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FREDERICK ALLEN HODGE for the DOCTOR OF PHILOSOPHY
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Title: THE RELATIONSHIP OF LYSOSOMAL STABILITY TO RADIATION-INDUCED DELAY OF CELL DIVISION IN *TETRAHYMENA PYRIFORMIS*

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D. Stuart Nachtwey

Cell division in a heat synchronized *Tetrahymena pyriformis* (GL-I) cell can be delayed if the cell is exposed to X-irradiation (300 kVp, 20 mA, HVL = 0.9 mm Cu, 25 R/sec) prior to a critical time after the end of the synchronizing treatment (EST). At the critical time, the cells undergo a rapid transition from a state of being sensitive to being delayed to one of relative insensitivity. After this time, the coming division is delayed little if at all; that is, there is an "all or none" transition to insensitivity to division delay. However, the second division is delayed, indicating that damage has occurred. The transition point (median critical time) is determined by interpolating the time after EST at which a given radiation exposure splits a population of cells into 50 percent delayed and 50 percent not delayed. The transition point is dose dependent in that the larger the exposure, the later the transition point (31 minutes after EST for 3kR

increasing gradually to 51 minutes after EST for 18.5 kR).

The division delay response is correlated with a resorption of the developing new mouth as determined from silver stained cells fixed every ten minutes after EST. The length of time for the oral primordium to be resorbed increases as the dose increases and varies with the time of irradiation relative to the transition point.

The "all or none" nature of the division delay response and the fact that cells irradiated after the transition point seemingly "ignore" the radiation damage until the second generation, together suggest that the damage produced by the irradiation may or may not trigger a cellular response that results in cell division delay and oral primordium resorption. These responses might suggest that preformed enzymes, possibly from lysosomes, could be involved. Therefore, cells were treated with a known lysosomal stabilizer (hydrocortisone) and a labilizer (vitamin A) continuously before, during, and after exposure to 7.5 kR of X-rays. Cells treated with hydrocortisone-21-phosphate (5.0 mM) showed an earlier transition point (36.5 minutes after EST) whereas cells treated with vitamin A-acetate (0.2 mM) showed a later transition point (50.5 minutes after EST) relative to the transition point for untreated, irradiated cells (43 minutes after EST). The time of the transition for cells treated with a lysosomal stabilizer (depresses enzyme release) and 7.5 kR corresponds to the transition point for untreated cells irradiated with only 5.0 kR,

suggesting less sensitivity. Conversely, the transition point for cells treated with a lysosomal labilizer (enhances enzyme release) and 7.5 kR corresponds to the time of transition for untreated cells exposed to 18.5 kR, suggesting greater sensitivity. However, in all cases, 7.5 kR delayed the divisions about the same amount, whether the agents were present or not, indicating that the agents do not act in a simple dose-modifying manner.

The data are consistent with the hypothesis that irradiation produces damage that may or may not trigger off a cellular response that leads to delay of division. The sensitivity of the cell to being triggered to delay division may involve lysosomes.

The Relationship of Lysosomal Stability to Radiation-Induced Delay of Cell Division in *Tetrahymena pyriformis*

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APPROVED:

Redacted for privacy

Associate Professor of Radiation Biology (Department of General Science)
in charge of major

Redacted for privacy

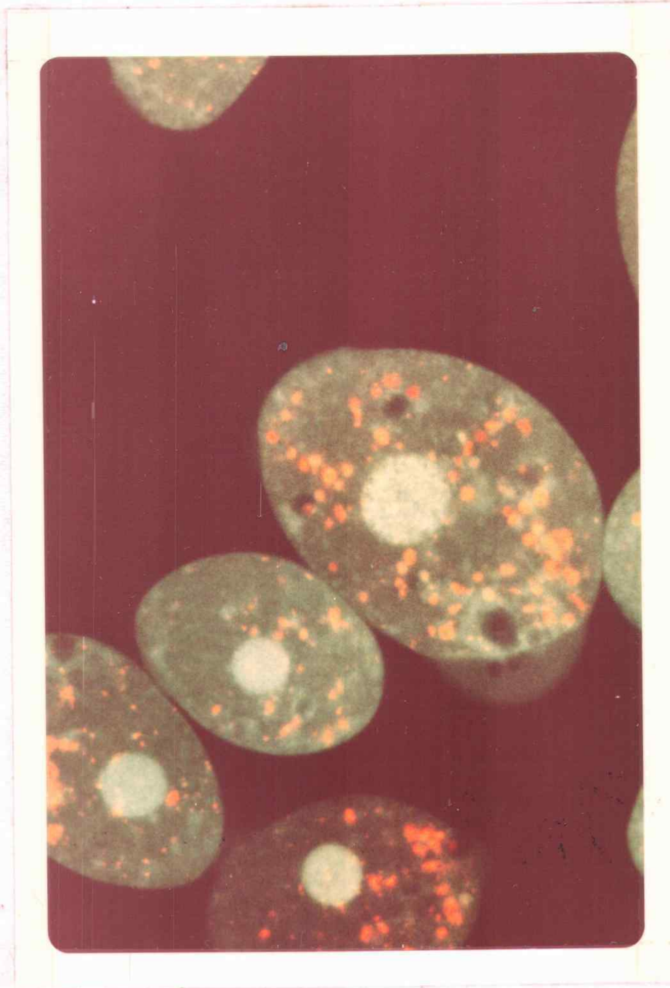
Chairman of Department of General Science

Redacted for privacy

Dean of Graduate School

Date thesis is presented 18 March 1971

Typed by Susie Kozlik for Frederick Allen Hodge



Frontispiece. Fluorescence photomicrograph of euchrysine stained Tetrahymena pyriformis (GL-I) cells (X 900). The large bright green objects are the macronuclei and the small, numerous bright red granules are lysosomes.

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THE RELATIONSHIP OF LYSOSOMAL STABILITY TO RADIATION-INDUCED DELAY OF CELL DIVISION IN *TETRAHYMENA PYRIFORMIS*

INTRODUCTION

The Problem

A fundamental property of life is its faculty for demonstrating irritability. Almost any noxious agent will stimulate living material to respond in some way. In the present study the living material is a ciliated protozoon, the noxious stimulant is ionizing radiation, and the response to be studied, although not ordinarily considered a stimulus-response phenomenon, is the delay of cell division.

A great many experiments have shown that, in general, sub-lethal exposure to ionizing radiation produces a temporary inhibition of cell division in all types of cells that divide (Lea, 1955; Giese, 1967; Elkind and Whitmore, 1967). The duration of this delay is dependent upon the type of cell, the radiation dose, and the age of the cell at the time of irradiation.

There may be several general ways by which radiation can delay cell division. For example, radiation might damage some specific organelle or substance that is essential for division. The delay of division might result from the time required to repair or replace the essential element. Or, the damage might decrease the rate of some

division-essential process which leads to a slowing of the rate of preparations for division. Or, radiation might alter the internal molecular environment of a cell such that progress toward division is slowed or stopped until the normal conditions can be restored. On the other hand, radiation acting as a stimulus may evoke a specific orderly sequence of events that has homeostatic value to the cell. That is, radiation may trigger a process, which has evolved in cells by natural selection, that prevents cell division until conditions are more favorable for successful reproduction of viable daughter cells.

This study is concerned with describing some of the underlying characteristics of radiation-induced division delay in synchronized cultures of the protozoon, *Tetrahymena pyriformis*. Evidence will be presented in support of the hypothesis that the damage produced by radiation acts as a stimulus that may or may not trigger a cellular response that leads to the delay of cell division and that the sensitivity of the cell to being triggered may involve lysosomes. It is postulated that irradiation occurring prior to some critical step in the cell's preparations for division, produces damage that is "recognized" and which triggers a sequence of events, possibly involving lysosomes, which returns the cell to an earlier physiological stage so that it can repair the defect before preparations for division can resume. Irradiation occurring after the critical step, produces damage that the cell seemingly "ignores"; it divides on schedule. The following or second

division, however, is delayed, possibly as a result of the damage triggering repair processes.

Since division delay is a prominant cellular response to ionizing radiation and may be important in other aspects of radiation biology such as the production of abnormal embryos and in allowing time for the repair of genetic damage, a more thorough understanding of it is valuable at a time when the use of radiation in medicine and industry is increasing.

For the non-radiation specialist, a brief review of the physical, chemical, and biological aspects of ionizing radiation follows.

Physical Aspects of Radiation

Ionizing radiation consists of electromagnetic radiation (X-rays and gamma rays) and corpuscular radiation such as beta rays (electrons), alpha rays (helium nuclei), protons (hydrogen nuclei), and neutrons. All of these produce electrically charged particles or ions in matter, hence their classification as ionizing radiations (Glasstone, 1967).

The primary means of energy deposition for both types of ionizing radiations is the ejection of an electron from its orbit around the positively charged nucleus of an atom; the energetic ejected electrons in turn eject more electrons until all of their energy is spent. The energetic electrons can also deposit energy by exciting molecules but this is thought to be a less biologically important mode. Almost all the

energy deposited by radiation is ultimately degraded to heat.

The biologically significant properties of ionizing radiation depend on: (1) the type and energy level of radiation; (2) the total amount of energy absorbed per gram of material; and (3) the rate of dose absorption. The physics involved in both the production and absorption of radiation will not be discussed here but a complete treatise of this subject may be found in Johns and Cunningham (1969).

The measurement of radiation dose can be based on energy deposition but it is more convenient and practical to measure the ionizations produced in some irradiated material such as air and talk in terms of exposure. Alternatively, the exposure values can be converted to energy absorbed in the biological material allowing one to use absorbed dose terminology. The exposure unit for ionizing radiation is the Roentgen (R) which is used to describe its ability to ionize air. The Roentgen is defined as the quantity of X-rays or gamma rays which will produce, as a consequence of ionization, one electrostatic unit (esu) of electricity, of either sign in 1 cc of dry air, at 0° C and standard temperature and pressure (ICRU Report 10a, 1962). The measurement is usually made with an air ionization chamber.

Since the Roentgen represents exposure and not absorbed dose in material other than air, the radiation absorbed dose (rad) was adopted by the International Commission on Radiation Units and Measurements in 1953 as the unit for absorbed dose of ionizing radiation. The rad is

defined as the dose of any ionizing radiation which is accompanied by the liberation of 100 ergs of energy per gram of absorbing material (ICRU report 10a, 1962). If one measures an exposure in air, a conversion to rad can be made by consulting a roentgen to rad table of conversion factors (f) (ICRU Report 10b, 1962) to find a value for the absorbed dose in materials other than air.

Chemical Aspects of Radiation

In addition to molecules ionized directly by electromagnetic radiation, the ionized particles resulting from the radiation exposure of some material produce free radicals which cause chemical reactions, including chain reactions, with many different molecules. Kernbaum (1909) and Duane and Scheuer (1913) were among the first to study the chemical effects of ionizing radiation on aqueous solutions. However, it was not until the studies of Fricke, Hart, and Smith (1938), working on organic chemical reactions in water and those of Dale (1940) working on irradiated aqueous enzyme solutions, that the importance of an indirect action of radiation began to be recognized.

Since the atoms in greatest numbers in cells are the hydrogen and oxygen atoms of water (roughly 80 percent of a cell is composed of water), reactions involving the radiolysis products of water, hydrogen ($H\cdot$), hydroxyl ($OH\cdot$), and peroxyl ($HO_2\cdot$) free radicals, become extremely important. The free radicals, defined as electrically neutral

atoms or molecules containing unpaired electrons, are not distributed uniformly throughout irradiated cells but are believed to occur in "spurs" or clusters along the track of absorbed radiation (Bacq and Alexander, 1961). The distribution of the reaction products is dependent upon the Linear Energy Transfer (LET)[1] of the incident particles (Matheson, 1964). Baxendale (1964) showed that the peroxyl radical and hydrogen peroxide (H_2O_2) were the primary oxidizing products of ionizing radiation when an abundance of oxygen was present.

Free radicals may react with an organic macromolecule, an enzyme for example, converting it to a free radical. This macromolecular free radical may react with another macromolecular free radical to form a cross-linked aggregate or with an oxygen molecule to form a peroxide. Macromolecular changes such as these might well account for altered functions involving such fundamental properties of cell constituents as solubility, viscosity, permeability, and chemical reactivity (Adelstein, 1965).

One can visualize the damaging consequences to cellular behavior if a critical molecule such as DNA is chemically altered. Alterations of the purine and pyrimidine bases of irradiated DNA are in fact the most common example of radiation induced change to a critical cellular molecule (Scholes, 1963). Single and double strand breaks of DNA are

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[1] A physical parameter for describing the rate of energy loss of penetrating particle along the length of its path. It is usually expressed in units of keV/micron.

also likely to be important types of damage produced by ionizing radiation (Okada, 1970).

Biological Aspects of Radiation

Almost immediately after the discovery of X-rays in 1895 by Wilhelm Conrad Röntgen and natural radioactivity by Henri Becquerel in 1896, scientists began studying the effects of ionizing radiation on biological material. Initial studies were not necessarily planned experiments *per se* but the unhappy results of radiation injury caused by laboratory accident and misuse by physicians.

There were, however, several early studies which were particularly significant, so much so in fact that they remain noteworthy even today. Heineke (1905) performed the first fairly complete analysis of the pathology of irradiated mice, guinea pigs, rabbits, and dogs. Bergonié and Tribondeau (1906) used irradiated rodent testes as a basis for their now famous "Law" of cellular radiosensitivity. Their "Law" states that the most radiosensitive cells are those which are the most mitotically active, undergo the most divisions, and are the least differentiated. Although there are exceptions to this rule today there are many cells which do fit this description of radiosensitivity. The works of Muller (1927) and Stadler (1928) on animals and plants, respectively, established the genetic effects of radiation and laid the foundation for research in radiation-induced mutations.

At about this same time period (the 1920's and earlier) experiments were being conducted which were to eventually result in the target theory. This theory assumes that radiation exposure has produced ionized particles within a small fraction of the cell's volume, the target.

The target theory, initially proposed by Crowther (1924) and further expanded by Lea (1955), suggests that an essential part of a cell is damaged by a direct hit by an electron. The theory allowed for the possibility that a cell might contain a number of targets each of which required inactivation to inactivate the cell. Target theory has been modified subsequently to include the indirect effects of ionizations very near to the target (Hutchinson and Pollard, 1961). More recent studies indicate that in some cells, a set amount of energy appears to be absorbed either as a single hit or in several hits, before inactivation occurs (Fowler, 1964).

Consequently, simple target theory has been expanded to include multi-hit-single-target and single-hit-multi-target situations (Lea, 1955; Powers, 1962). Although there are exceptions and nonconformities to target theory, it remains as a means for mathematically describing survival curves and the concepts of targets.

Opinions vary as to the nature of radiation damage to cells that might result from a direct hit on a vulnerable target and, also, regarding the specific chemical changes initiated by free radicals formed

from water. However, it is clear that the two theories (direct _vs_ indirect) are not mutually exclusive and that opportunities for the formation of excited, free radicals exist with both direct and indirect hits. Regardless of whether radiation damage occurs predominantly by direct or indirect action, the end result may include subtle changes in the permeability of cellular, nuclear, mitochondrial, or lysosomal membranes, in enzyme activity, in the sensitivity of cells to various chemicals, in polymerization of small molecules into non-functional aggregates, and in other functions characteristic of living cells. Any one of these defects may perturb the cell's normal functioning and/or stimulate the cell to respond in some observable way.

Cellular Responses to Radiation

A cell's response to radiation may be classified in two categories, lethal and nonlethal. Although neither type can be termed simple, the latter is by far the more complex and pertinent to this study. There are, however, certain lethal aspects of particular importance to this discussion.

Radiation-induced cellular lethality or cell death can occur in two ways, interphase death and reproductive death (Little, 1968). Interphase death occurs soon after, or in some cases during, radiation exposure. This type of response can be obtained merely by exposing cells to such large doses of radiation that death occurs at any stage of

the cell cycle and before the cell divides. It is sometimes referred to as molecular death since massive and irreparable chemical and structural damage occurs (Bond, Fliedner, and Archambeau, 1965).

Reproductive death, sometimes termed mitotic inhibition, is of much greater importance. Cells exposed to moderately low doses of radiation (a few hundred to a few thousand roentgens) initially show little or no visible damage. However, these cells eventually die because of their inability to undergo continued cell division (Lea, 1955).

Puck, Marcus, and Cieciura (1956) developed a technique for culturing single mammalian cells as clonal colonies. This led to the procedure used by Puck and Marcus (1956) for establishing some of the details of the action of radiation on cell division. They showed that some cells (HeLa S-3) that received up to 850 rads survived but were delayed in dividing whereas others divided a few times and then died. At higher doses they found still other cells that neither died nor divided but continued metabolizing and grew to form giant cells. This classic study was instrumental in initiating more experiments on the quantitative study of radiation-induced division delay and reproductive death.

A third type of radiation-induced cell lethality is genetic damage. Radiation can cause either single or double strand breaks in the DNA of chromosomes.

Depending on where in the cell cycle the irradiation occurred, chromosome or chromatid aberrations can be produced. Such

aberrations, which result from the various rejoining configurations (or lack of rejoining) of broken chromosome or chromatids, can lead to immediate reproductive failure or some malfunction at a later time which results in the death of the cell or as sometimes happens, no observable defect except continued presence of the aberration (Davies and Evans, 1966).

Although chromosomes probably do contain areas lethally-sensitive to irradiation, they are not the only major mechanism of cell death (Little, 1968). Indeed there is probably no single mechanism leading to cell lethality. A more probable explanation might include combinations of all the various mechanisms of radiation damage which lead to the death of a cell.

Of the non-lethal responses to radiation, cell division delay is by far the most common and observable. It is probably universal for all proliferating cells. Using the early work of Henshaw (1932; 1940a; 1940b) on X-irradiated sea urchin eggs (*Arbacia*) and the work of Cook (1939) on irradiated *Ascaris* eggs, Lea (1955) suggested that temporary blockage of cell division was associated with a metabolic recovery from the radiation damage. This hypothesis has been repeatedly supported (Ducoff, 1957; Elkind, Moses, and Sutton-Gilbert, 1967). However, a delicate dissection of the mechanisms involved remains somewhat elusive. For example, the position of the cell in its cell cycle at the time or irradiation is extremely important in determining the type of division delay.

It will be convenient to recall Stanners and Till's (1960) widely used diagram of Howard and Pelc's (1953) stages of the cell cycle:

The cycle starts at cytokinesis, the division of one cell into two. Preparations for the next cell division consist of a presynthetic stage (G_1), followed by a period of DNA synthesis (S), and then another growth stage prior to division (G_2). In general, G_2 is the most sensitive and easily blocked stage whereas cells irradiated in G_1 are the least sensitive (Terasima and Tolmach, 1963). Cells irradiated in G_1 move through S and into G_2 but are delayed for a dose-dependent-time from going on to division. Cells irradiated in S may show a delay in entering G_2 (i.e. a prolongation of S, and also a G_2 delay). The duration of the delay of cell division, therefore, is dependent upon the position of the cell in the cycle at the time of irradiation. The occurrence and duration of radiation-induced division delay has been shown by Elkind, Han, and Volz (1963) to be similar for both surviving and non-surviving cells, which suggests that the mechanisms producing non-lethal division delay and reproductive death differ.

Although the mechanisms for radiation-induced division delay have remained obscure, several alternative hypotheses have been proposed. Some of the possible sites of action include DNA, a long

lived messenger RNA, and various intracellular fine structures and membranes (Hutchinson, 1966; Alexander _et al._, 1965). Little (1968) has suggested that division delay may be related to the phenomenon of chromosome "stickiness". Doida and Okada (1969) concluded that radiation-induced division delay in cultured mouse leukemic cells (L5178Y) was caused by a complete but transient blockage of cell progress in the middle of the G_2 stage, and that it involved the inhibition of protein synthesis. Recently, Scaife (1970) suggested that the mechanism responsible for mitotic delay following X-irradiation is not damaged DNA _per se_. This conclusion is based on the fact that division delay can be demonstrated at doses much less than those at which DNA strand scission can be demonstrated. Walters and Petersen (1968) equated X-ray induced division delay with a defect in protein synthesis at the translational level. Translation refers to the translation of the nucleotide language into the amino acid language during protein synthesis (Luria, 1970). Scaife and Brohée (1969) suggest the possibility that division delay requires the involvement of the condensation mechanism for prophase chromosomes. In the final analysis, the mechanism responsible for division delay still remains obscure. There is, therefore, much room for some speculative thoughts and new alternative hypotheses.

