	0.25	0.23	0.20

Group:	2	12	10	6	7	9	5	8	4
Sacrifice Time:	1300_1	1600_2	1000_{2b}	0100_1	0400_1	1000_{2a}	2200_1	0700_1	1900_1
MI(%):	0.19	0.11	0.07	0.05	0.04	0.04	0.03	0.03	0.01

Note: A subscript of 1 on the sacrifice time indicates that the group of newts was killed within 24 hours postirradiation. Subscripts of 2 or 3 indicate that the newts were killed between 24 and 48 hours or after 48 hours, respectively.

CONCLUSION:

The sample mean mitotic index of group 19 (56 hours postexposure) was significantly higher than those from groups 4, 5, 6, 7, 8, 9, 10 and 12.