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Gary L. Whitson, Major Professor

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# CYCLIC NUCLEOTIDE CHANGES IN X-IRRADIATED SYNCHRONIZED TETRAHYMENA

A Thesis

Presented for the

Master of Science

Degree

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Paul A. Charp

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ABSTRACT

The purpose of this investigation was to determine the effects of 5000 r X-irradiation on the ciliated protozoan Tetrahymena pyriformis, specifically the levels of cyclic nucleotides, cyclic adenosine monophosphate and cyclic guanosine monophosphate, as well as the cyclic nucleotide phosphodiesterase associated with each compound and the relationship of these compounds to synchronized cell division.

Tetrahymena was selected for this study because it displays a rapid growth rate, and can easily be synchronized by a series of hot and cold temperature shifts and also shows an extremely high resistance to ionizing radiation.

The procedures used in this study involved basic physical and biochemical methods. These included radioimmunoassays for cyclic nucleotides, competitive protein-binding studies and X-irradiation. The results show that there is a definite correlation between cell division and the fluctuation of levels of the cyclic nucleotides investigated. However, immediate changes in the levels of cyclic AMP preceding cell division do not appear to be closely related to cell division. Results from both control and irradiated cultures indicated that levels of cyclic AMP decreased even if cell division did not immediately follow. However, changes in cyclic GMP showed a close correlation to cell division. Increase in cGMP occurred only during the initiation of cell division. Phosphodiesterase studies showed that the enzyme was not affected by

radiation but showed altered patterns of activity related to different times in the synchronized division cycle.

The increase in the levels of cyclic GMP signals the initiation of cell division in Tetrahymena while the decrease in the levels of cyclic AMP is important to some other physiological process roles other than the initiation of division.

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# CHAPTER I

# INTRODUCTION

## I. Background

### General Description of the Organism

The ciliated protozoan Tetrahymena pyriformis is an ideal organism for the study of biochemical and radiological effects of ionizing radiation. It can be grown axenically in a chemically defined medium (Kidder and Dewey, 1951) or in a simple proteose peptone-based medium supplemented with a few inorganic salts (Whitson et al., 1967).

In appearance, pyriformis is a pear-shaped ciliated protozoan, which reaches the size of 40 by 60 μm, has a varied number of meridianal rows (Figure 1). The oral apparatus is located at the anterior end of the organism and contains four membranellar structures from which the organism derives its name, Tetrahymena. The development and formation of these membranelles occurs prior and during the formation of the fission-furrow (Frankel, 1964). When all membranelles are completely developed, the organism is near the end of the division cycle.

### Synchronization

Cell division in Tetrahymena can be synchronized by a series of seven heat shocks, each of 30 minutes duration, of 33.9° C alternating with seven recovery 30 minute periods at 28.5° C (Scherbaum and Zeuthen, 1954). During the heat shocks cells do not divide, but at approximately 90 minutes after the last heat shock (EHS) over 80% of the cells are in

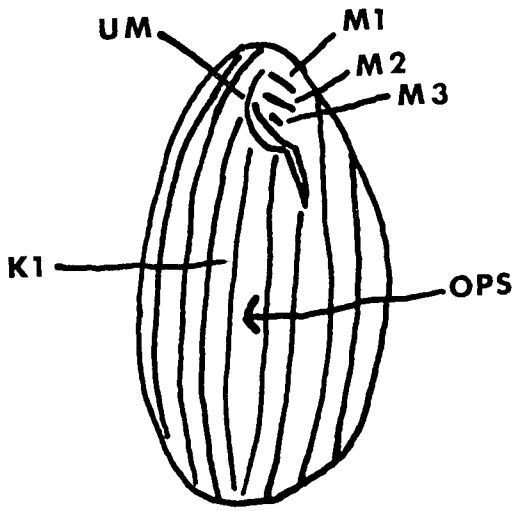



Figure 1. General morphology of Tetrahymena pyriformis GL-C. (UM--Undulating Membrane; M1--Membranelle 1; M2--Membranelle 2; M3--Membranelle 3; OPS--Oral Primordial Site; K1--First Ciliary Row)

division. It is believed that the heat shocks used to synchronize cell division denatures some substance necessary for cell division to occur. It has been suggested that this substance is a "division protein" (Williams and Scherbaum, 1959; Zeuthen, 1961; and Watanabe and Ikeda, 1965). The latter investigators using DEAE-cellulose chromatography indicated a qualitative change in protein content occurs just prior to cell division. It is their supposition that during the recovery periods between successive heat shocks that a partial reconstruction of the "division protein" which when not completely denatured can allow for a small amount of cell division to occur between each heat shock. It has also been suggested that the "division protein" is associated with the formation of the oral apparatus in the developing Tetrahymena (Williams, 1964). In addition, Frankel (1964) observed development was blocked by an agent such as high temperature (34° C), no cell division occurred at this time.