Lysosomes and Radiation

One of the earliest biochemical changes that can be seen in cells given moderate doses of ionizing radiation is an increase in enzyme activity. This finding led early investigators to propose that for cell death the primary lesion was an alteration in the permeability of certain intracellular structures such as lysosomes. As a consequence, Bacq and Alexander (1961) proposed their well known "enzyme-release" hypothesis. They suggest that radiation affects the permeability of lysosomal membranes allowing the release of hydrolytic enzymes, which in turn have a deleterious effect on the cell.

Lysosomes are tiny, usually spherical, intracellular organelles, about 0.25 to 1.0 microns in diameter. They contain a variety of enzymes which can break down virtually all the major constituents of living cells. They were visually discovered as distinct cytoplasmic particles in 1955 by Christian deDuve and his colleagues at the Catholic University of Louvain, Belgium.

The enzymes in lysosomes can digest food particles entering the cell, the entire cell itself (or its parts), or tissues outside the cell. Consequently, lysosome functions or malfunctions are probably involved in many vital processes. They have been implicated in the fertilization of the egg, the development and death of cells, in certain diseases such as silicosis and gout, in cell division (deDuve and Wattiaux, 1966), in the intra-cellular digestion of macromolecules (Davies, Lloyd, and

Beck, 1969), in the immune process, in chromosome activities such as mitotic configurations (Allison, 1967), and finally, in the problem at hand, in responses to radiation (deDuve, 1963).

In isolation (from cell fractionations), lysosomes require more than 10,000 rads of ionizing radiation before leakage of enzymes can be detected (Casarett, 1968). There is, however, a distinct possibility that enzymatic release from lysosomes occurs within a living cell after much lower doses of irradiation. Such release could be mediated through the activation of some naturally occurring substance that controls lysosomal enzyme release. For example, radiation may set up a pattern of synergistic or complimentary activities resulting in premature enzyme-release which in turn causes some form of inhibition to the normal activities of the cell (e.g. cell division delay).

Weissman (1964) described several studies in which various chemicals appeared to augment or inhibit the release of enzymes from lysosomes. Some of the agents which increase the permeability of the lysosome membrane include bacterial toxins, vitamin A, and various pyrogenic steroids such as progesterone. Collectively, these agents are termed lysosomal labilizers. The labilizing effects can be nullified by agents classed as lysosomal stabilizers. Some of the stabilizing agents include several forms of cortisone, chloroquine, and some antihistamines.

If a cell is presented with a lysosomal labilizer and then

irradiated with a sublethal dose of ionizing radiation, one might expect the lysosomes to be more vulnerable to the radiation. Conversely, if the cell is presented with a lysosomal stabilizer followed by irradiation one might expect that a higher dose would be required to affect the lysosome. Since lysosomes contain enzymes which can hydrolyze phosphate esters in DNA and RNA, proteins, polysaccharides, glycosides, and sulfate esters it follows that an enhanced release of these enzymes after irradiation could be detrimental to cell division. Similarly, an inhibition of enzyme release might minimize the radiation damage to the cell. An attempt was made to test these hypotheses in this study.

MATERIALS AND METHODS

The Organism

The organism used in this study was an amicronucleate strain (GL-I) of the ciliated protozoon, Tetrahymena pyriformis (Ehrenberg, 1830). According to the latest classification of the phylum Protozoa (Honigberg et al., 1964), T. pyriformis is in the suborder Tetrahymenina, order Hymenostomatida of the subclass Holotrichia, class Ciliatea, and subphylum Ciliophora.

The initial culture was obtained from Dr. William Balamuth at the University of California, Berkeley, California. This strain is a subculture from the original GL stock maintained axenically in the laboratory of Andre Lwoff in Paris since 1925. The history of the GL-I strain (Frankel, 1964) is somewhat circuitous. It was originally obtained from Lwoff in 1946 by G. W. Kidder at Amherst, Massachusetts. Erik Zeuthen from the Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark (in California at the time) obtained a sample of Kidder's stock in 1956. This sample served as the foundation of the stock that has been maintained since then by Balamuth. Balamuth sent a subculture to Joseph Frankel at the University of Iowa in 1962. Frankel (1964) found that it differed somewhat in physiological characteristics from the strain he had been using in Zeuthen's laboratory, so he designated it GL-I (I=Iowa) and Zeuthen's

strain GL-C (C=Copenhagen) even though both were from the same original stock.

T. pyriformis GL is normally piriform (pear-shaped) to ovoid, about 30 microns wide by 50 microns long, and contains 17 - 21 longitudinal ciliary rows or meridians. Two of the meridians, termed the post oral meridians, have their anterior termination at the posterior margin of the oral area, while the remainder terminate near the apex of the cell. (The meridians are generally numbered from left to right beginning with the furthest right of the post oral meridians.) The mouth or cytostome is located near the anterior end and consists of an undulating membrane on the right side and three membranellae on the left of the buccal cavity (see Figures 16 and 17 in the Results Section). There are typically two contractile vacuole pores located near the posterior ends of ciliary meridians five and six. The macronucleus is typically ovoid to irregularly spherical, about 9.5 X 11 microns, and is located in the central portion of the ciliates' body, slightly posterior to the midline. It is a polyenergid structure with a DNA-ploidy level of approximately 40-84 (Nilsson, 1970). The macronucleus divides amitotically immediately prior to cytokinesis. The binary fissions are preceeded by the development of a new oral apparatus. The site of oral morphogenesis is on the mid-ventral surface of the cell posterior to the old mouth on ciliary meridian number one. At cleavage the old mouth is retained by the anterior half (proter) while the posterior half

(opisthe) receives the new structure. No reproductive or resting cysts have ever been reported for this species.

T. pyriformis is widely distributed throughout the world. It is commonly found in fresh water habitats as one of many microphagous (bacterial-feeder) species.

Culture Methods

The *Tetrahymena* were maintained axenically as logarithmically growing stock cultures at 24° C $\pm$ 1° C in glass tubes containing six milliliters of a nutrient medium. The ingredients for the medium, designated TYESS, are shown in Table 1. Transfers were made every other day, with a bacteriological loop using aseptic technique, to maintain the cells in the logarithmic growth phase.

Synchronization of Cell Division

Cell divisions were synchronized by a modification of the method of Zeuthen and Scherbaum (1954). This procedure consisted of subjecting log phase cells to nine temperature cycles each consisting of a five minute transition from 28° C to 34° C, a 27 minute heat shock at 34° C, a four minute transition down to 28° C, and 24 minutes at 28° C. The cells were synchronized in 10 ml volumes of fresh medium in 25 ml Erlenmeyer flasks. Fifty ml volumes in 250 ml (75 cm^{2}) tissue culture flasks (Falcon Plastics) were used for experiments requiring

Table 1. TYESS culture medium[1]

Ingredient	Quantity needed to make one liter
Tryptone[2]	2.5 grams
Basamin-Busch Yeast Extract[3]	5.0 grams
Soluble Starch[2]	5.0 grams
Balanced Salt Stock Solution (see below)	5.0 milliliters
Glass Distilled Water	995.0 milliliters

Stock Solution for Osterhout's Balanced Salt[4] Solution (200X)	
NaCl	2.08 grams
$MgSO_4 \cdot 7H_2O$	0.164 grams
$MgCl_2 \cdot 6H_2O$	0.22 gram
KCl	0.46 gram
$CaCl_2$	0.02 gram
Glass Distilled Water	100.0 milliliters

1 Although no buffer was added to the medium, the pH was invariably 6.8 to 7.0.

2 Difco Laboratories, Detroit, Michigan

3 Anheuser-Busch Inc., St. Louis, Missouri

4 All salts from Mallinckrodt Chemical Works, St. Louis, Missouri (reagent grade).

a large number of cells. The flasks were partially submerged in a controlled temperature water bath equipped with a timing mechanism for turning the heat shocks on and off (see Figures 1 and 2). The flasks were gently agitated thoughout the synchronizing procedure to insure ample oxygenation of the culture. Immediately after the last heat shock of the synchronizing treatment, the cultures were returned to 24° C.

Cell Division

In order to determine an accurate time of cell division, the cells were handled individually using the single cell technique of Nachtwey and Dickinson (1967). This procedure consisted of "spotting" several (usually 25 per dish) one microliter drops by touching a capillary tube (1 mm diameter) containing the appropriate medium to a Falcon Plastics tissue culture dish (35 X 10 mm) and then covering the drops with a thin layer of paraffin oil to prevent evaporation. One cell was then pipetted into each drop with the aid of a very finely drawn out pasteur pipette attached to three feet of surgical tubing and a mouthpiece. The fine bore of the pipette allows delicate manipulations by alternately blowing and sucking. The tip can be placed into the microdrop through the paraffin oil without picking up any of the oil (See Figure 3).

The drops, each containing one cell, were then examined at frequent intervals for evidence that division had occurred (the presence

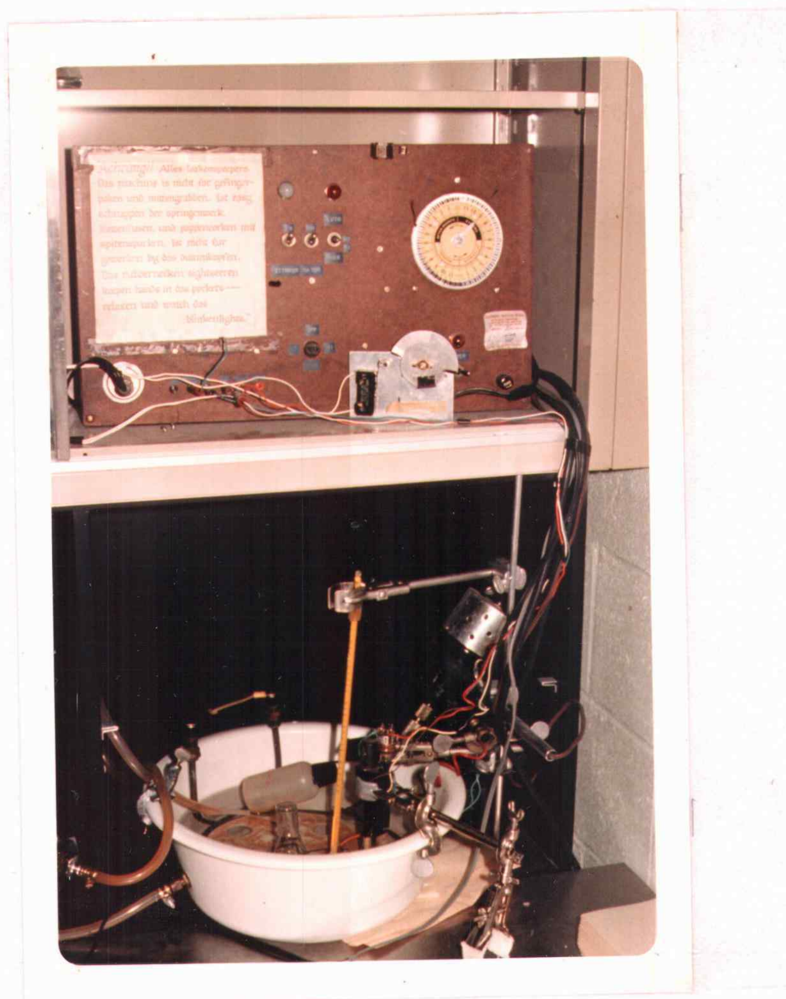

Figure 1. Synchronizer, showing the timing mechanism for the heat shocks and the water bath with a 25 ml Erlenmeyer flask in position.

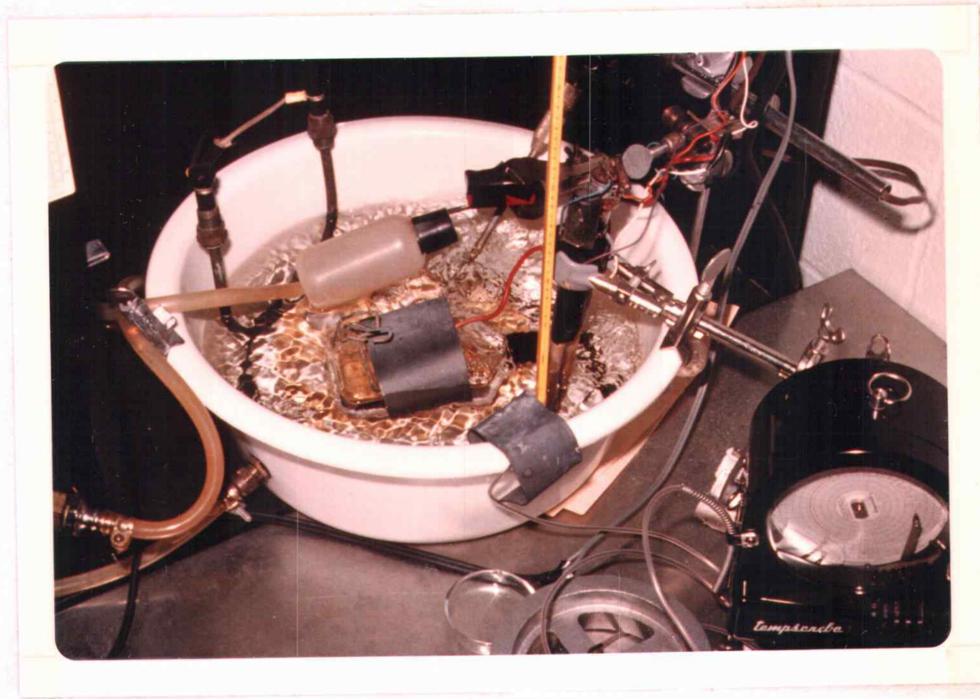

Figure 2. Synchronizer water bath with a 250 ml tissue culture flask in position.

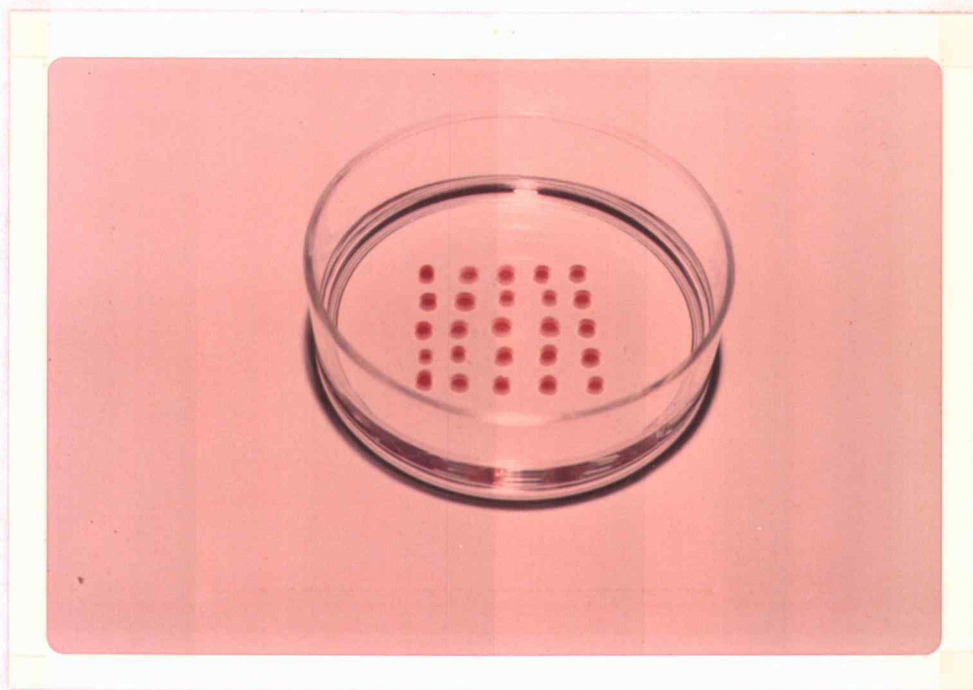

Figure 3. Petri dish (35 X 10 mm) containing 25 microdrops (colored water) covered with paraffin oil.

of two cells). Each time a drop containing a newly divided cell was observed, it was added to the total number of divided cells from the last observation time. Thus, an accumulated total number of divided cells was recorded for each observation time. These results were converted to the percent cells divided and plotted against the time of observation on linear coordinates (see the curve labelled control, unirradiated, in Figure 12). The time at which 50 percent of the cells divided could be interpolated and used as the reference point for the time of division of a sample. The 50 percent division time will be referred to as the DT_{50}. Since the control (unirradiated) DT_{50} varied slightly for each experiment, to facilitate comparisons, it was normalized to 100 minutes and the DT_{50}'s of irradiated samples and the irradiation times were normalized in each experiment.

A measure of the quality of synchronization of each batch of cells was obtained from the control curves (like that shown in Figure 12) by observing the percentage reached before the slope of the line began to decrease. This value ranged from 80 percent to 96 percent with a mean of 93 percent.

Determination of Division Delay

The amount of division delay that resulted after exposing the cells to X-irradiation was obtained by subtracting the DT_{50} of the irradiated cells from that of the unirradiated cells. This was most

easily accomplished by plotting the percentage of cells that had divided against the time after EST at which the cells were observed. (See Figure 12 in the Results Section.)

Silver Staining

The developing oral apparatuses (oral primordia) were observed by processing cells according to Frankel and Heckmann's (1968) modification of the Chatton and Lwoff (1930) silver impregnation technique. This technique makes the basal regions of the cilia visible so that the developing oral primordium may be observed. There are three major steps in this procedure, fixation, slide preparation, and slide processing.

Fixation

The ciliates were concentrated in a 15 ml conical centrifuge tube by 15 moderately fast turns of a standard hand centrifuge. The supernatant medium was aspirated off, and Champy's fixative (Table 2) was added at five times the volume. After five - ten minutes, the cells were again concentrated by centrifugation, the Champy's fixative aspirated off (in a fume hood because of the osmium), and 10 ml of DaFano's fixative (Table 2) added. Two washes in DaFano's fixative were required to remove any traces of Champy's fixative. The cells were then stored in DaFano's fixative for at least two hours until slide preparation.

Table 2. Ingredients of the solutions used in the silver staining procedure.

NAME (Storage Conditions)	INGREDIENTS	FINAL CONCENTRATION
Champy's Fixative (freshly prepared)	Potassium dichromate[1]	7 parts of a 3% stock solution in distilled water
	Chromic acid[1] (Chromic trioxide)	7 parts of a 1% stock solution in distilled water
	Osmic acid[1] (Osmium tetroxide)	4 parts of a 2% stock solution in distilled water
DaFano's Fixative (stored at 5° C indefinitely)	Cobalt nitrate[1]	1 gram
	Sodium chloride[2]	1 gram
	Formalin[3]	10 milliliters
	Glass distilled water	90 milliliters
Saline Gelatin (stored at 5° C indefinitely)	Gelatin[4]	10 grams
	Sodium chloride[2]	0.05 gram
	Glass distilled water	100 milliliters

1 Matheson Coleman & Bell, Los Angeles, California

2 Mallinckrodt Chemical Works, St. Louis, Missouri

3 J. T. Baker Chemical Co., Phillipsburg, New Jersey

4 Laboratoires du Bois de Boulogne, 33 Rue Voltaire, Puteaux, Seine, France

Slide Preparation

The slides were made on a slide warmer set at 40° - 45° C. A tiny drop of concentrated cells in DaFano's fixative was placed on a warm slide, and a larger drop (three times the volume) of liquefied (40° C) saline-gelatin (Table 2) added on top of the cells. A warm glass rod was used to stir and spread the drop over a large area of the slide, the thickness of which was regulated by the degree of spreading. The slide was then quickly transferred from the slide warmer to a flat area of crushed ice to solidify the gelatin. After the gelatin had solidified, the slides were rinsed with cold distilled water from a squeeze bottle and transferred to cold (10° C) freshly prepared three percent silver nitrate in a coplin jar for 1/2 to 24 hours in the dark. The dissolved gelatin was stored in the refrigerator in small, sterile, screw cap vials.

Slide Processing

The slides were removed from the silver nitrate, rinsed with cold distilled water from a squeeze bottle, and dehydrated through cold dehydration solutions (in coplin jars in an ice bath) which consisted of 70 percent, 80 percent, 95 percent, and two 100 percent ethanol solutions. The slides were strongly illuminated (mercury vapor light) throughout dehydration. The slides were then processed through a one

to one mixture of 100 percent ethanol and xylene, and two changes of xylene. The slides were left in each solution for ten minutes. Cover slips (24 X 50 mm) were then added to the slides using Preservaslide (Van Waters and Rogers) as the mounting medium, placed on a white surface, and exposed on both sides, alternately, to bright light for 12 - 24 hours in order to completely reduce the impregnated silver.

Analysis of Oral Morphogenesis Data

Silver stained slides fixed at 10 - 15 minute intervals after EST were scanned under a compound microscope (400X). Each appropriately oriented[2] cell was scored as to its stage of development according to Frankel's (1962) staging criteria, until a total of 100 cells had been tallied. The various stages are diagramatically represented in the top of Figure 21; photographs of the various stages appear in Figures 16 and 17. The number of cells in each of Frankel's stages at each time interval was converted to the number of cells that had reached each stage by each interval; for example, the number of cells in stages IV, V, and VI were added to the number of cells in stage III to obtain the cumulative percent of cells having reached stage III. The data were then analyzed with the aid of a quantal response analysis computer program.

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[2] Due to the random orientation of the cells in the gelatin, only those cells which clearly showed both the old oral apparatus and the developing primordium were scored.

This program was adapted to the Oregon State University computer (CDC-3300) and operated from an on-line teletype. The program is based on probit analysis (Finney, 1952) as modified for computer by Aitchison and Brown (1957). It employs the maximum likelihood method, an iterative procedure that determines the line of best fit to transformed data by applying weights that decrease toward the extremes of the distribution. If one assumes the data to be lognormally distributed, the program transforms the sampling times to the natural logarithm of the time and transforms the percentage of cells having reached each stage at each sampling time to a weighted normal equivalent deviate (NED) value (NED = probit minus 5). A linear regression is then analyzed for the maximum likelihood of being the best fit line. New weights are assigned and the computation is iterated with the new weighted NED values. Testing, weighing, and iteration continue until the values for the Y intercept and the slope of the regression line do not differ from previous values by more than 0.001 (at which time the values have "converged"). The confidence limits are based on a statistical parameter for probability of 0.05. Curves generated in this manner are shown in Figures 18 and 19. Tables 4 and 5 give the statistical values obtained.

Food Vacuole Formation

In order to determine if either the old or new oral apparatus

was functional after being irradiated, India ink particles (0.1percent Higgins #4425 black drawing ink) were added to some cultures and the cells observed microscopically for food vacuole formation. This concentration of ink particles had no apparent effect on the organisms.

Fluorescence Microscopy

In order to visualize lysosomes, both control and experimental cells were vitally stained with euchrysine, a purified aminoacridine dye, and observed under a fluorescence microscope. With this stain, the lysosomes fluoresce bright red and the macronuclei bright green (see frontispiece). A stock solution of euchrysine (#4851 K & K Laboratories, Plainview, New York) was made up at a concentration of 1 mg/ml in a brown bottle and diluted with the cells in TYESS to a final concentration of 0.03 mg/ml. Because of the photodynamic nature of the dye, avoidance of light prior to observation was necessary. The use of minimal yellow light (General Electric "Buglite") provided enough light to make the necessary dilutions and prepare the slides.

The fluorescence microscope set-up consisted of a Zeiss RA microscope; a Zeiss-housed, high pressure, mercury vapor light source (OSRAM HBO 200-w/4); and Zeiss excitation filters, BG-3 and BG-12. The light source and filters provided near UV light at a predominant wave length of 365 nanometers. Excellent visualization of

the fluorescent cells on a black field was obtained by using a dark field condenser and a yellow barrier filter.

Colored photomicrographs were made with a Nikon AFM microscope-camera assembly. In order to obtain good photographs with exposures of 4 - 5 seconds, high speed color film (Anscochrome 500, ASA = 500) was used. It was necessary to make the exposure times as short as possible because the cells were alive and moving and the stained cells were photodynamically sensitive to the light source.

Radiation

A General Electric Maxitron-300 X-ray machine was used to irradiate the cells (Figures 4 and 6). The operating parameters for all the experiments were 300 kVp, 20 mA, internal filtration - 0.25 mm of aluminum, added external filtration - 0.25 mm of copper to harden the beam (absorption of soft X-rays), and a target-subject-distance of 20 cm. The half value layer, a measure of beam quality, was found to be 0.91 $\pm$ 0.02 mm of copper (Ellett, personal communication).

The cells were irradiated either in drops of TYESS medium under oil or in about 5 ml of TYESS medium in 35 X 10 mm plastic petri dishes which were placed in 35 mm holes cut in a 38.1 X 38.1 X 0.635 cm pressed board: six 35 mm holes were arranged in a 15.2 cm circle around a seventh hole in the center. See Figure 5. The

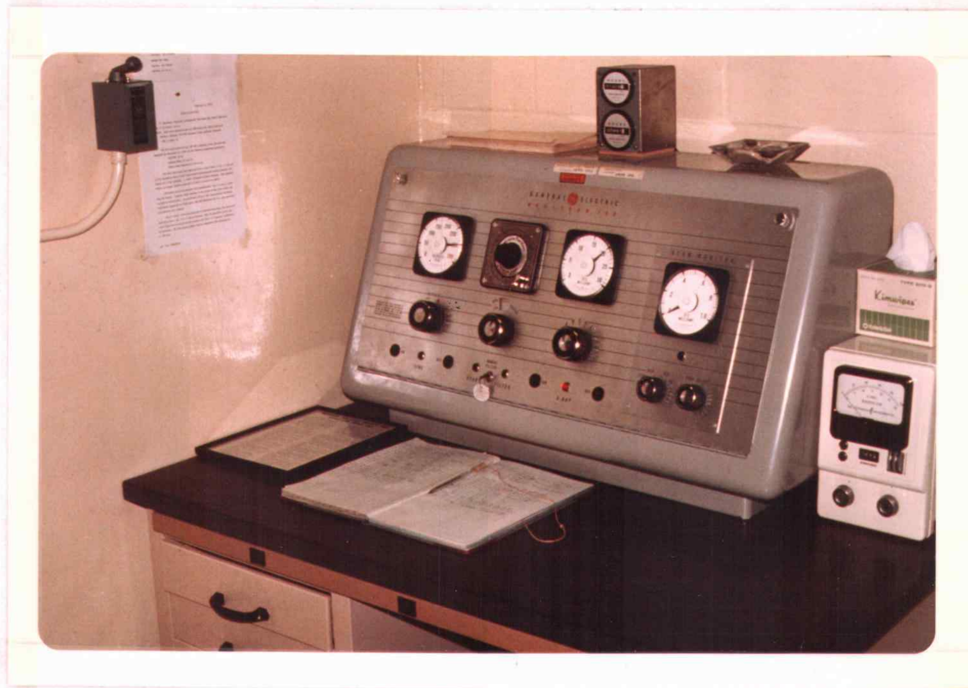


Figure 4. Control panel for General Electric Maxitron 300 X-Ray Machine.

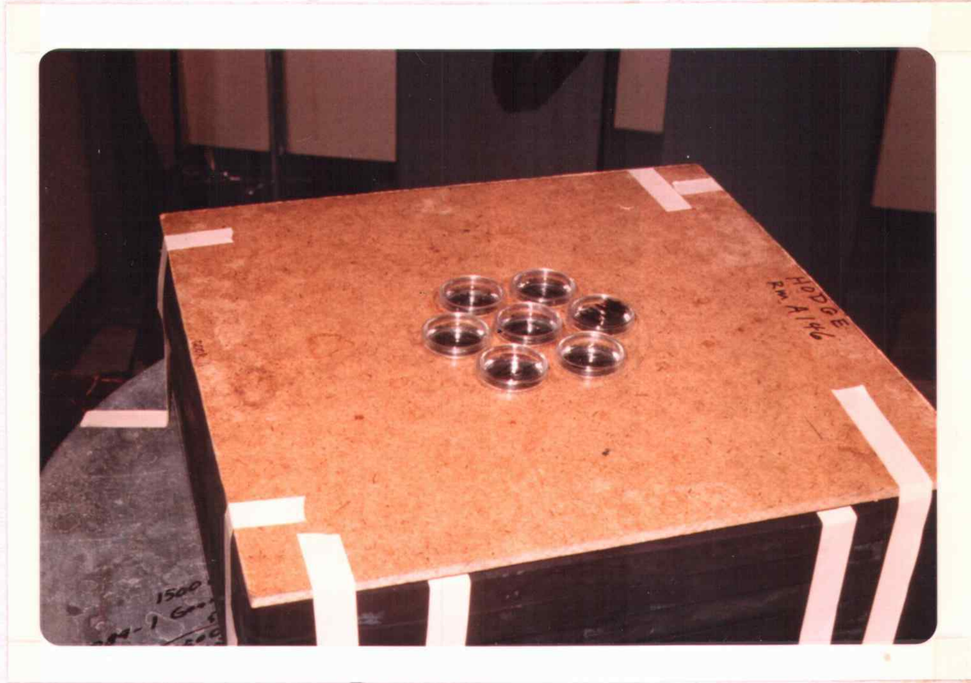


Figure 5. Pressed board petri dish holder for irradiation.

perimeters of all seven holes were 1 cm apart at their closest points. The board was then placed on a stack of six 38.1 X 38.1 X 2.5 cm rubber (tissue equivalent material) mats. This setup provided maximum backscatter geometry. The mats and board were placed on a turntable rotating at 1.0 RPM in order to obtain uniform exposures for all dish positions (Figure 6). When less than seven samples were to be irradiated, a blank dish containing the same volume of medium was inserted into each position not used.

The size of the beam field was determined radiographically using Kodak RP/s X-OMAT (RPS #54) rapid processing medical x-ray film in a leaded cardboard cassette. The cassette was placed under the pressed board petri dish holder and on top of the stack of tissue equivalent material 20 cm from the target (the distance used for irradiating the cells). A small chip of lead was placed in the center of each petri dish for better visualization. Exposures of 0.5 seconds yielded readable films. The size of the usable radiation field under these conditions was 16 cm in diameter.

Dosimetry

Initial measurements were made with a Victoreen R chamber (model #154 - 250 R) read on a Victoreen Condenser R meter (model 570). The Victoreen R chamber is a polystyrene capacitor connected in parallel with a thin-walled ionization chamber. Its response to

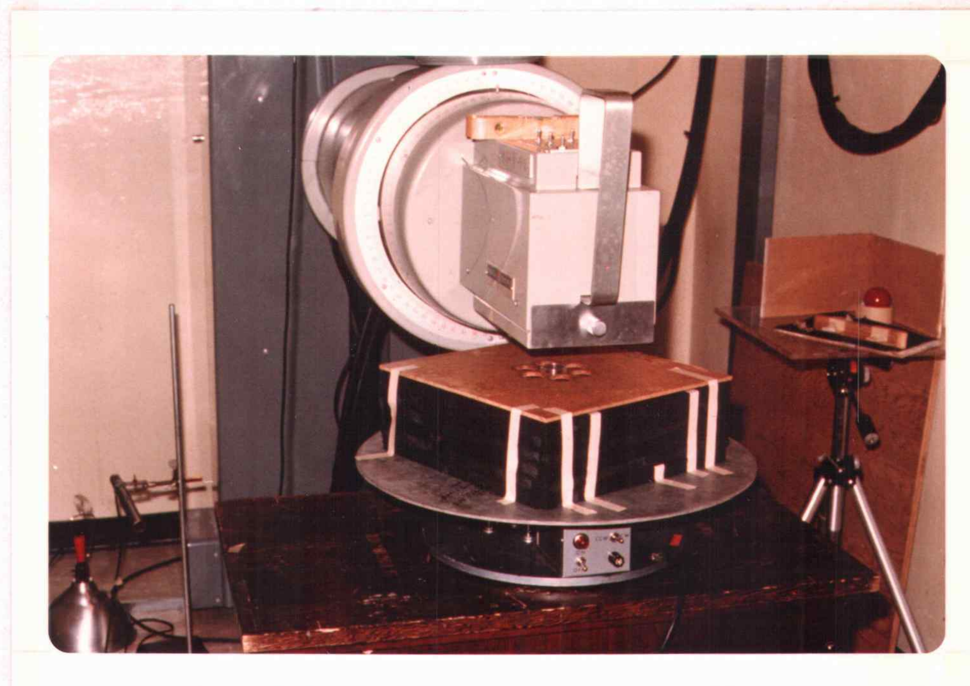


Figure 6. X-ray machine head in position over petri dish holder, black rubber tissue equivalent material, and turn table.

radiation is affected by temperature, pressure, and inherent factors. The actual exposure in R can be calculated by the relationship,

$$\frac{273 + t^{o}\ C}{295} \times \frac{760}{x\ mm\ Hg} \times (0.98) \times (\text{R meter reading})$$

in which the first term corrects for temperature, the second for pressure, and the third for the factors inherent in the particular chamber employed (as determined by calibration against a standard chamber).

The R chamber was placed immediately above, but not touching, the lid of a petri dish (Figure 7) on the revolving turntable and exposed at a target to sample distance of 20 cm. The exposure rates under the conditions used ranged from 21.8 R/sec to 25.0 R/sec with a mean of 23.5 $\pm$ 0.7 R/sec.

Since air ionization chambers are considered accurate only in free air, and due to the small volume of the petri dishes, thermoluminescent dosimetry (TLD) was utilized to determine more accurately the exposure inside the petri dishes. Pure crystals of lithium fluoride (LiF) pressed into tiny (3 X 3 X 1 mm) square chips (see Figure 8) were used. (These were obtained as extruded Lithium Fluoride Chips, model TLD-100, from Harshaw Chemical Co., Cleveland, Ohio.) These crystals contain electrons that can be excited by the energy from the ionizing radiation (i.e. raised to a higher energy level) which allows them to migrate until they reach a region of the crystal containing an impurity at which point they can become

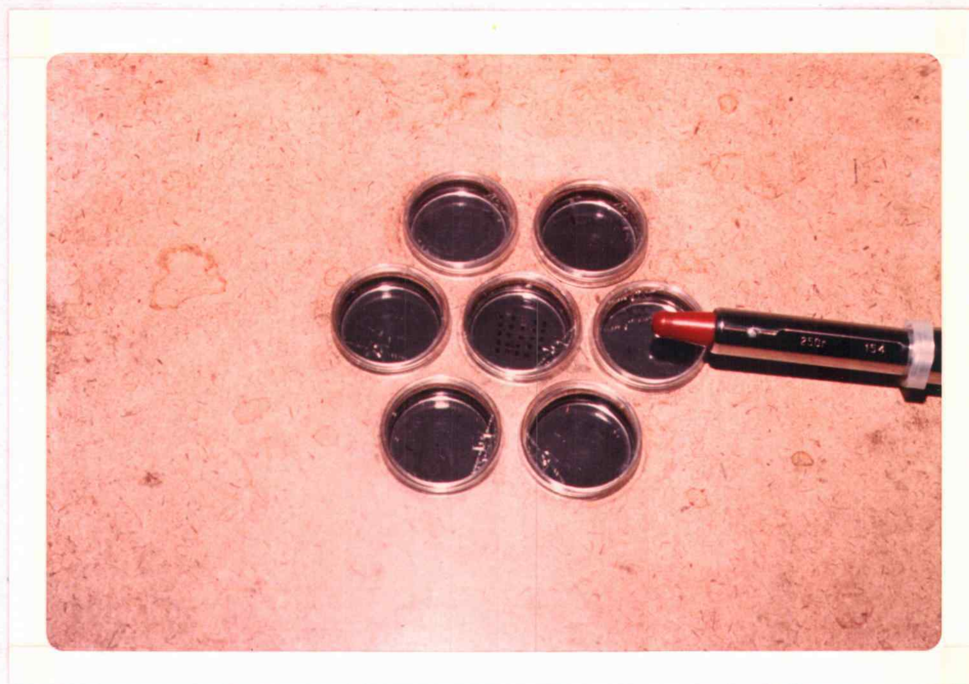


Figure 7. Victoreen R chamber in position immediately over a petri dish.

Figure 8. Five Lithium Fluoride TLD dosimeters next to a Victoreen R Chamber.

trapped. After irradiation the crystals are heated causing the electrons to rise again into the conduction band, migrate and then fall back to their lower energy level and in so doing emit photons of light which can be measured with a photomultiplier tube. A TLD reader (model E-IV, Madison Research, Inc., Middleton, Wisconsin), containing the photomultiplier, was operated at 800 volts (Figure 9). The peak temperature was 250° C with a heating time of 30 seconds. The appropriate heating temperature and heating time were determined from a glow curve obtained by heating an irradiated dosimeter in the TLD reader attached to a strip chart recorder. The temperature was determined at which the peak number electrons became untrapped, i.e., emitted the greatest amount of light.

In order to prepare the TLD chips for irradiation, it was necessary to remove previously trapped electrons. To do this, the chips were annealed for one hour at 389° C in an electric muffle oven, rapidly cooled to room temperature, and returned to an 80° C oven for 20 hours. This annealing regimen yielded background counts below the electronic noise level of the TLD reader.

The LiF chips were calibrated on a thin sheet of mylar film supported by a plastic frame. A 250 R Victoreen R chamber was placed immediately adjacent to the chips (Figure 10) 60 cm from the X-ray target and 60 cm from the floor. Under these conditions, a one minute exposure yielded a Victoreen reading of 114.6 R and a TLD

Figure 9. Thermoluminescent Dosimeter Reader.

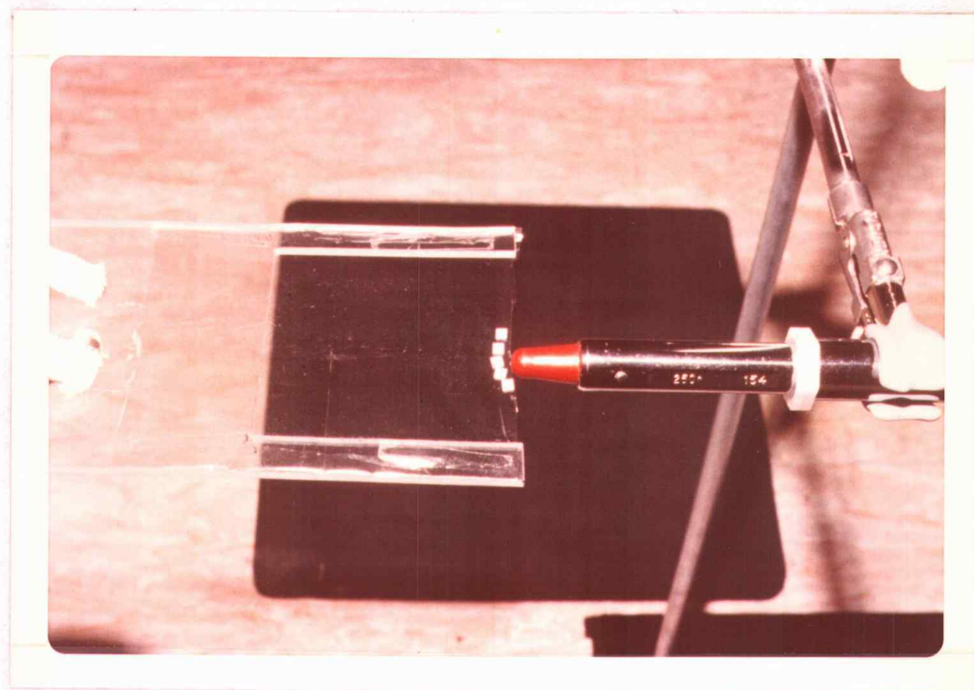


Figure 10. Victoreen R chamber and LiF chips in position on Mylar film for calibration in air.

reader count of 325 with a standard deviation of ± 18. In order to measure the exposure for the cells, one LiF chip was placed in the bottom of each of the petri dishes under maximum backscatter conditions and exposed for one minute at 20 cm (see Figure 11). The mean TLD reader count and its standard deviation obtained from the irradiated LiF chips was 541 ± 23 or, converted to Roentgens, 1522 ± 12 R which corresponds to an exposure rate of 25.3 ± 0.2 R/sec.