## Irradiation

The elucidation of the mechanism which control cell division in cells is of considerable interest and one of the more widely used methods for exploring these mechanisms is radiation. For example, in X-irradiated cell populations, one of the major events easily predicted and measured is the delay of cell division (Hollaender, 1955) and associated damage to the mitotic apparatus as observed in HeLa cells (Brent et al., 1966). Griffiths and Tolmach (1975) reported that the magnitude of DNA synthesis in irradiated HeLa cells is age-dependent. Cells at $G_1$/S boundary show

the greatest suppression of DNA synthesis; whereas, cells in early $G_2$ or late S-early $G_2$ have the least amount of deficient DNA synthesis. Bacchetti and Sinclair (1971) observed in irradiated Chinese hamster cells that rates of RNA and protein synthesis in experimental cultures were higher than the rates in control cultures.

Ciliates are of interest in regard to ionizing radiation because they have been shown to be among the most radiation-resistant organisms. The dosage in kiloroentgens necessary to kill 50% of a cell population in a 24-hour time period for some selected ciliates are: Paramecium calkinsi, 400 Kr; Colpidium colpoda, 330 Kr; and Paramecium polycarum, 210 Kr (Giese, 1967). Tetrahymena has been reported to be one of the most radioresistant cells known with an $LD_{50}$ estimated to be 482 Krads (Calkins, 1964). It is not known precisely why this cell is so radioresistant but it is believed to be due in part to the fact that the organism has a very efficient DNA repair mechanism for the removal of ultraviolet-induced pyrimidine dimers (Whitson et al., 1968). Another possible explanation for Tetrahymena's high radioresistance is that as a ciliate, Tetrahymena is highly polyploid and contains hundreds of duplicate copies of its genome (Whitson, 1972).

Studies involving X-irradiation of Tetrahymena in association with cell division and macromolecular synthesis have shown that there is a critical time period associated with EHS in which there is no division delay for the first synchronous division regardless of the amount of radiation the organism receives up to its lethal dose of approximately 482 Krads. Before this time point, the time of radiation-induced delay

depends on when after EHS, the cells are irradiated. This delay is dose dependent, the larger the dose, the greater the delay (Hodge and Nachtway, 1972). Paul and Zimmerman (1973) have observed in heat-shocked synchronized Tetrahymena that after 4 Krads of ionizing radiation, the rate of DNA synthesis exceeded that of the controls until the onset of division. At that time the rate of thymidine incorporation was less than that of the controls; whereas, uridine and amino acid studies showed that the rates of RNA and protein synthesis were less than or equal to that found for control cultures at all times during synchrony.

## Cyclic Nucleotides

Since the discovery of cyclic adenosine 3', 4' monophosphate (cAMP) in 1958, (Rall and Sutherland, 1958; Sutherland and Rall, 1958), this compound has been implicated in many physiological processes in cells ranging from bacteria to human cells (Drummond et al., 1975). Increased levels of cAMP and its analogs, when present in the cell, paralleled a decrease in the rate of DNA synthesis (Froehlich and Rachmeler, 1972, 1974). Conversely, if cAMP is added to a cell system in very low amounts, there can be a stimulation of DNA and RNA synthesis (Abell and Monahan, 1973). The cyclic nucleotide has also been shown to important in growth, development and morphological changes in Chinese hamster ovary cells (O'Neill et al., 1976). These investigators observed that increases in intracellular cAMP resulted in cell elongation through the polymerization of microtubule subunits. The resulting microtubules then orientate along the long axis of the cell, changing the growth patterns and normal cell morphology.