Chemical Treatment

Chemicals known to enhance or depress lysosomal enzyme release, called labilizers and stabilizers, were added to the cultures for some of the experiments. The agents were added to the cells prior to the last heat shock of the synchronizing treatment in order to insure that the agents were in contact with the cells long enough to get in, yet not so long as to have an undesirable effect on the cells. Labilizers used included Progesterone and Vitamin A acetate, both obtained from Calbiochem, Los Angeles, California. The stabilizers used were Chloroquine diphosphate and Hydrocortisone-21-phosphate from Sigma Chemical Co., St. Louis, Missouri. The various concentrations and dilutions that were used are shown in Table 7 in the Results Section.

Figure 11. Lithium Fluoride chips in petri dishes, note small polyethylene containers for LiF chips.

RESULTS

Division Time

In order to study the division times of experimentally treated cells, it was necessary to firmly establish the division times for untreated cells. As was mentioned in the Materials and Methods Section, the 50 percent division time (DT_{50}) was used as the reference point. The mean DT_{50} and its standard deviation for heat-synchronized *Tetrahymena pyriformis* GL-I in this laboratory was found to be 93.35 $\pm$ 4.82 minutes after the end of the synchronizing treatment (EST).

Division Delay

To study radiation-induced division delay, synchronized *T. pyriformis* cells were irradiated at varying times after EST at six exposures ranging from 1.5 kR to 18.5 kR. These exposures are sublethal: after the division delay, the cells completely recover and continue to proliferate in a normal fashion. For comparative purposes, the $LD_{50/48 \text{ hours}}$ (the dose required to kill 50 percent of the population within 48 hours) for heat synchronized *T. pyriformis* GL X-irradiated at six minutes after EST, was found to be 174 krads (Nachtwey, 1968). Figure 12, showing a representative set of curves from these experiments, presents the effects of 7.5 kR on the division pattern. Note that, at some irradiation times, not all of the cells are similarly

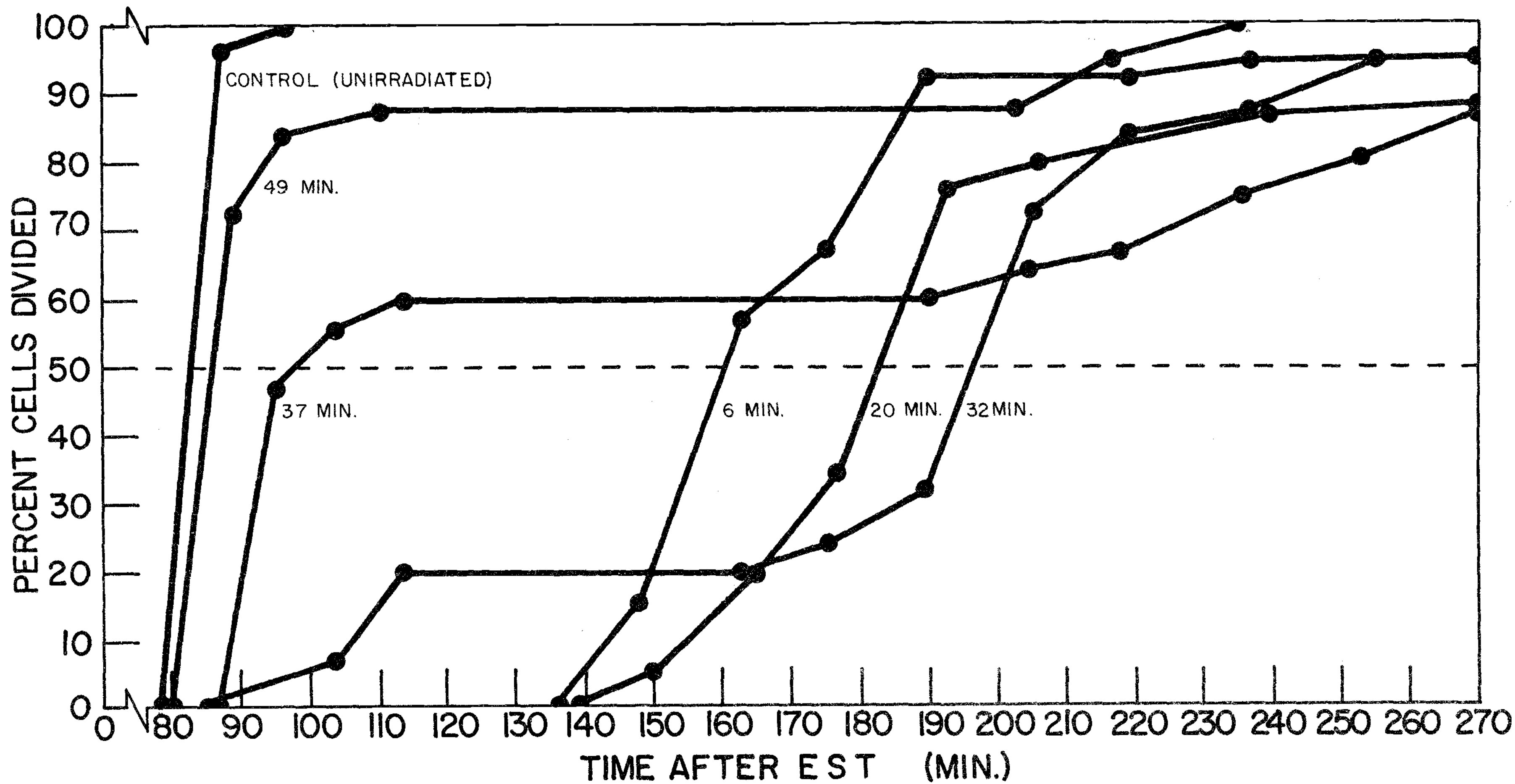


Figure 12. Cell division in synchronized Tetrahymena pyriformis after X-irradiation (7.5 kR at 25 R/sec., 300 kVp X-rays) at indicated times after the end of the synchronizing treatment (EST).

affected. Nonetheless, the DT_{50} may be used as a single point criterion for the time of division of the majority of the population. Table 3 gives the delays of the DT_{50}'s for cells given different exposures starting at different times after EST. The data in Table 3 were obtained from sets of curves like those in Figure 12. Some of the division delay data from Table 3 are plotted in Figure 13 against the time of irradiation.

From the data in Figure 13 and Table 3, one can see that at each exposure level the amount of division delay increases with the time of irradiation up to a point, at which time the amount of delay abruptly decreases toward zero. This abrupt shift indicates that the majority of the cells have undergone a transition from being sensitive to division delay to being relatively insensitive (minimally delayed).

Transition Point

The time at which individual cells undergo such a transition varies from cell to cell but it falls within a fairly short range of times characteristic for each exposure level. As can be seen in Figure 12, the curves for cells irradiated at 32 and 37 minutes show little delay for part of the population and great delay for the rest of the cells (indicated by the plateaus at 20 percent and 60 percent respectively). That is, the populations are split into maximally and minimally delayed cells. By obtaining the percentages of cells that were minimally

Table 3. Division delay (DT_{50} for irradiated cells minus the DT_{50} for unirradiated cells) of synchronized <u>Tetrahymena</u> irradiated at various times after the end of the synchronizing treatment (EST) at various exposure levels (25 R/sec, 300 kVp X-rays).

RADIATION EXPOSURE (ROENTGENS)	DIVISION DELAY (min)									
	TIME OF IRRADIATION (MINUTES AFTER EST)									
	5	20	25	30	35	40	45	50	55	60
1500	27	26	17	11	9	4		2		
3000	46	71	78	80	13	10		6		
6000	70	82		111	113	16	8		3	
7500	77	99	103	114		14	5	3		
12000	121	135		151		166	150		7	
18500	139	155		160		186		187	13	7

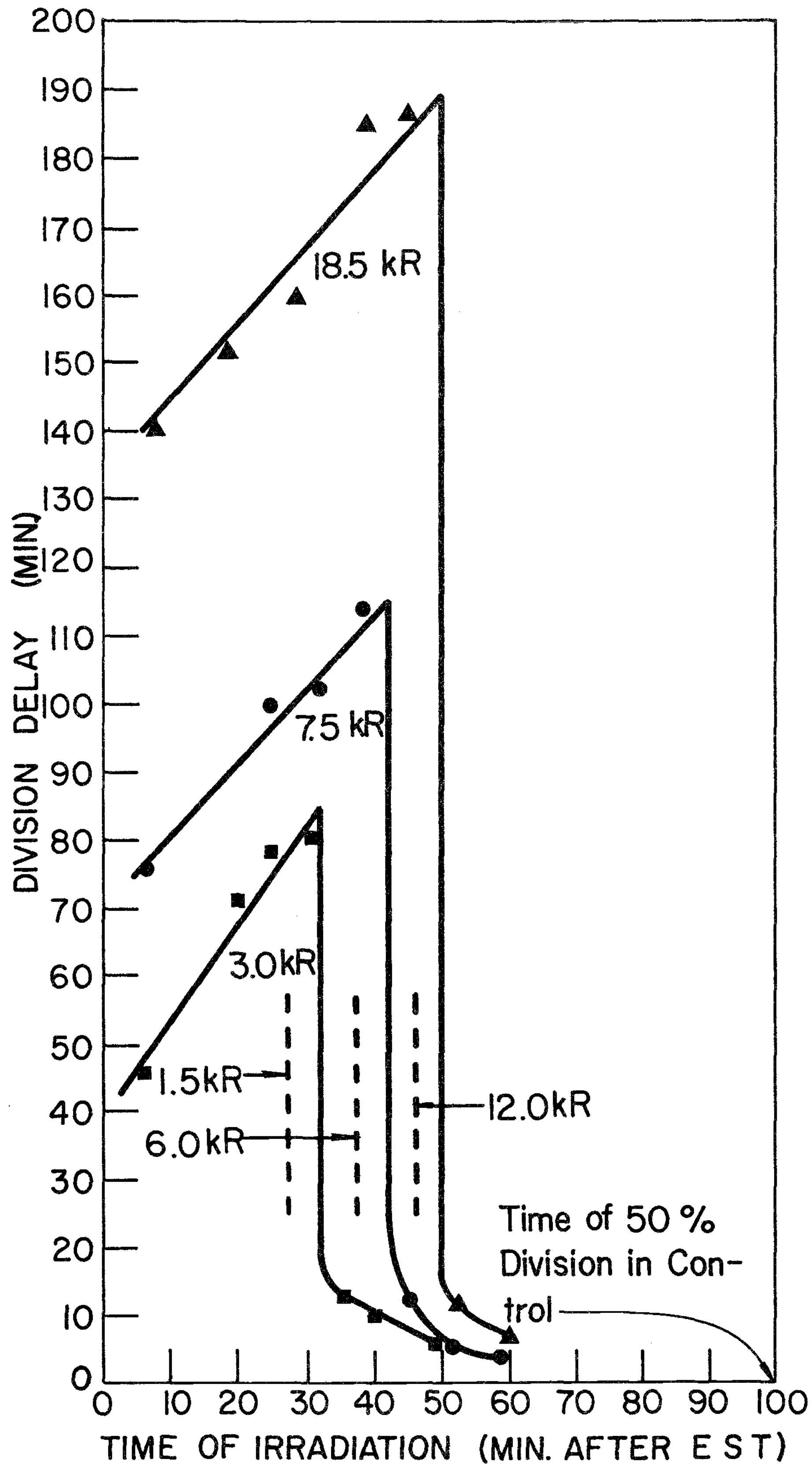


Figure 13. Delay of 50 percent division time of synchronized Tetrahymena irradiated at various times after the end of the synchronizing treatment (25 R/sec., 300 kVp X-rays).

delayed at each irradiation time, the time at which 50 percent of a population undergoes the transition to insensitivity (the transition point) can be interpolated from a line drawn between the two points bracketing the 50 percent point (percentage divided at the plateau level _vs_ time of irradiation). The transition point, then, is defined as the interpolated point in time after the end of the synchronizing treatment at which a given exposure splits the population of cells into 50 percent maximally delayed and 50 percent minimally delayed. The transition points for the six exposure levels are indicated in Figure 13 as vertical lines. They were obtained from curves like those in Figure 12.

The curves in Figure 13 indicate that the transition point is somewhat dose dependent: it becomes later and later with higher and higher exposures up to a certain critical time at about 55 minutes after EST. After this point even very high exposures (32 kR) do not delay the first division. By plotting the transition points against radiation exposure (Figure 14), one can see that as the radiation exposure increases, the time of the transition approaches asymptotically the critical time of 55 minutes. This critical time of 55 minutes is apparently a time when almost no insult, short of killing the cell, can delay the first division (Frankel, 1962).

Although the first division is not delayed in cells irradiated after the transition point or after 55 minutes, damage has been done as indicated by the fact that the second division is delayed (see Figure 15).

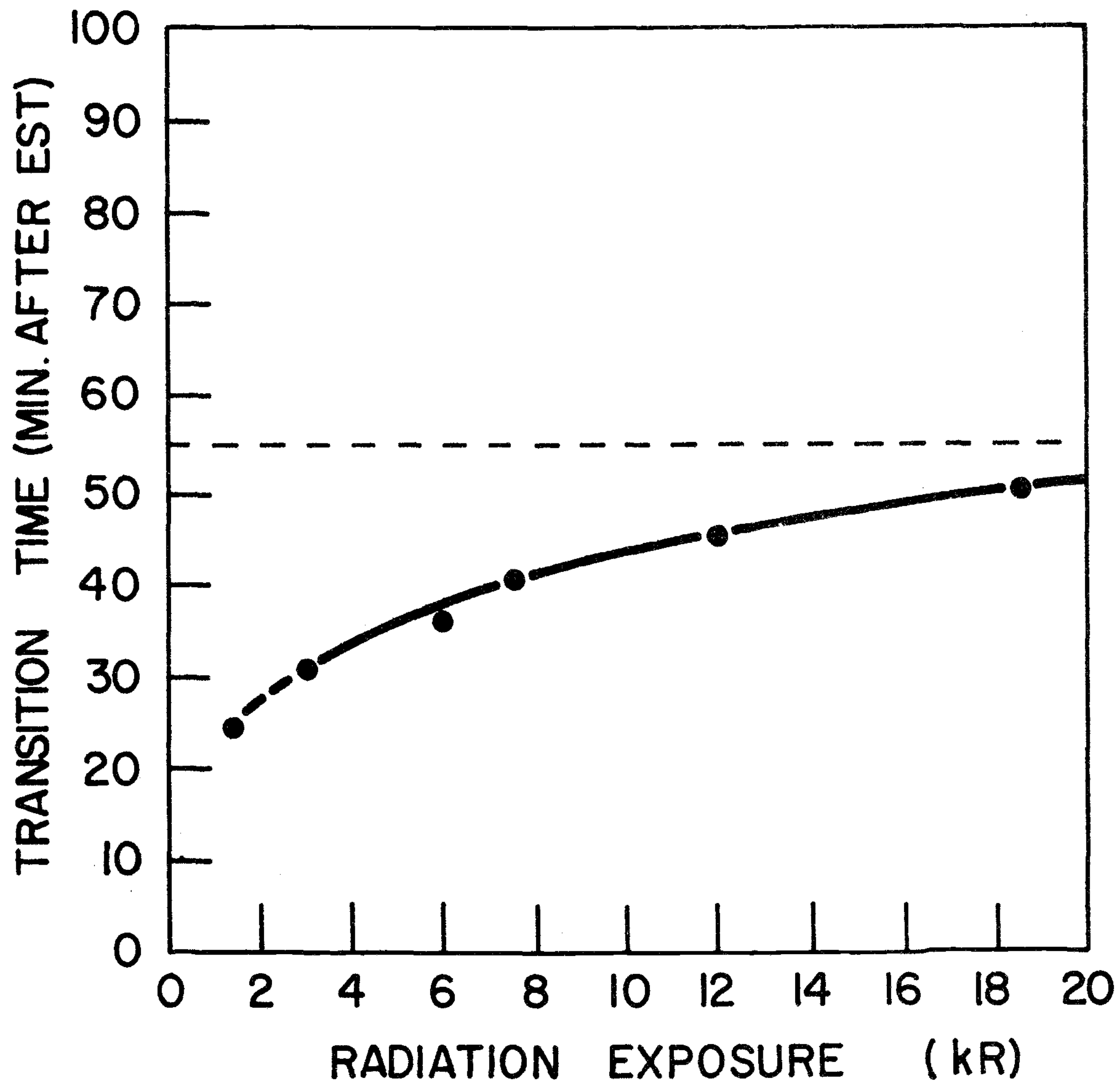


Figure 14. Transition point *vs.* radiation exposure (25 R/sec. 300 kVp X-rays). The light dashed line represents the 55 minute point. The heavy dashed line represents an interpolation between 1.5 kR and 3.0 kR.

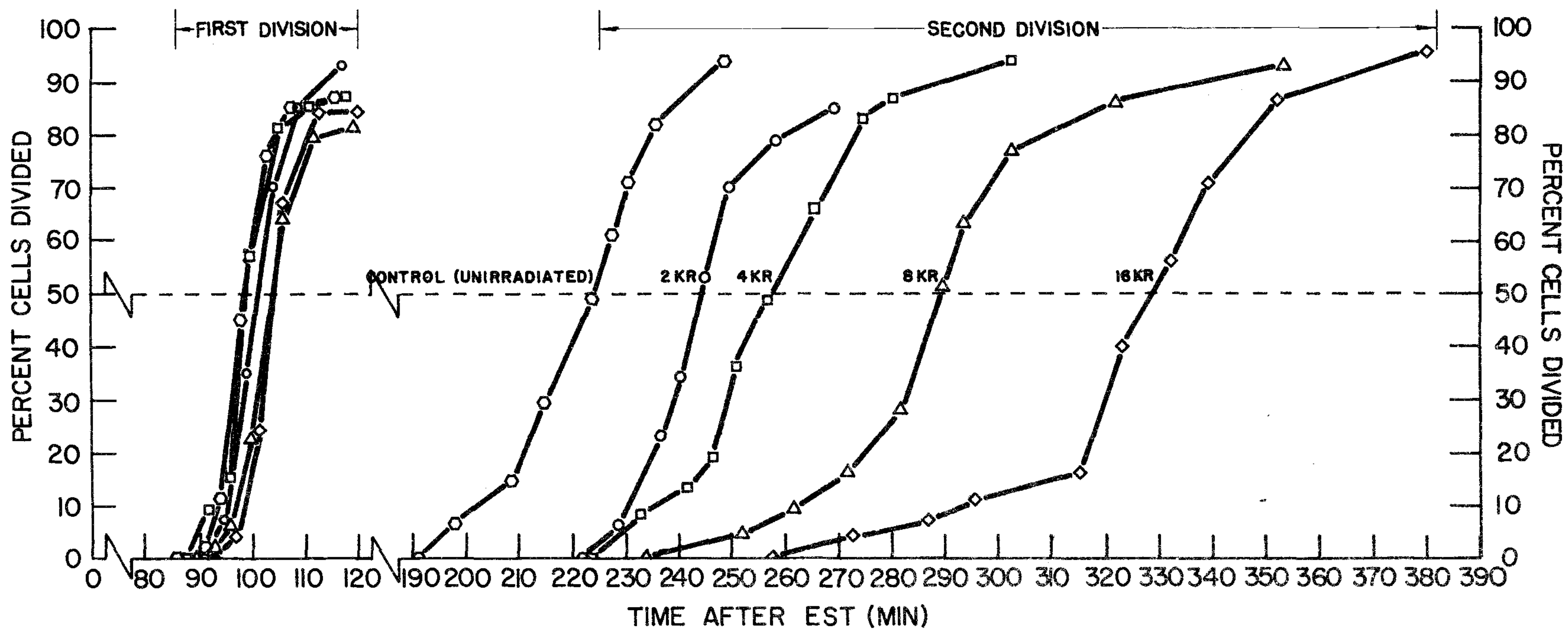


Table 15. Delay of second division in synchronized Tetrahymena exposed to X-irradiation after the transition points.

Oral Morphogenesis

In an attempt to gain some insight into the underlying mechanisms of this type of radiation-induced division delay, oral morphogenesis, which is correlated with cell division in *Tetrahymena*, was studied by the silver impregnation technique.

The silver impregnation technique makes visible the basal regions of the cilia (or infraciliature), allowing one to follow the development of a new oral apparatus prior to and in preparation for cell division. The site of the developing new mouth is on the mid-ventral surface of the cell, posterior to the old mouth. At division, the old mouth is retained by the anterior half while the posterior half receives the new structure. Frankel (1962) has subdivided oral primordium development into six stages. The sequence of Frankel's stages from EST through cell division for unirradiated cells are shown in the photomicrographs in Figures 16 and 17 and diagramatically in the top section of Figure 21.