Cyclic AMP was first identified in Tetrahymena pyriformis E by Voichick and coworkers (1973). They reported that changing levels of cAMP were related to different states of carbohydrate metabolism in this organism. When cells were grown in the absence of glucose in the medium, cAMP levels were six times greater than when cells were grown in the presence of glucose. Presently, it is still unclear how cAMP can function in so many divergent processes, but it has been postulated to act as a second messenger in many of the cell physiological processes. The stimulation of formation of cAMP has been associated with insulin binding, muscle contraction, and even neurosecretory stimulation such as release of serotonin (Robison et al., 1971; Weissmann and Claiborne, 1975). This process of stimulation of specific functions by cAMP has been called the "second messenger concept" by Robison, et al. (1971) because the substance that activates its synthesis works on the external surface of the cell, not getting inside, but the catalysis of this cyclic nucleotide occurs inside which results in divergent and corresponding physiological changes.

Another cyclic nucleotide, guanosine 3', 5'-monophosphate (cGMP) appears to function antagonistically to cAMP. For instance, it has been shown in differentiated, beating heart cells that a 2-fold increase in cAMP results in an increase in contractibility of the heart; whereas, a 2-fold increase in cGMP results in a decrease in the rate of contraction. Other cellular responses which show an antagonistic response between cGMP and cAMP are leukocyte motility which is increased by cGMP and slowed by cAMP. Lipolysis is stimulated by cAMP and lysosomal enzyme release is stimulated by cGMP (Goldberg, 1975). In general the levels of cGMP in

most differentiated, nondividing cells, levels of cGMP are at least ten times less than the amount of cAMP in the same cells (Robison et al., 1971). Although the relative proportions of the cyclic nucleotides and not the absolute amounts that determine their actions. Some functions of cGMP include changes in muscle contraction, promotion of cell growth and stimulation of blood clotting (Goldberg et al., 1975). For instance, in rats when cGMP is in high concentration with respect to growth hormone, there is an increase in the release of this hormone; whereas, analogs of cAMP show no stimulatory effect (Peake et al., 1972). Ferrendelli and coworkers (1970) reported that in the mouse cerebellum and cerebral cortex, cGMP and cAMP have independent functions in the central nervous system and cGMP plays a role in cholinergic neurotransmission. When cGMP levels rise in the cortex, cAMP levels drop.

There are some reports which indicate that intracellular changes in cyclic nucleotides may also be related to cell growth and cell division. Hadden et al. (1972) reported that lymphocytes treated with phytohemagglutin showed increased levels of both cGMP and cAMP. However, cGMP showed a 12-fold increase over controls and cAMP showed a 70% increase over that found in controls. After treatment of these cells with concanavalin A which stimulates cell division, the levels of cAMP remained the same; whereas, cGMP levels increased 50 times. These findings suggested that cAMP may not play a direct role in cellular proliferation but indicated instead that cGMP might function as a "second messenger" stimulating cell growth and division.

Cyclic Nucleotide Phosphodiesterase

The function of cyclic nucleotide phosphodiesterase is to degrade cyclic 3',5'-monophosphate nucleotides to the 5' nucleotide form. In liver extracts, there appears to be three distinct fractions of this enzyme. As eluted from a salt gradient column, the first and third fractions of phosphodiesterase are a cGMP phosphodiesterase and cAMP specific phosphodiesterase respectively. The second fraction hydrolyzes both cyclic nucleotides at equal rates (Appleman and Terasaki, 1975).

Phosphodiesterase activity in Tetrahymena was first reported by Lazarus and Scherbaum (1967). They observed that 95% of the enzyme activity was associated with the ribosomes and required the divalent cations calcium and magnesium. It must be noted that the assays as performed were not specific for either cAMP or cGMP. Cyclic AMP phosphodiesterase in Tetrahymena is composed of three distince fractions as reported by Ramanathan and Chou (1973) which are similar to the phosphodiesterase fractions found in rats as reported by Appleman and Terasaki (1975). Ramanathan and Chou, eluting the fractions by gel filtration, observed that only fractions one and two hydrolyzed cAMP; whereas, the third fraction did not. Consequently, Ramanathan and Chou suggest that the enzyme in Tetrahymena is more closely related to mamalian phosphodiesterase rather than that found in microorganisms which normally is only a singular form of phosphodiesterase.

## II. Purpose

The main purpose of this present investigation was to measure the levels of cyclic nucleotides in relation to the division cycle in

synchronized Tetrahymena. All of the background information supported the idea that differences in amounts of these compounds might occur with alternating states of cell division and inhibition of cell division in synchronized Tetrahymena.