By classifying silver-stained cells fixed every ten minutes after EST according to Frankel's (1962) six stages of oral primordium development, one can obtain a morphological time-map of the cell's progress toward division: Figure 18 shows the percentages of cells that have reached a particular stage of oral primordium development at various times after EST. (The curves result from the computer

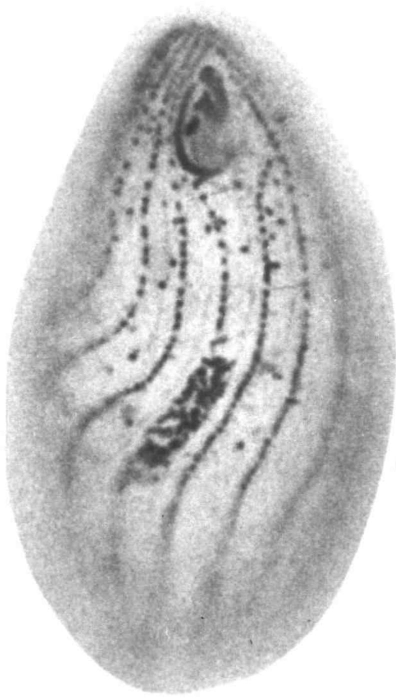
Figure 16. Photomicrographs of silver impregnated *Tetrahymena pyriformis* GL showing normal development of the oral primordium (X 1500).

A. Stage I 30 minutes after EST

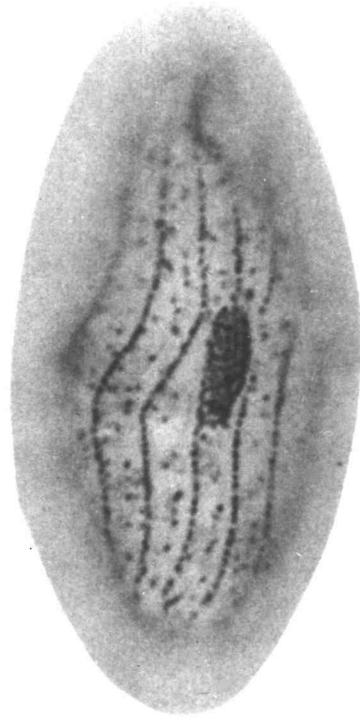
B. Stage II 41 minutes after EST

C. Stage III 51 minutes after EST

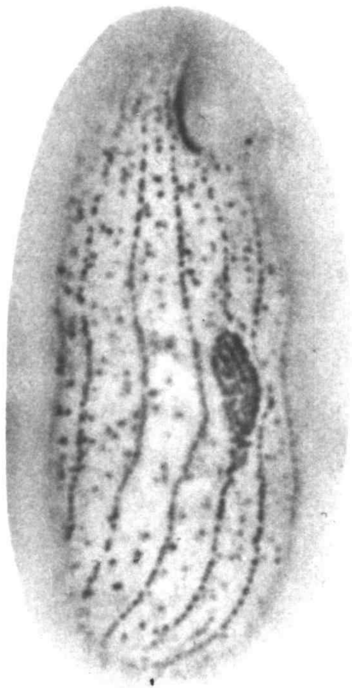
D. Stage IV 60 minutes after EST



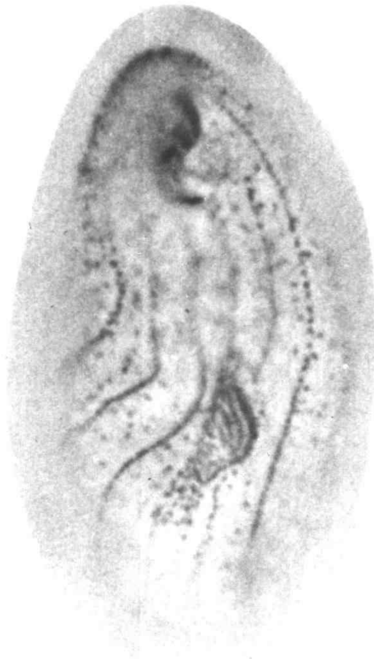
A



B



C



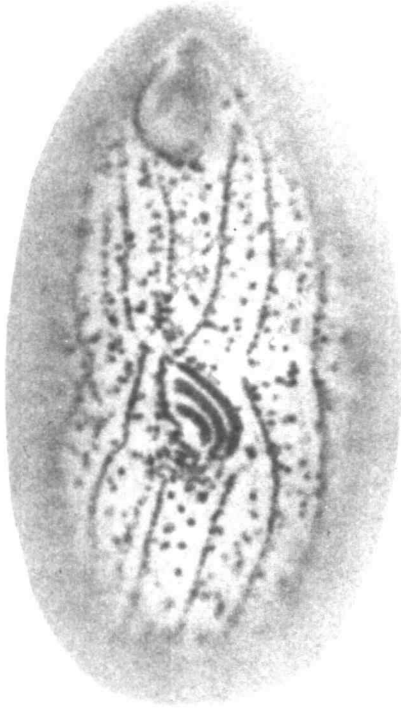
D

Figure 17. Photomicrographs of silver impregnated *Tetrahymena pyriformis* GL showing normal development of the oral primordium (X 1500).

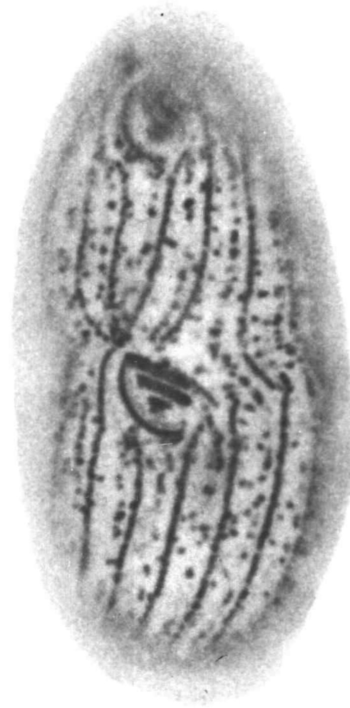
A. Stage V 75 minutes after EST

B. Stage VI 85 minutes after EST

C. Late stage VI 95 minutes after EST



A



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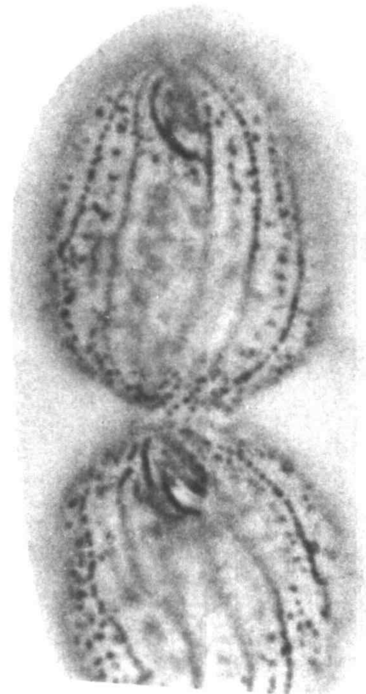

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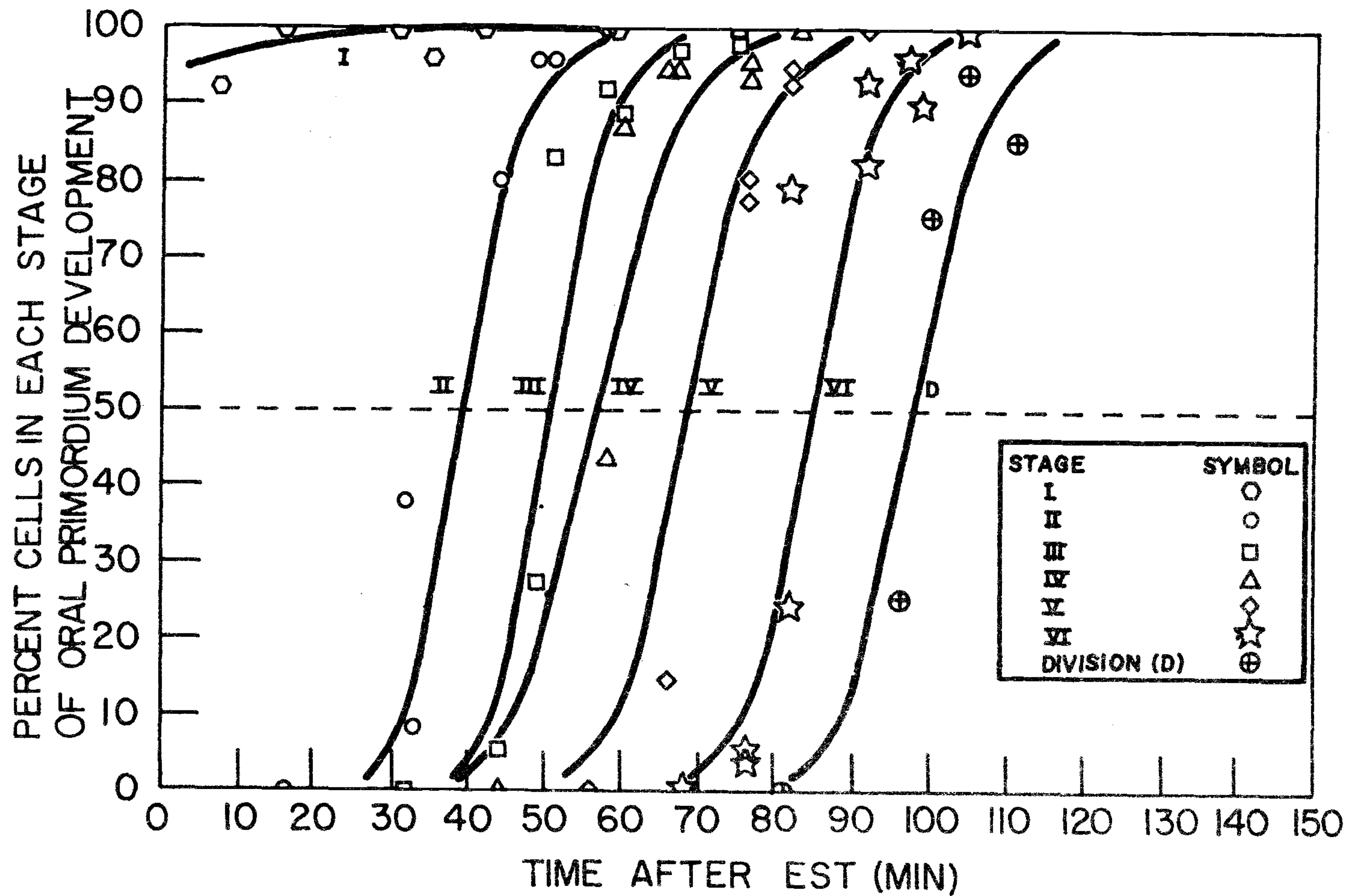


Figure 18. Oral primordium development in unirradiated Tetrahymena. Cumulative percent cells in each stage of oral primordium development after the end of the synchronizing treatment (EST). These curves were generated from a computer analysis.

analysis outlined in the Materials and Methods section.) The times at which 10, 50, and 90 percent of the cells have reached each stage are presented in Table 4 along with their upper and lower 95 percent confidence limits. The times by which 50 percent of the cells have reached each stage serve to quantitatively subdivide on a morphological basis the period during which the cells are preparing for division. That is, they form a morphological time-map. (The times by which 10 percent and 90 percent of the cells have reached a particular stage provide a measure of the degree of synchrony of the process.)

Frankel (1962; 1965; 1967a) has shown that division delay, induced with heat shocks, cold shocks, and various chemicals, was correlated with a resorption of the developing oral primordium. Several silver stain experiments were performed with irradiated cells on the assumption that a similar correlation might be found with radiation-induced division delay. A representative of this type of experiment is shown in Figure 19. The results of a computer analysis of the data are shown in Table 5.

In the cells represented by the curves in Figure 19, the oral primordium appears to progress more slowly but normally after the end of the irradiation exposure until stage III is reached. At this time one begins to see oral primordium resorption, a gradual "deterioration" and disappearance of the organization of the oral primordium. As shown in photomicrographs A and B of Figure 20, it is almost as

Table 4. The times at which 10, 50, and 90 percent of the unirradiated cells are in a particular stage of oral primordium development.

Stage of Oral Primordium Development	Percent Cells in Stage	Time (Min After EST)	Lower 95% Confidence Limit (Min)	Upper 95% Confidence Limit (Min)
	10	32.4	31.3	33.4
II *	50	39.4	38.5	40.3
	90	47.8	46.4	49.6
	10	43.0	41.4	44.4
III	50	50.4	49.3	51.4
	90	59.0	57.5	60.8
	10	45.5	42.9	47.7
IV	50	57.2	55.6	58.7
	90	68.9	66.3	70.9
	10	59.7	57.8	61.3
V	50	68.7	67.5	69.8
	90	79.0	77.5	80.8
	10	75.6	74.2	76.8
VI	50	84.1	83.1	85.1
	90	93.6	92.2	95.2
	10	89.3	86.5	91.4
DIVISION	50	98.1	96.6	99.3
	90	107.7	105.9	110.0

* The cells are already in stage I at EST, therefore stage I values are not shown.

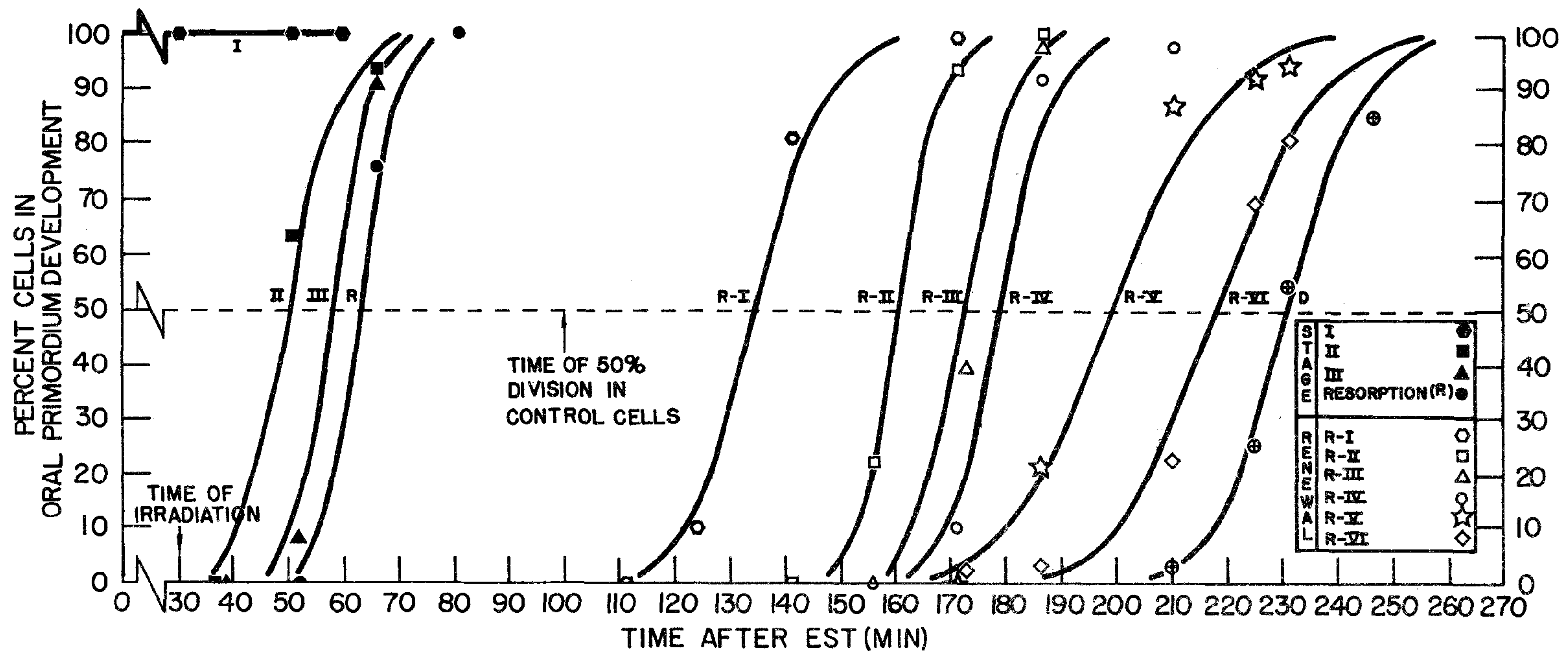


Figure 19. Oral primordium development in irradiated Tetrahymena. Cumulative percent cells in each stage of oral primordium development after the end of the synchronizing treatment (EST). The cells were exposed to 7.5 kR X-irradiation at 30 minutes after EST.

Table 5. The times at which 10, 50, and 90 percent of the irradiated cells (7.5 kR at 30 min after EST) are in a particular stage of oral primordium development.

Stage of Oral Primordium Development	Percent Cells in Stage	Time (Min After EST)	Lower 95% Confidence Limit (Min)	Upper 95% Confidence Limit (Min)
II *	10	40.7	38.1	42.7
	50	49.5	47.8	51.1
	90	60.2	57.8	63.5
III	10	51.4	49.5	52.9
	50	58.0	56.6	59.4
	90	65.4	63.6	68.0
RESORPTION	10	56.2	53.6	58.1
	50	63.1	61.5	64.5
	90	70.8	69.0	73.3
RENEWAL (R)				
R-I	10	122.4	119.3	124.8
	50	134.5	132.6	136.6
	90	147.8	152.3	144.6
R-II	10	153.2	150.9	154.9
	50	160.9	159.4	162.3
	90	168.9	167.0	171.6
R-III	10	164.6	161.8	166.5
	50	172.4	170.9	173.9
	90	180.6	178.5	183.7
R-IV	10	169.2	166.4	171.3
	50	178.7	177.1	180.4
	90	188.8	186.4	192.0
R-V	10	179.9	175.9	183.2
	50	198.8	196.2	201.3
	90	219.8	216.4	223.9
R-VI	10	199.7	194.6	203.3
	50	218.1	215.7	220.3
	90	238.1	234.3	243.5
delayed DIVISION	10	217.6	213.5	220.2
	50	230.9	228.9	233.7
	90	244.9	240.2	253.3

* The cells are already in stage I at EST, therefore stage I values are not shown.

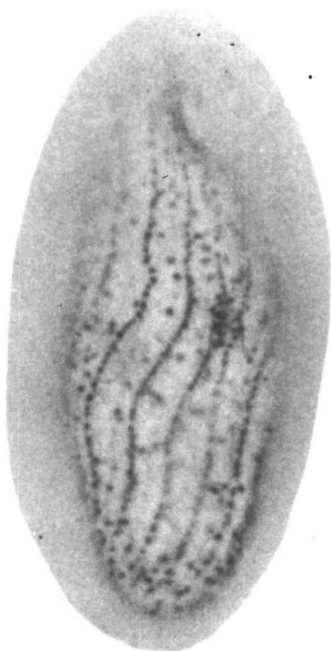
Figure 20. Photomicrographs of silver impregnated *Tetrahymena pyriformis* GL irradiated at 30 minutes after EST with 7.5 kR (X 1500).

A. cell fixed 81 minutes after EST in the process of resorption.

B. cell fixed at 130 minutes after EST, resorption completed.

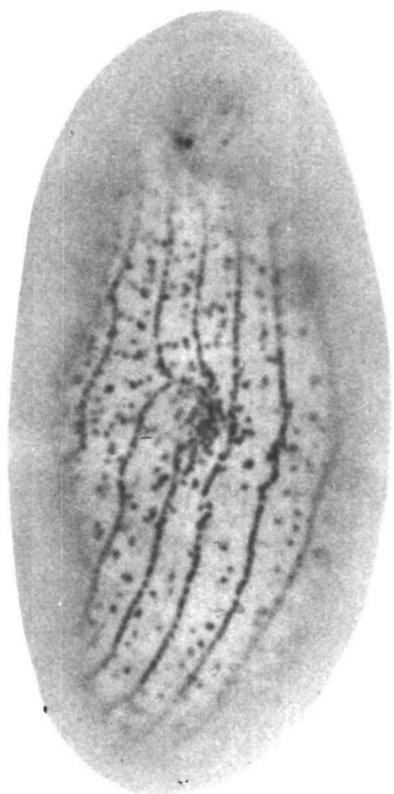
C. cell fixed at 145 minutes after EST, resorption completed, with a new stage I oral primordium developing.

D. cell fixed at 165 minutes after EST, new stage II oral primordium developing.



A

B



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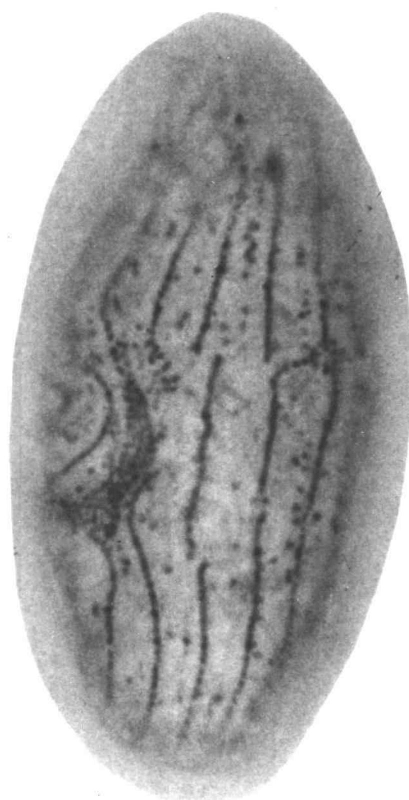


D

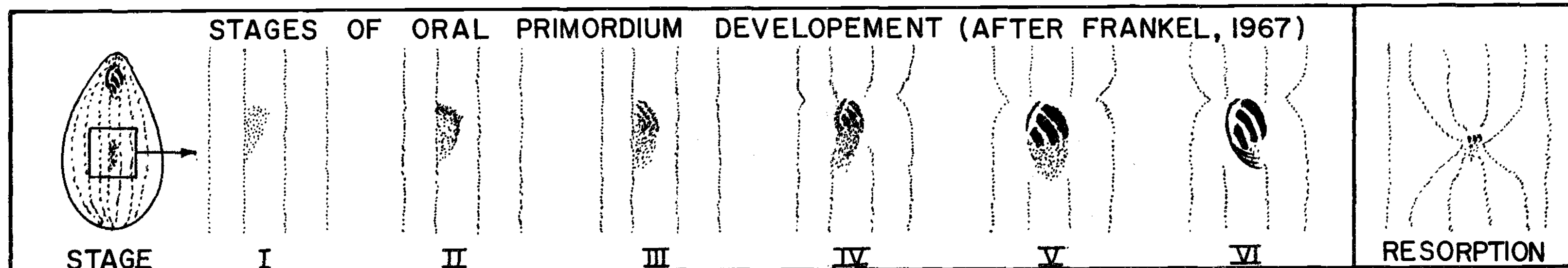
if the development process had reversed itself. At the time when one would expect to see a stage IV or V primordium, only a disorganized spot of basal bodies is seen.

Photomicrographs C and D in Figure 20 show that in cells fixed at a later time, the oral primordium begins developing again, signaling the end of resorption. The end of resorption is characterized by a new loose anarchic field of ciliary basal bodies (kinetosomes) below the "scar" of resorption. This marks the beginning of stage I of the renewal period.

The stages of development of the renewal period soon become almost indistinguishable from those of the untreated controls in their appearance at each stage as well as in the timing of each stage. Indeed, there is very little difference between renewal division time (the time from the end of resorption to division) and the control division time (the time from EST to division).

Comparisons between treated and control cells can be more clearly made by using Figure 21 where the results of four experiments like the one presented in Figure 19 are summarized. (The data for the three additional experiments are presented in Table 6. Computer analysis was not performed because graphic interpolation was judged to be adequate.)

In the experiments shown in Figure 21, cells were irradiated with two exposures at each of two times after EST: 7.5 kR and 13.8 kR



0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160

DIVISION DELAY (MIN. AFTER CONTROL DIVISION)

CONTROL

STAGE	I	II	III	IV	V	VI
DURATION	37	12	7	12	14	18

X-RAY (7.5 kR) ON OFF — RESORPTION — RENEWAL

STAGE	I			RESORPTION	I	II	III	IV	V	VI
DURATION	6	6	20	45	101					

X-RAY (13.8 kR) ON OFF — RESORPTION — RENEWAL

STAGE	I			II	RESORPTION	I	II	III	IV	V	VI
DURATION	6	20	40		66	98					

X-RAY (7.5 kR) ON OFF — RESORPTION — RENEWAL

STAGE	I			II	III	RESORPTION	I	II	III	IV	V	VI
DURATION	30	6	27			70	96					

X-RAY (13.8 kR) ON OFF — RESORPTION — RENEWAL

STAGE	I			II	III	RESORPTION	I	II	III	IV	V	VI
DURATION	30	10	30			94	90					

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260

TIME (MIN. AFTER E S T)

Table 6. Oral primordium development in *Tetrahymena* after exposure to 7.5 and 13.8 kR X-rays at 6 and 30 minutes after the end of the synchronizing treatment (EST).

Time and Level of Exposure	Sampling Time (Min after EST)	Percentage of Cells in Given Stage of Oral Primordium Development[1]							
		R^2	I	II	III	IV	V	VI	D/2
	6		100						
			(100)*						
	30	52	48						
7.5 kR			(92)	(8)					
at	60	100							
6 min					(6)	(80)	(14)		
after EST	90	5	95**						
							(5)	(10)	(85)
	135			12	7	34	43	4	
	150			5	2	28	21	44	
	180				1	2	10	25	62
	30		100						
			(88)	(12)					
	50		24	74	2				
13.8 kR			(6)	(73)	(20)	(1)			
at	80	100			(1)	(11)	(48)	(37)	(3)
6 min	130	8	91	1					
after EST	150		23	67	10				
	190			2	4	8	47	23	16
	230					4	5	17	74

Time and Level of Exposure	Sampling Time (Min after EST)	Percentage of Cells in Given Stage of Oral Primordium Development[1]							
		R[2]	I	II	III	IV	V	VI	D/2
7.5 kR at 30 min after EST	35		99	1					
			(95)	(5)					
	60	76	6	3	15				
				(3)	(3)	(14)	(76)	(4)	
	90	100				(1)	(2)	(57)	(40)
	120	18	82						
	150	4	74	22					
	180			1	7	71	18	3	
	220					4	7	19	70
13.8 kR at 30 min after EST	40		86	14					
			(19)	(76)	(5)				
	70	56		1	40	3			
				(2)	(2)	(24)	(66)	(6)	
	100	96					3	1	
								(12)	(88)
	190	1	6	84	7	2			
	220			3	15	58	23	1	
	250					2	12	71	15
	260						1	13	86

1 Stages of oral primordium according to Frankel's (1962) classification. D/2 = one half the number of newly divided cells. One hundred cells were scored at each sampling time.

2 R = resorption of the developing primordium.

* unirradiated control cells are represented by the values in parentheses.

** The underlined values represent irradiated cells that have undergone resorption and are developing new oral primordia; the values not underlined represent irradiated cells developing prior to resorption.

at six minutes (much before the transition) and 7.5 kR and 13.8 kR at 30 minutes (closer to the transition), and morphological time-maps constructed. The time-maps are represented by bars in the figure and the time intervals are the times by which 50 percent of the cells have reached each stage. The control (unirradiated) bar shown in Figure 21 represents the pooled and normalized results from the four control populations that were sampled in parallel with each of the irradiated populations. The times of the various stages in the control populations compare favorably with those found by Frankel (1962).

Although Figure 21 shows only the time intervals for the 50 percent points (the time at which 50 percent of the cells have reached a particular stage), several conclusions can be drawn: (1) Irradiation does not immediately trigger resorption; the cells progress, seemingly normally, for a variable time before the onset of resorption. (2) The resorption time increases with dose and with the time of irradiation just as does the division delay. Since the resoprtion time is not a fixed amount, resorption is not simply a process which once initiated goes at its own inherent rate. (3) The resorption time does not account for all of the division delay, but it does account for a fairly constant fraction of it: 0.58, 0.55, 0.54, and 0.61 for 7.5 kR at six minutes, 13.8 at six minutes, 7.5 kR at 30 minutes, and 13.8 kR at 30 minutes, respectively. (Although these fractions are close to one another, it is not certain if the resorption time is always a constant

fraction of the delay time.) (4) Once resorption is complete, the renewed oral primordium development appears normal and proceeds at the same rate as in the unirradiated control population. (5) Since the renewal time is practically constant, the division delay is completely accounted for by the time from EST to the end of resorption. These latter two observations indicate that all of the seemingly normal progress made toward division before, during, and after irradiation is essentially wasted.

Cells irradiated after the transition point, which showed essentially no delay in the first division, did not demonstrate oral primordium resorption even at high exposures. In fact, no visible abnormalities were observed in either the old or the new oral apparatus when irradiation occurred after the transition.

Food Vacuole Formation Following Irradiation

In order to determine whether the resorbed and renewed oral apparatus was functional or not, some experiments involving food vacuole formation were initiated.

India ink particles are readily engulfed by *Tetrahymena* without adversely affecting their normal behavior. Cells begin forming black food vacuoles that are clearly visible three minutes after adding the ink to a culture. By observing control and irradiated cells that have been exposed to India ink, the number of cells forming food vacuoles

at any particular time can be determined. Figure 22 shows the results of some of these experiments. It may be seen that most unirradiated cells (solid line) cease forming food vacuoles around the time of furrowing (notched areas on the midline of the cell marking the beginning of cytokinesis). Food vacuole formation remains stopped for about 30 minutes before and five minutes after cell separation. These results are in good agreement with those of Nachtwey and Dickinson (1967). Similarly, irradiated cells (dashed line in Figure 22) show a cessation of food vacuole formation around the time of their delayed division. (There is also a slight decrease in food vacuole formation in the irradiated group that corresponds in time with the period of oral primordium resorption. However, this slight decrease may not be significant.) That the irradiated cells, although delayed, eventually divide and resume normal food vacuole formation indicates that the renewed oral apparatuses are complete. The results also indicate that the old oral apparatus did not sustain any damage when the cells were irradiated prior to the transition point. If either the old or the new oral structures had been functionally damaged the percentage of cells forming food vacuoles would not have been greater than 50 percent. Damage to both, of course, would have resulted in less than 50 percent food vacuole formation.

Cells irradiated after the transition (7.5 kR given at 45 minutes after EST) were allowed to divide before the ink particles were added.

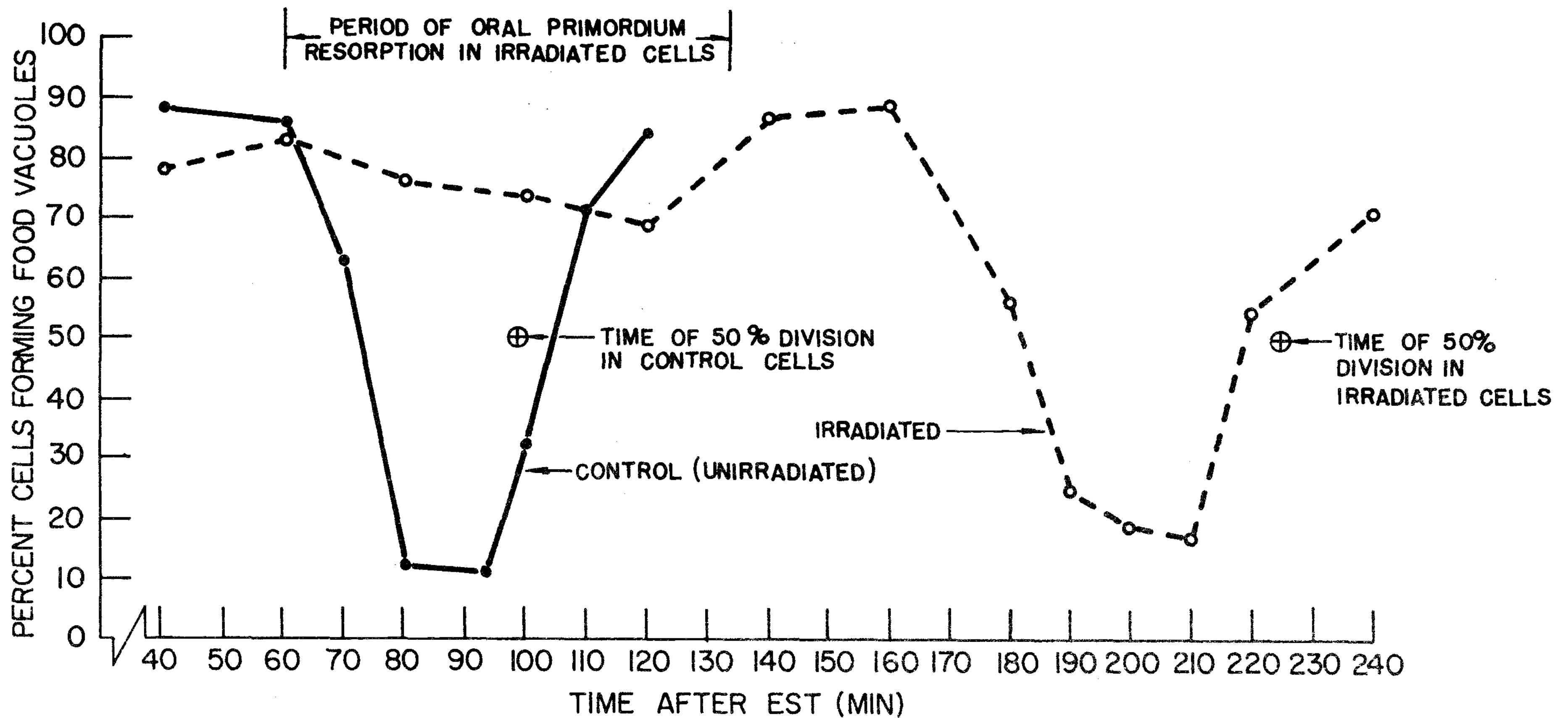


Figure 22. Food vacuole formation in synchronized Tetrahymena. Unirradiated cells and those exposed to 7.5 kR X-irradiation at 30 minutes after the end of the synchronizing treatment (EST) are shown.

These cells showed little or no division delay and normal food vacuole formation resumed in both daughter cells after division.

Cells from both pre- and post-transition-point irradiations showed the same number of food vacuoles formed per cell per unit time as unirradiated cells. All of these results taken together indicate that the oral structures are not permanently damaged by radiation occurring either before or after the transition.

Lysosomes

The results so far, indicate that the cells are in some way damaged by sublethal irradiation and that the cell responds to this damage by delaying division and by triggering the resorption of the oral primordium. Since lysosomes contain hydrolytic enzymes that could be used by the cell in the resorption phenomenon and that might be involved in the division delay phenomenon, some experiments to test the involvement of lysosomes in radiation-induced division delay were initiated. Known lysosomal labilizers (which produce an enhanced enzyme release) and stabilizers (which produce a depressed enzyme release) were added to the synchronized cultures of *Tetrahymena* prior to X-ray exposure.

A series of experiments using different concentrations were performed prior to the irradiation experiments in order to determine the highest concentration that did not cause any significant division

delay in unirradiated cells. The effect of low concentrations of ethanol was also tested, because stock solutions of the labilizers, being insoluble in water or TYESS, were made with ethanol and diluted with TYESS to yield acceptably concentrated suspensions. No effect of ethanol was observed with concentrations up to and including 1.0 percent. Even so, the final concentration of ethanol used with the labilizers never exceeded 0.1 percent. From the results shown in Table 7, the final concentrations for the labilizers and stabilizers used in the irradiation experiments were chosen; these were 0.03 mM for progesterone, 0.2 mM for vitamin A, and 5.0 mM for both chloroquine and hydrocortisone.

Figure 23 represents the division delay and transition points for cells treated with labilizing and stabilizing agents prior to exposure to 7.5 kR X-irradiation (and prior to the last heat shock of the synchronizing treatment). These curves were obtained using the procedure already described for the division delay experiment. Noteworthy in Figure 23 is the shift of the transition point to an earlier time for the stabilizer-treated cells and to a later time for the labilizer-treated cells. By adding the labilizer and stabilizer transition points to the curve in Figure 24, two conclusions become obvious. The time of the transition for cells treated with a stabilizer and irradiated with 7.5 kR corresponds to the transition point for untreated cells irradiated with 5.0 kR, suggesting less sensitivity. Conversely, the transition

Table 7. Division delay in synchronized *Tetrahymena* treated with various concentrations of lysosomal labilizers and stabilizers.

CLASSIFICATION	CHEMICAL	MOLECULAR WEIGHT	DIVISION DELAY (MIN) CONCENTRATIONS (MILLIMOLAR) 5.0	1.0	0.5	0.3	0.1	0.05	0.01
STABILIZER	HYDROCORTISONE -21-PHOSPHATE	486.5	2	1			0		
	CHLOROQUINE-DIPHOSPHATE	515.9	5	3			0		
LABILIZER	VITAMIN A-ACETATE	328.5			All Died	15	1		0
	PROGESTERONE	314.5		All Died			119	99	0

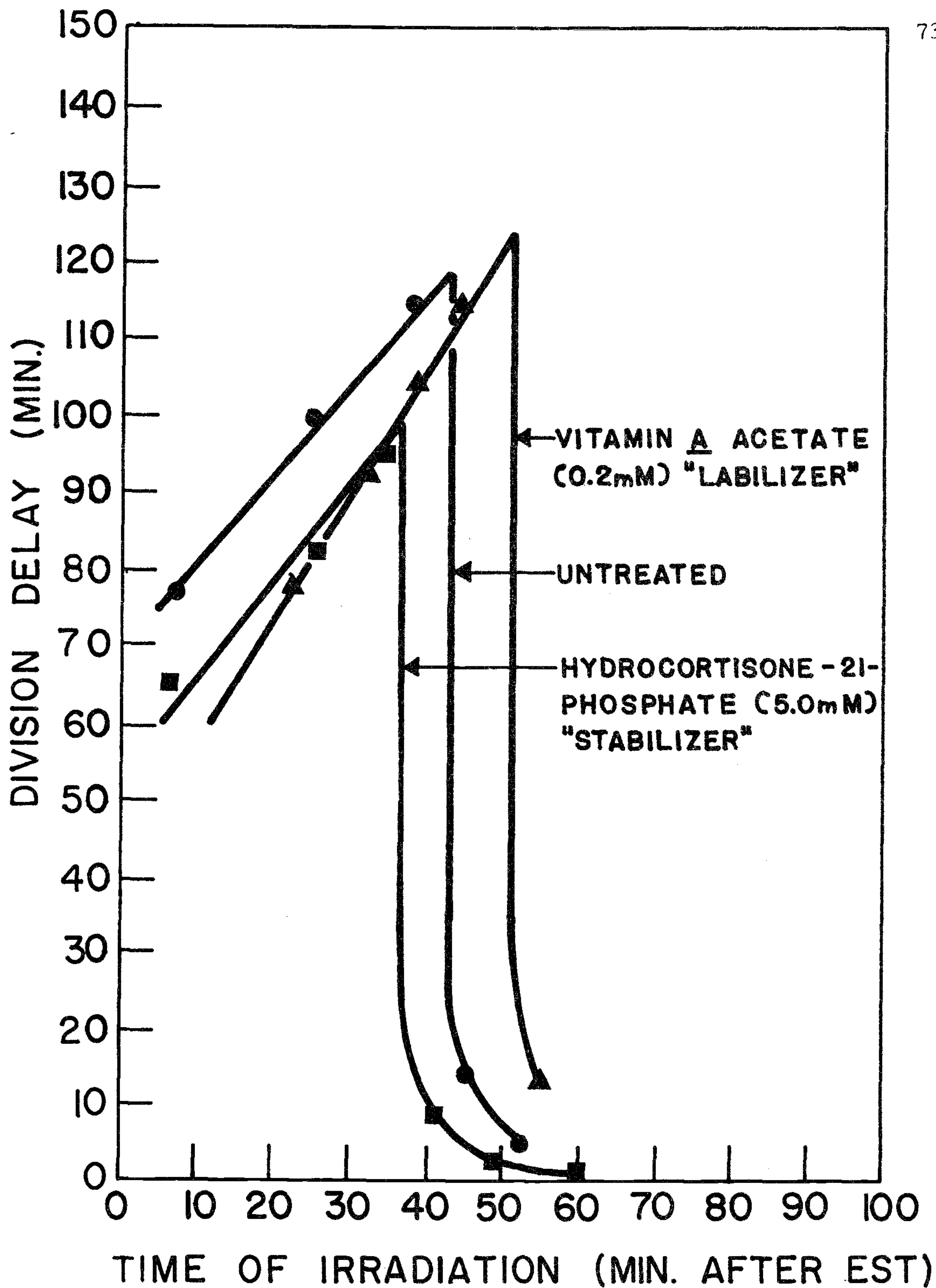


Figure 23. Delay of 50 percent division time of synchronized Tetrahymena untreated and treated with lysosomal labilizer and stabilizer prior to the last heat shock of the synchronizing treatment. Cells were exposed to 7.5 kR X-irradiation at various times after the end of the synchronizing treatment (EST).