For these reasons, it was decided to use Tetrahymena pyriformis as a test organism to study the role of cyclic nucleotides in relation to radiation-induced division delay. Not only is it an ideal organism for both biochemical and cell-synchrony experiments, its high resistance to radiation damage allows it to be exposed to doses that ordinarily would kill mammalian cells. The present investigation was carried out using synchronized Tetrahymena in order to study the fluctuations of cAMP, cGMP and the cyclic nucleotide phosphodiesterases at various times correlating with the times of cell division in both normal and X-irradiated cells.

The main purpose of this investigation was an attempt to answer the following fundamental questions:

1. Is the onset of cell division in some way signaled by the changes in cGMP and/or cAMP levels. Are specific levels of these cyclic nucleotides closely coupled with the events leading to cell division?

2. Do the levels of the cyclic nucleotides change regardless of whether cells divide at regularly scheduled times after synchronization? If so, are there any concomitant changes in activity of the phosphodiesterases associated with specific cyclic nucleotides?

3. X-irradiation is known to delay cell division in synchronized Tetrahymena. Therefore, does irradiation alter levels or patterns of

cyclic nucleotides during the times associated with delays in the synchronized division cycle. If not, then what role could changing levels of these compounds play in cell division?

# CHAPTER II

## MATERIALS AND METHODS

### I. Culture Methods

The organism used in this study was the ciliated protozoan Tetrahymena pyriformis GL-C. This ciliate is an amicronucleated strain reaching a size of 40 by 60 μm when fully grown. Stock cultures were grown at 22° C in glass screw cap culture tubes, 25 ml capacity, containing 10 ml of autoclaved media containing 0.2% liver extract concentrate (Nutritional Biochemical Corp.), 2.0% proteose peptone #3 (Difco) and 0.5% dextrose (Merck) in glass-distilled water (Whitson, 1968). Cultures were aseptically transferred under ultraviolet light by sterile pipette every 48 hours to maintain cells in the logarithmic growth phase.

### II. Growth Studies

Determination of generation time was performed in the following manner. An aliquot of cells was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of growth media supplemented with penicillin, streptomycin and fungizone (GIBCO) at the following concentrations: 100 U/ml; 100 ug/ml and 2.5 ug/ml respectively. At various time intervals after inoculation, 1 ml of cells was aseptically removed and diluted to a final volume of 10 ml with 0.3% NaCl containing 1.0% formaldehyde in glass-distilled water, which was then counted using a Coulter Particle

Counter Model B equipped with 100 μm aperature to determine the cell numbers. Cell numbers were displayed on a log scale versus time and generation times were calculated using the following equation of Buchanan and Fulmer (1928):

$$\text{Generation time} = \frac{T \times \log 2}{\log (b/a)}$$

where T is the elapsed time between the final cell number "b" and the initial point "a" providing these points are on the straight line portion of the graph.

## III. Synchronization Procedure

Only cells from one day old cultures were used in the subsequent experiments. Approximately 1 ml of cells, $3 \times 10^4$ cells per milliliter, were transferred to 125 ml Erlenmeyer flasks containing 50 ml of growth media supplemented with antibiotics as described in Section II. Synchronization was obtained by the heat-shock method originally described by Scherbaum and Zeuthen (1954) which was modified so that seven 30 minute heat shocks of 33.9° C were alternated with six 30 minute cold shocks at 28.5° C. These were controlled by an automated synchronizing system consisting of two water baths regulated by electric timers connected to solenoid valves regulating the flow of water to a heat transfer apparatus containing the flasks of cell cultures. The cultures were allowed to grow at 28.5° C for a minimum of 14 hours and a maximum of 16 hours before the onset of the heat shocks.

## IV. Radiation Exposure

Synchronized cells were irradiated in 5 cm x 10 cm round glass dishes (Fisher) using either a General Electric Maxitron 300 or Maxitron 250 X-Ray machine. The depth of the media was 8 mm. The physical parameters of irradiation were 250 KvP, 20 mA with 0.13 mm copper as added filtration. The distance to target of the samples was 23 cm. Exposure time was determined using a Radocon dosimeter (Victoreen of Cleveland, Ohio), with dose rate corrected for temperature and pressure. The average corrected dose rate was 1019 R/minute with the midpoint of the irradiation occurring at the end of the last heat shock.