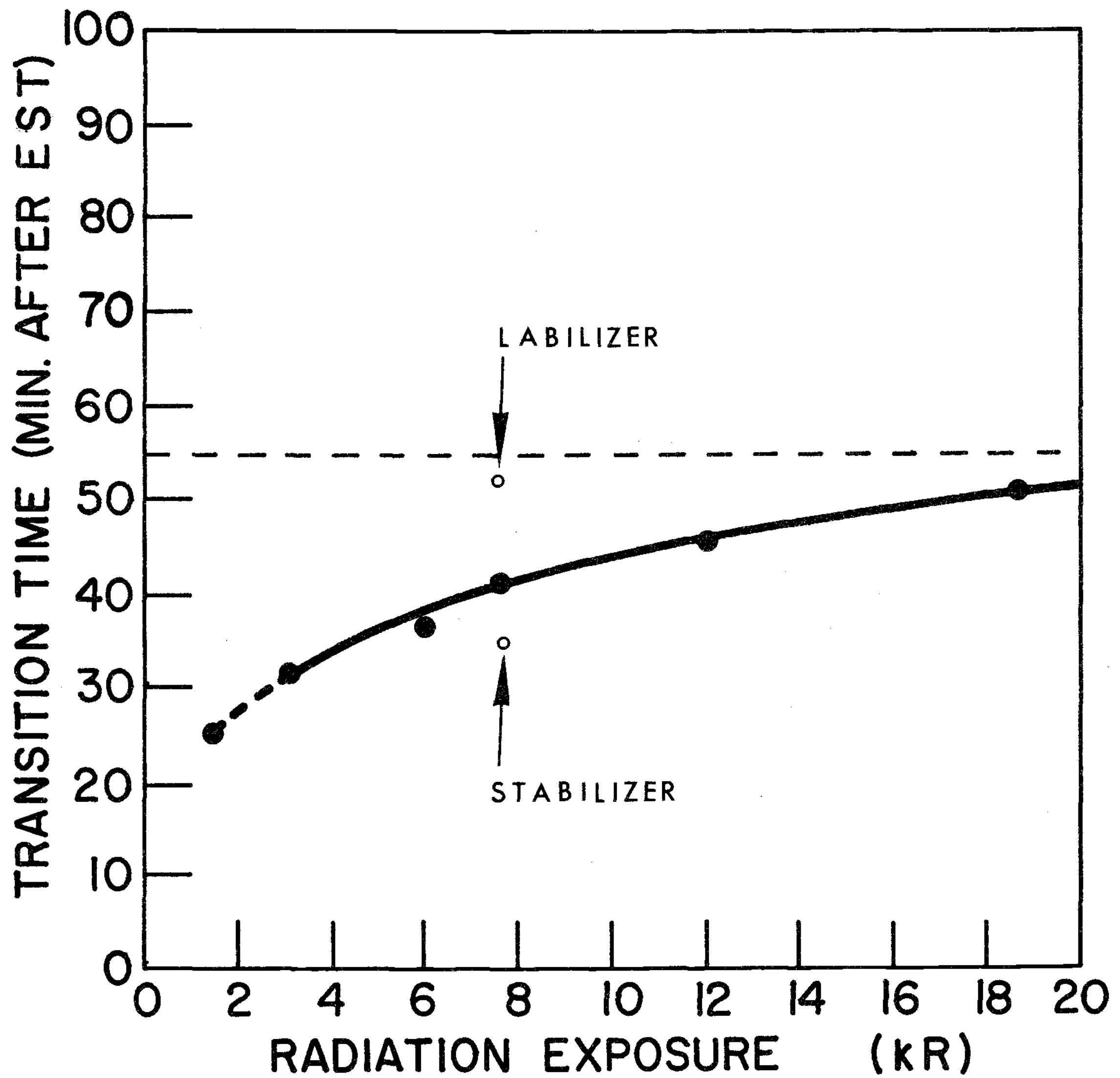


Figure 24. Effects of lysosomal labilizer and stabilizer on the transition point at 7.5 kR superimposed on curve from Figure 14, transition point vs radiation exposure (25 R/sec, 300 kVp X-rays).

point for cells treated with a labilizer and exposed to 7.5 kR corresponds to the time of the transition for untreated cells exposed to 18.5 kR, suggesting greater sensitivity. That is, the time at which the cells become insensitive to radiation (insensitive in terms of the first division being delayed) occurs earlier in stabilizer-treated cells and later in labilizer-treated cells. It should be emphasized, however, that very little effect on the amount of delay was observed with either agent indicating that the agents do not act in a simple dose-modifying manner.

Although vitamin A and hydrocortisone were effective in shifting the transition point, they had no effect on the timing of events during oral morphogenesis. This tends to rule out the possibility that the shift of the transition point results from a "slowing-down" or "speeding-up" of the development. The only difference noticed in silver-stained preparations of cells treated with either of these agents was a slight disorientation of the arrangement of basal bodies in stages I through III of the vitamin A treated cells. These cells regained their normal appearance in stage IV and a normal looking and functional oral apparatus developed.

There was no change in the transition point for irradiated cells pretreated with the lysosomal labilizing agent progesterone. On the other hand, chloroquine, a supposed lysosomal stabilizer, caused a greatly increased division delay (a labilizing effect?). However, since

Allison, O'Brien, and Hahn (1965) have shown that chloroquine complexes with DNA and Arnold, McHaro, and Allison (1969) have shown that chloroquine has protein binding characteristics, the value of chloroquine as a specific lysosomal stabilizer is decreased.

Euchrysine Staining

If lysosomes are involved in division delay and in resorption of the developing oral apparatus in irradiated *Tetrahymena*, then it might be possible to observe some change in the appearance of the lysosomes at the time of resorption or at some other critical time. Therefore, normal and irradiated cells were stained with the vital dye, euchrysine, and observed with a fluorescence microscope. The cells pictured in the frontispiece are typical of the fluorescence obtained with euchrysine. The nuclear material (macronucleus) fluoresces bright green and the lysosomes bright red (the tiny red granules are probably primary lysosomes and the larger granules, secondary lysosomes and phagosomes). The specificity of the euchrysine-induced red fluorescence of the lysosomes of living material has been repeatedly confirmed (Barrett and Dingle, 1968; Allison and Young, 1969).

There was no observable difference in the appearance of the red fluorescence of any of the four groups of euchrysine stained cells (unirradiated; irradiated with 7.5 kR at 30 minutes after EST; vitamin A treated-irradiated; hydrocortisone treated-irradiated).

Differences in appearance of the green fluorescent areas were noted, however: the macronucleus of the irradiated but otherwise untreated cells appeared to have a dark halo around it (Figure 25 -A and -B). The vitamin A-treated cells had somewhat of a granular appearance, presumably due to particles of ingested vitamin A.

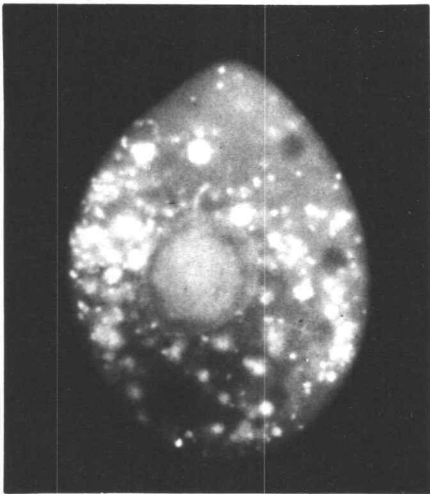
While observing the unirradiated cells throughout the division cycle (from EST through division) an interesting phenomenon was noticed. The red fluorescing granules (RFG) were randomly distributed throughout the cell (Frontispiece and Figure 25-C) from EST until the cell started to divide (division is marked by the formation of a "notch" or division furrow anterior to the developing oral primordium at about 65 minutes after EST). At this time, the RFG's migrated to the macronucleus and completely surrounded it (Figure 26-A). As the cell progressed toward division, the macronucleus began to elongate with the RFG's still surrounding it, but concentrated somewhat along a line in the middle of the cell. That is, there was a concentration of RFG's at the site of the division furrow (Figure 26-B). The configuration of red fluorescence assumed an "H" or figure 8 shape as the macronucleus split in two and the division furrow became more pronounced (Figure 26-C and -D). (Soon after the macronucleus divided, one or two small, spherical, bright green-fluorescing bodies were sometimes seen. These small spheres are probably the extrusion bodies of "excess" DNA described by Scherbaum, Louderback, and

Figure 25. Fluorescence photomicrographs of euchrysine stained *Tetrahymena pyriformis* cells (X 900). In these black and white prints made from the original color slides, the large bright objects are the green fluorescing macronuclei and the small, numerous bright spots are red fluorescing granules.

A. Irradiated with 7.5 kR at 30 minutes after EST, photographed at 95 minutes after EST (Note halo around macronucleus.)

B. Irradiated with 7.5 kR at 30 minutes after EST, photographed at 110 minutes after EST (Note halo around the macronucleus).

C. Control (unirradiated) cells photographed at 10 minutes after EST (Note random distribution of the red fluorescing granules (bright dots) throughout the cytoplasm.



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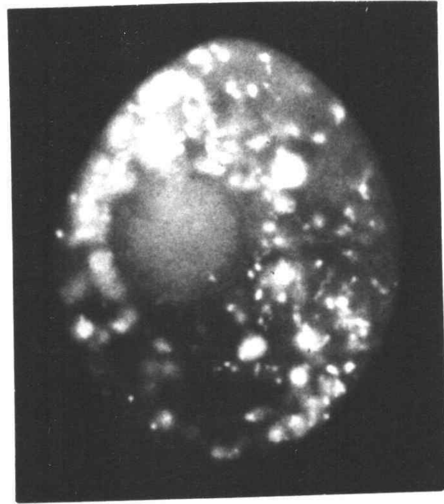

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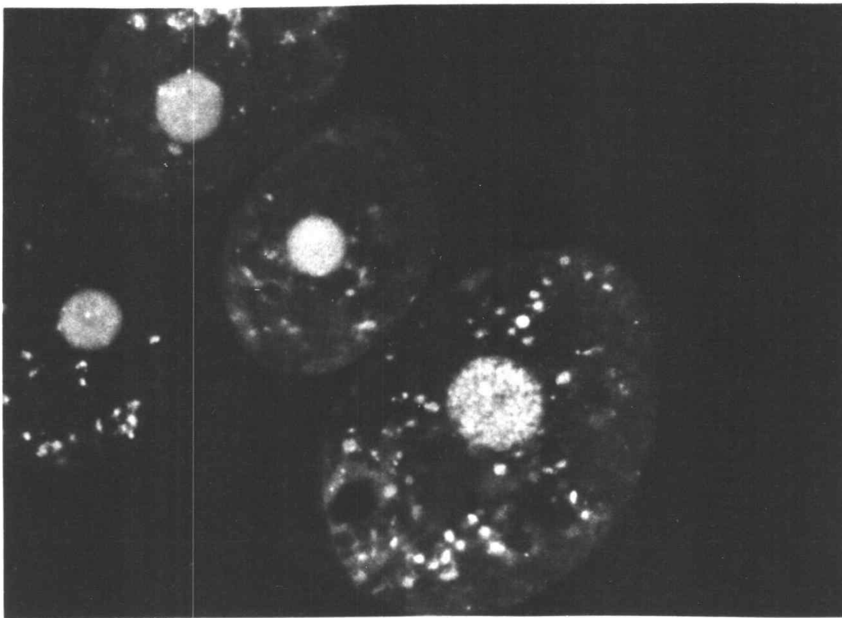


C

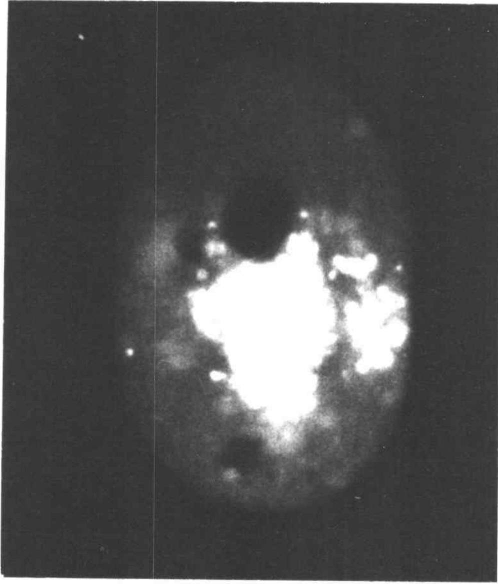
Figure 26. Fluorescence photomicrographs of euchrysine stained *Tetrahymena pyriformis* cells photographed during division (X 1200). In these black and white prints made from the original color slides, the large bright objects are the green fluorescing macronuclei and the small bright spots are red fluorescing granules.

A. 50 minutes after EST. Note the concentration of red fluorescing granules (RFG's) around the macronucleus and in the area of the division furrow.

B. 60 minutes after EST. Note the macronucleus beginning to elongate with RFG's continuing to surround it.

C. 80 minutes after EST. Note that the macronucleus has split with the RFG's surrounding the two nuclei and aligning on the division furrow.

D. 90 minutes after EST. Note the figure "8" configuration of the RFG's around the two daughter macronuclei.



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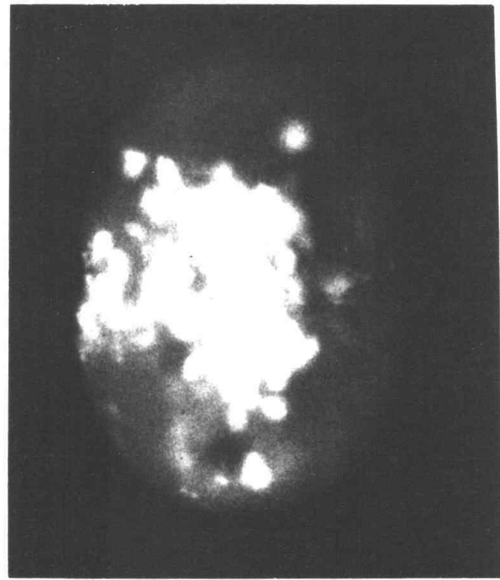

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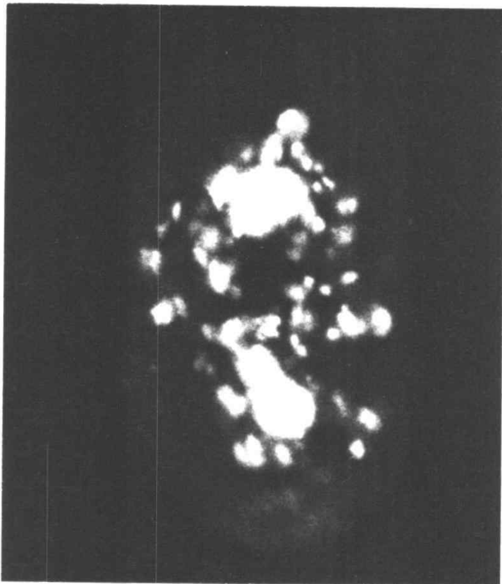

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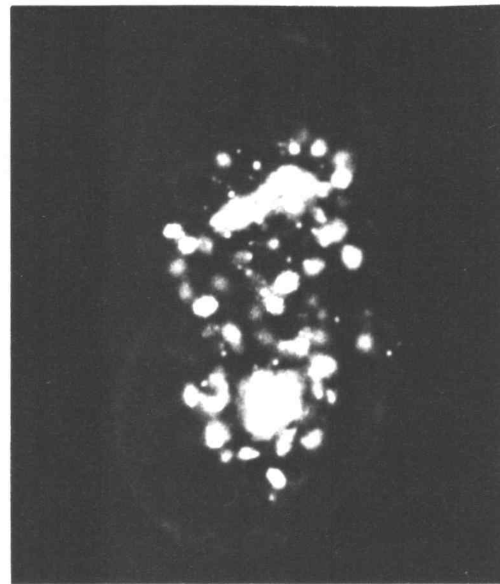

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Jahn (1958). After the two daughter cells completely separated, the RFG's once again returned to a random distribution throughout the cell.

The phenomenon just described for synchronized cells was also seen in logarithmically growing cultures and in the delayed divisions of irradiated cells. It is therefore, thought to be a normal behavior of division in this strain of *Tetrahymena*.

DISCUSSION

In the present study, it was found that X-irradiation delays cell division in sychronized *Tetrahymena* if they are exposed at any time prior to a critical time after the end of the synchronizing treatment. The critical time, or transition point, is a point in time when the cells undergo a rapid transition from sensitivity to being delayed to relative insensitivity. The transition point becomes later with increasing dose and approaches asymptotically an ultimate critical time of 55 minutes beyond which very high doses do not delay the coming division. Irradiation after the transition point does, however, delay the second division. Accompanying the delay of the first division is a morphological regression of the oral primordium that *Tetrahymena* cells develop during the preparations for division. The transition point for division delay is correlated with a "stabilization" of the oral primordium such that irradiation does not induce its resorption. By treating the cells with lysosomal stabilizers and labilizers, the transition point can be shifted to earlier and later times, respectively.

The data presented suggest that the damage produced by the X-rays acts as a stimulus that may or may not trigger off a cellular response that leads to division delay and morphological regression and that the sensitivity of the cell to being triggered may involve lysosomes.

Division Delay Response

The degree of synchrony and the time of division that was found for unirradiated, synchronized Tetrahymena pyriformis (GL-I) compares favorably with that found by other workers (Frankel, 1962; Nachtwey and Dickinson, 1967). Since the time of division after the end of the synchronizing treatment (EST) is so stable, one can rely on knowing what phase of development the cells are in at any time from EST to cell division; and one can consider delays of division in excess of ten minutes as being significant. Therefore, comparisons can confidently be made between different experiments in which cells are exposed to various agents at varying times after EST.

The data in Table 3 and Figure 13 indicate that the amount of delay induced by an exposure at any given time is dependent upon the amount of radiation given. A dose-dependent relationship between the amount of division delay and the dose of some agent is logical from the standpoint that the "harder" the cell is "hit", the greater the delay.

In addition to a dose dependency, there is also a time-dependency: up to the transition point, the amount of delay induced by a given exposure increases with the time after EST at which the cells are irradiated. The amount of the increase is approximately one minute additional delay for each additional minute since EST. For example, cells exposed to 7.5 kR at seven minutes after EST are delayed 77

minutes whereas those exposed at 38 minutes are delayed 114 minutes; the difference in the delays produced in cells irradiated 31 minutes apart is 37 minutes. Looked at another way, cells given a particular exposure all take about the same amount of time after irradiation to reach division, independently of when after EST they were irradiated. There may be a small tendency for cells irradiated later to take a little longer than those irradiated earlier, but the maximum differences in times from the start of irradiation to division (DT_{50}) are small: 12 minutes in the 3 kR experiment, 6 minutes in the 7.5 kR experiment, and 15 minutes in the 18.5 kR experiment.

The relationship described above suggests that any progress toward division that the cell has made since EST is eliminated by the irradiation; this response to irradiation is similar to that found by Zeuthen (1958) with heat shocks and which he called "set-back". It appears that the cells are "set-back" in physiological time to a state resembling that at EST. The delay is more than just a loss of progress already made, however, otherwise the cells would require the same amount of time after irradiation to reach division as the unirradiated controls require after EST, namely, 100 minutes. The time to division is greater than this by a dose-dependent amount. The extra time is presumably the amount of time required to recover from the radiation damage.

The apparent "set-back" in physiological time is also reflected

in the morphologically detectable return to a state that resembles the interphase condition, i.e. to one in which the oral primordium is lacking.

The above considerations apply only to cells that are delayed by irradiation. Cells irradiated after the transition point are not delayed and do not resorb the developing oral primordium. However, they are damaged by the irradiation as evidenced by the delay of the second division. The time at which any particular cell undergoes a transition to relative insensitivity varies from cell to cell. However, within each individual cell, the transition appears to be practically instantaneous as judged by the lack of intermediate delays of division in cells irradiated near the transition point. (In Figure 12, note the long horizontal plateaus separating the cells that are not appreciably delayed from those that are.) The division delay response appears to be almost all-or-none.

Preparations for Division of Synchronized *Tetrahymena*

Numerous studies with presumably specific inhibitors have demonstrated division delay responses and transition points in synchronized *Tetrahymena* somewhat similar to those demonstrated with irradiation. Some of the agents studied include the following: p-fluorophenylalanine (Frankel, 1962; Rasmussen and Zeuthen, 1962; Frankel, 1965); fluorodeoxyuridine and fluorouridine (Frankel, 1965);

fluoride, fluoroacetate, and azide (Hamburger, 1962); mercaptoethanol (Gavin and Frankel, 1965; Mazia and Zuethen, 1966); actinomycin-D (Frankel, 1965; Nachtwey and Dickinson, 1967); puromycin (Frankel, 1967b); cycloheximide (Frankel, 1969); dinitrophenol (Frankel, 1967a); exposure to anaerobiosis (Rasmussen, 1963); emacronucleation (Nachtwey, 1965); colchicine (Nelsen, 1970); and high hydrostatic pressure (Lowe-Jinde and Zimmerman, 1969; Simpson and Williams, 1970).

From these studies the following understanding of the sequence of preparative events has emerged: (1) the essential preparations for division after EST involve mainly protein synthesis (see Zeuthen, 1964 for review and references) and such synthesis can proceed using stored precursors; no nutrients are required (Hamburger and Zeuthen, 1957). Such protein synthesis proceeds up to about 55 minutes after EST. After this time further protein synthesis, though it occurs, is not essential. (2) DNA synthesis during this time is not required (Cerroni and Zeuthen, 1962) but RNA synthesis, presumably messenger RNA synthesis, is required for most of the period of essential protein synthesis. However, it is not required during the last few minutes of the period of required protein synthesis (Nachtwey and Dickinson, 1967), presumably because enough has been synthesized to support the synthesis of essential protein. (3) About this same time, even the nucleus itself is not required for division to occur

(Nachtwey, 1965). (4) During all of the protein synthesis period, energy producing metabolism is required (Hamburger, 1962; Rasmussen, 1963; Frankel, 1967a). (5) After the synthetic period, it is likely that the structures into which the essential proteins are built are not complete: high hydrostatic pressure, an agent that interferes with the assembly of microtubule proteins, can affect division (Simpson and Williams, 1970). Colchicine, another agent that can interfere with assembly of microtubule proteins can also affect the completion of oral primordium development and macronuclear division but not cytokinesis (Nelsen, 1970).

Oral Primordium Involvement

Since division delay caused by a number of agents has been correlated with oral primordium resorption (Figure 21), the protein synthesis involved in oral morphogenesis might be implicated as the division-essential process. Indeed, in all cases studied, if oral development is blocked by an agent prior to the transition point, the cell resorbs the structures already present and begins development again. However, it does not follow that oral primordium resorption causes division delay. The resorption phenomenon may be only an observable manifestation of an underlying process which includes a set-back of the entire cell plus a delay required to replace or repair damage induced by an agent. In fact, Nelsen (1970) and Simpson and Williams

(1970) have presented evidence that cell division and oral morphogenesis are not absolutely tied together.

Nelsen (1970), using colchicine-treated Tetrahymena, has shown that the assembly of microtubules prior to the transition point is a necessary requirement for both oral morphogenesis and division. However, she has also shown that oral morphogenesis can be blocked by colchicine after the transition point without stopping cell division. In other words the completion of oral morphogenesis is not necessarily an absolute prerequisite for cell division.

Furthermore, Simpson and Williams (1970) have recently shown with high hydrostatic pressure applied to synchronized Tetrahymena, that resorption of the oral primordium and delayed division are separable phenomena: they found that high hydrostatic pressure administered to cells after the usual critical point at 55 minutes did induce a division delay and also resorption of the oral apparatus in both the proter and opisthe. However, the important point is that such resorption appeared to span the division process such that resorption was not completed and renewal of the oral apparatus did not commence until after the delayed division. This finding shows that oral primordium resorption is not necessarily tied to division delay. Thus, the effect of various agents on oral morphogenesis may reflect an effect on the cell as a whole rather than indicating the organelle responsible for division delay.

Possible Mechanisms of Radiation-Induced Delay

Viewed in the context of what is known about the course of events leading to division of *T. pyriformis*, ionizing radiation may induce a division delay by interfering in some way with the protein synthesis necessary for division. Irradiation has been shown to inhibit induced enzyme synthesis in bacterial systems while having little effect on total protein synthesis (Okada, 1970). A similar inhibition of induced division protein synthesis may occur in irradiated *Tetrahymena*. However, *by itself*, inhibition of division protein synthesis is not likely to account for the division delay response observed. It is difficult to see how inhibition of any synthetic reaction could lead to the set-back response; one would expect a general slowing down of progress toward division and a shorter delay the closer the cell is to division before it is irradiated.

The set-back response, in which progress already made prior to irradiation is seemingly "wiped out", and the oral resorption phenomenon, both point to an effect of radiation greater than just inhibition of a synthetic reaction. The shifting of the transition point by lysosomal labilizers and stabilizers suggests that the "wiping out" of progress made may be mediated via lysosomal enzyme release.

Lysosome Involvement

Lysosomes in the cortical regions of *Tetrahymena* have been demonstrated by Goldfischer, Carasso, and Favard (1963) and Elliott (1965) and Müller, Baudhuin, and deDuve (1966). Lysosomes contain hydrolytic enzymes that are normally utilized in digestion of ingested food particles, and also in self digestion of parts of the cell such as worn out or unneeded mitochondria (autophagy). The conclusion that lysosomes may be involved in radiation-induced division delay is dependent upon the specificity of the labilizing and stabilizing agents for lysosomes.

Among the physiologically active compounds known to labilize isolated lysosomes, vitamin A has been studied most extensively (Dingle, 1961; deDuve, Wattiaux, and Wibo, 1962; Dingle and Lucy, 1965). Weissman and Thomas (1963) demonstrated *in vivo* that vitamin A enhanced the release of acid hydrolases from rabbit liver lysosomes and that this enhanced release could be diminished by the addition of hydrocortisone. Although these two agents have been shown in this way to be lysosomal labilizers and stabilizers, respectively, there is some evidence that their action may be different for different cells or even for different "types" of lysosomes within one cell (Iqbal and Wynn, 1970). This could explain why some labilizers and stabilizers are effective and others are not. (There was a considerable

difference noticed, for example, between vitamin A and progesterone in the present study.)

If the changes in the transition point brought about by lysosomal labilizers and stabilizers do in fact indicate lysosomal involvement in division delay and oral primordium resorption, then the question of mechanism arises.

Irradiation could induce division delay via lysosomes and enzymatic degradation by two possible mechanisms, one direct and one indirect: (1) irradiation could have a direct effect on lysosomes causing either an immediate or a delayed release of enzymes into the cytoplasm or nucleoplasm resulting in damage via self digestion. (2) Irradiation could produce damage to some constituent or process and the cell, "recognizing" the damage, could activate the lysosomes via normal physiological mechanisms as one phase of a repair process.

Various experiments have shown that both ionizing and ultraviolet light irradiation can induce the release of enzymes from isolated lysosomes (Desai, Sawant, and Tappel, 1964; Wills and Wilkinson, 1966). Koenig (1969) suggests that ionizing radiation affects lysosomal membranes through the action of free radicals and peroxides. The release of enzymes after irradiation can be enhanced or depressed by labilizers and stabilizers such as the vitamin A and hydrocortisone used in the present experiments (Weissman and Dingle, 1961). Although high doses are usually required to elicit an effect on

isolated lysosomes, this does not preclude lysosomes from being a sensitive target when within cells. Harris (1966b) has demonstrated that the sensitivity of isolated lysosomes is dependent upon the method and technique of isolation. Lysosomes prepared with minimal damage were relatively sensitive to radiation and effects were observed after doses as low as 200 rads.

In the experiments reported here, there was a considerable lag after irradiation before the onset of resorption. This might be taken as evidence against a direct effect on lysosomes that results in the immediate release of enzymes. However, the possibility exists that lysosomes may be subtly damaged by low doses of irradiation which might predispose them to rupture or leak at a later time. Richards, Schoeket, and Michaelis (1969) provided some _in vivo_ evidence for this "latency" by showing a marked decrease in acid phosphatase concentration in the germinative zone of the lens epithelium of rabbits 28 days after a cataractogenic X-ray dose. This decrease progressed at 42 and 60 days and was shown to be due to fewer acid phosphatase granules (lysosomes) in the cells.

The point is that direct radiation effects on lysosomes _in vivo_ may occur at lower doses than required for _in vitro_ effects, and may not necessarily be expressed immediately.

Let us assume for the present that radiation does directly affect lysosomes in _Tetrahymena_ but produces latent damage that doesn't

yield an observable effect until later. If the effect is direct one might reasonably assume that the dose dependency of the division delay results from an increasing number of lysosomes affected with increasing dose. Since the transition point shifts with dose, one may postulate that the lysosomes change in their sensitivity to latent damage induction as the cell progresses toward division. Since lysosomal labilizers and stabilizers shift the transition point for a given exposure, it is reasonable to assume that these agents can modify the sensitivity of the lysosomes to damage. However, there was no appreciable increase or decrease in the amount of delay as one would expect from a dose modifying agent that altered the sensitivity to a direct effect. That is, if irradiation delays cell division in Tetrahymena by having a direct effect on lysosomes, then one would expect a longer delay in cells treated with a lysosomal labilizer prior to irradiation, and a shorter delay in those treated with a stabilizer.

From the foregoing considerations one can see that the available evidence for a direct effect of irradiation on lysosomes in Tetrahymena is not strong. Furthermore, when lysosomes are irradiated within cells, modifying factors may be present that cloud the interpretation. For example, Harris (1966a), studying irradiated rabbit leukocytes, found that under certain conditions calcium ions increased the permeability of lysosomal membranes. Since injured cells accumulate calcium he concluded that this might form a basis for lysosomal

activation in irradiated cells. Thus, even though lysosomal permeability *in vivo* can be affected by an agent, the effect might still be indirect. This finding has a bearing on the conclusions of Müller *et al.* (1966) and Allison and Malluci (1964). Müller *et al.* (1966) found that exposure of *Tetrahymena* to lowered osmotic pressure, to freezing and thawing, or to heat or cold shocks activated five lysosome-bound hydrolases (acid phosphatase, ribonuclease, deoxyribonuclease, proteinase, and amylase). They concluded that these conditions affected lysosomes directly. Similar studies by Allison and Malluci (1964) led them to suggest that the activation of enzymes resulted from changes in lysosomal permeability associated with changes in temperature. (They further suggested that such changes contribute to the temperature shock synchronization of *Tetrahymena*.) These experiments, however, do not really allow one to distinguish between a direct effect on lysosomes and an indirect effect (such as through the accumulation of calcium) produced by non-specific altered internal conditions.

Even if we accept the possibility that irradiation may affect the lysosomes of *Tetrahymena* indirectly via some non-specific effect on the internal conditions, it would still not account for the fact that there is essentially no difference in delays in the presence or absence of labilizers and stabilizers. Without a great number of unwarranted assumptions, the results presented are very difficult to explain by the

hypothesis that the amount of division delay is completely a result of a dose-dependent activation, either directly or indirectly, of a certain number of lysosomes.

A more probable explanation is that activation of lysosomes is a secondary effect mediated by the cell as a specific response to some primary damage. The cell in some way "recognizes" the damage and triggers a sequence of events that involves lysosomes. Quite possibly, the lysosomes play a role in the return of the cell to an earlier physiological stage at which repair can take place before proceeding toward division. Or, what is also plausible, the cell attempts to repair the damage while continuing to progress toward division but the delicate balance of synthetic reactions and assembly processes has been perturbed. (Such perturbation could result from damage to some critical substance and/or function while leaving others unaffected.) Eventually the imbalance is so severe that preparations can not proceed normally. At this point, as a homeostatic response to the imbalance, the resorption phenomenon is triggered and all progress so far made (synthesis and assembly) starts to be destroyed. The rate of resorption is geared to the dose-dependent repair time. When resorption and repair are completed, the cell starts anew in preparing for division.

The above hypothesis is supported in part by the following observations made with silver stained cells (see Figure 21). (The morphological time-map based on oral morphogenesis is used mainly

as an indicator of progress toward division and radiation-induced regression and not as an indicator of the target organelle.) Progress after irradiation continues for a variable time period, depending on exposure level and time of irradiation, before resorption is triggered. The duration of resorption is variable but seemingly a constant fraction of the delay time. The delay time itself is equal to all of the time since EST, i. e., progress made before irradiation plus the abortive progress made during and after irradiation, plus the time to complete resorption. It should be emphasized that the resorption time is variable and somewhat dependent on dose which indicates that it is not a clearcut physiological process requiring a fixed length of time once initiated. This variation in resorption time may result from either a dose-dependent effect of irradiation on the resorption mechanism, which would inhibit its activity, or the result of the resorption mechanism being rate limited by some other underlying factor, i. e., it is tied to another process such as repair. In any event, Figure 21 shows that the radiation-induced delay of division occurs in a limited period between irradiation and the end of the resorption; it does not result from a general slowing down of the preparations for division. Evidence for this conclusion is that once renewal starts it proceeds normally and at the rate shown by the control population.

The idea that division delay and oral primordium resorption might be triggered by an imbalanced state is supported by the fact

that a number of relatively non-specific agents can bring about the same response: heat and cold shocks (Thormar, 1959; Frankel, 1962 and 1967a), osmotic shocks (Nachtwey, unpublished), ultraviolet light irradiation (Nachtwey and Giese, 1968). These agents can produce essentially the same effects on division and primordium resorption as the large number of more specific agents listed earlier. It seems unlikely that this diversity of agents all act on the same process in the same way. It seems more likely that the agents, by acting on different processes, could lead to the same general effect - imbalance.

More pertinent evidence for the imbalance hypothesis is found in studies with temperature shocks. Cells cold shocked at 1° C require longer exposure than do those exposed at 9° C to induce division delay (Nachtwey, 1967). This result suggests that total metabolism at the lower temperature is so slowed that a longer time to become unbalanced is required than at the higher temperature. Moreover, Frankel (1967a) has shown that cells progress further after a 1° C shock before resorption is initiated than those after a 9° C shock.

Nachtwey (unpublished) has shown that a short (five minutes) heat shock, which by itself is insufficient to induce a division delay response, plus irradiation after the transition point, i.e. at a time when it does not induce division delay, together will induce division delay. That is, two very different subeffective stimuli added together become effective in inducing division delay.

If the process affected by irradiation is synthesis of division protein, then the imbalance hypothesis could account for the fact that the transition point shifts with increasing dose. At a low exposure level, 3 kR, at early times before much of the protein has been synthesized, the damage interferes in some way with the production of the protein and so the rate of production falls off. The progress toward division continues on, utilizing previously synthesized protein and the protein slowly synthesized subsequent to irradiation. However, eventually the cell runs out of protein or the process becomes a bottleneck and an imbalance occurs. Not being able to sustain the normal rate, the resorption process is triggered. After the transition point for low exposures, the amount of the essential protein already synthesized and the remaining capacity to synthesize is sufficient to sustain completion so resorption is not triggered. At higher exposures though, more machinery is damaged so the rate of production falls off even more, so that completion is prevented and resorption is triggered. At the ultimate critical time, the amount of protein needed has been synthesized so, even complete inhibition of further synthesis does not block division and the new oral apparatus is developed.

The lysosomes play a role in bringing about oral primordium resorption and set-back. They are triggered in an all-or-none manner by the cell in response to the imbalanced state produced by

irradiation inhibiting the division-essential process. The effect of lysosomal labilizers and stabilizers is simply to make them more or less sensitive to the triggering conditions, i.e. labilizers or stabilizers lower or raise the threshold at which the cell's physiological signal evokes a response. Once triggered, the response is the same - physiological set-back, and oral primordium resorption. The amount of delay, when it is triggered, is not influenced by the lysosomes; the amount of delay depends solely on the time required to repair the radiation damage or replace damaged organelles or substances such that conditions are favorable for successful division to occur.

It may be pertinent in this regard that Brunk (1967) has shown that X-irradiated Tetrahymena perform an extensive excision-repair of damaged DNA.

As often happens in science, seeking an answer to one question leads to the formulation of more questions and more alternative hypotheses.. Therefore, before these hypotheses can be accepted or rejected, further studies must be made. Some suggestions for further study include the following: (1) a sequential study of the ultrastructure of irradiated Tetrahymena to determine whether lysosomes are visibly damaged or concentrated in the area of oral primordium resorption; (2) specific enzyme identification such as acid phosphatase (Gomori, 1952) to localize lysosome-bound enzymes as well as released enzymes; (3) the use of lysosomal labilizers and stabilizers in

conjunction with electron microscope and enzyme identification studies; (4) positive identification of the euchrysine staining granules in fluorescence microscopy would not only be useful for the radiation studies but also help to understand the behavior of the red fluorescing granules seen at division; (5) further studies on the delay of the second division from post-transition point irradiation, especially silver stain studies to determine whether oral primordium resorption is involved in the second division delay; and (6) studies like the present one and the above suggested experiments using other cell types such as cultured mammalian cells.

SUMMARY

1. The present study represents an attempt to gain a better understanding of some of the underlying characteristics of sublethal X-irradiation-induced division delay in heat synchronized cultures of the ciliated protozoon Tetrahymena pyriformis (GL-I).

2. The problem was approached by utilizing the hypothesis that the damage produced by radiation acts as a stimulus that may or may not trigger off a cellular response that leads to the delay of cell division and that the sensitivity of the cell to being triggered may involve lysosomes.

3. One objective of this study was to determine the times after the end of the synchronizing treatment (EST) at which the cells undergo a rapid transition from a state of being sensitive to being delayed to one of relative insensitivity. This transition point was found to be dependent upon the amount (dose) of radiation: the higher the dose the later the transition point, up to an ultimate critical time of 55 minutes. After 55 minutes, even very high exposures (32 kR) do not delay the coming division. The second division is, however, delayed.

4. Another objective of this study was to correlate the radiation-induced division delay with some observable phenomenon: resorption of the developing new mouth. From silver stained cells, it was found that the length of time for the oral primordium to be resorbed increases

as the dose increases and varies with the time of irradiation relative to the transition point. However, the length of the division time is not completely accounted for by the resorption time; the division delay is greater in all cases than the resorption time. Once resorption has been completed the cell proceeds toward division normally as indicated by the similarity between the lengths of the renewal times (the time from the end of resorption to division) and the lengths of the control division times (the time from EST to division). Cells exposed to irradiation after the transition point, which showed essentially no delay in the first division, did not demonstrate oral primordium resorption even at high exposures. As observed from experiments involving food vacuole formation, cells exhibiting pre-transition point division delay (involving both oral primordium resorption and a delay of the first division) or post-transition point division delay (involving neither oral primordium resorption nor a delay of the first division) did not sustain any permanent damage to their oral apparatuses.

5. Another objective of the present study was to ascertain whether lysosomes were involved in either the radiation-induced division delay or oral primordium resorption. Direct evidence from fluorescence microscopy of euchrysine stained cells for the involvement of lysosomes in either phenomenon was not obtained. However, evidence for an indirect action of lysosomes was obtained from shifts in the transition point after treating the cells with lysosomal

labilizers (enhanced enzyme release) and stabilizers (depressed enzyme release) prior to irradiation. The transition point for cells treated with stabilizer (hydrocortisone) was shifted to an earlier time (to a transition point normally shown by cells exposed to a lower dose of radiation), and for cells treated with labilizer (vitamin A) it was shifted to a later time (to a transition point normally shown by cells exposed to a higher dose of radiation). This suggests lesser and greater sensitivities, respectively.

6. The results and conclusions are discussed in relation to other stimuli which induce division delay, emphasizing some alternative hypotheses for explaining radiation-induced division delay.

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