## V. Assay Procedure for Cyclic Nucleotides

The following procedures were used for all experiments in which cyclic nucleotides were assayed. Ten minutes before the first samples were removed, an aliquot of cells was counted to determine the volume of cell suspension needed to give $10^5$ cells. For every time point thereafter, the same volume of cells was used but a cell count was taken as previously described in Section II. The sampling times after the last heat shock were 50, 75, 100, 110, 120 and 130 minutes.

### Cyclic AMP

Cyclic adenosine 3', 5'-monophosphate was determined by the procedure of Gilman (1970) and obtained in kit form from Amersham/Searle. The assay as run, was a competitive protein-binding assay. If unlabeled cAMP is added to a known amount of $^3$H-cAMP which is bound to a

cAMP-dependent binding protein, the unlabeled cAMP replaces the $^{3}$H-cAMP in a linear fashion. Approximately $10^{5}$ cells were centrifuged in an IEC clinical centrifuge for four minutes at 1840 x g. After decanting, 5 ml of phosphate buffer ($KH_2PO_4$, 7.35 M; $Na_2HPO_4$, 7.04 M; $MgCl_2$, .32 M; and NaCl, 3.42 M/ml)pH 6.75 were added and the resulting suspension was recentrifuged. At the end of the centrifugation, the buffer was decanted and 0.5 ml of cold 6% TCA was added. Samples were mixed by vortexing and frozen in a dry ice-acetone bath and stored at -20° C. After thawing, cells were homogenized on ice using a teflon-glass Potter-Elvehjem homogenizer, centrifuged and the supernatant was retained. After three ether extractions with two volumes of ether, samples were concentrated to dryness in a 65° C water bath in a stream of air. The samples were reconstituted in 1.0 ml of 0.05 M Tris buffer pH 7.5 containing 4 mM EDTA (disodium salt). Fifty microliters of the reconstituted solution were used in the assay and added to duplicate tubes. Next 50 ul of 3H-labeled cAMP were added to each tube followed by the addition of 100 ul of the binding protein. After mixing each sample in a vortex mixer, samples were incubated for one hour in ice water. During the incubation period, Millipore filters (0.45 μm) were soaked in the Tris-EDTA buffer at 0° C. At the end of an hour 2.5 ml of the Tris-EDTA buffer were added to four tubes, vortexed and poured on a filter. To each of the four tubes, 5 ml of the buffer were added and then poured on the corresponding filter. Each filter was then washed in buffer, heat dried and counted in 10 ml of scintillation cocktail containing BBOT in toluene (4 g/l) using a Beckman LS230 liquid scintillation system. Levels of cAMP were determined from the standard curve.

Cyclic GMP

Samples for cyclic guanosine 3', 5'-monophosphate were prepared as those of cAMP; however, the dried homogenized samples were reconstituted in 1.0 ml of 0.05 M sodium acetate pH 6.2 and 200 ul were radio-immunoassayed according to the procedure of Steiner et al. (1972). The antiserum and label were purchased from Schwarz/Mann (Orangeburg, New York). The assay was performed as follows. To the 200 ul of the unknown cGMP, 100 ul of 125 I-labeled succinyl cyclic GMP tyrosine methyl ester and 100 ul of antiserum were added and each tube was vortexed. After covering, tubes were incubated at 4° C for 20 hours. After incubation. all tubes were placed in an ice water bath and 2.5 ml of 60% saturated ammonium sulfate were added. After vortexing, tubes were incubated for ten minutes. Tubes were then centrifuged for 20 minutes in an IEC centrifuge at 1240 x g at 0° C. The supernatant was discarded and any trace of the supernatant was removed with a cotton tipped applicator. Radioactivity was counted in an Intertechnique solid crystal gamma counter. Levels of cGMP were determined from the standard curve.

## VI. Phosphodiesterase Assay

After centrifuging the cells and decanting the buffer, 1.0 ml of fresh phosphate buffer (described above) was added to each sample instead of TCA. Tubes were then immersed in the dry ice-acetone bath and stored at -70° C. After thawing and homogenizing, total enzyme activity for either cAMP phosphodiesterase or cGMP phosphodiesterase was measured as described by Thompson and Appleman (1971). Samples were

processed in duplicate using the solutions listed in Table I. The assay procedure was as follows. Te each tube 0.1 ml of solution I was added. If the assay specified cAMP, then 0.1 ml of solution IIa was added. Similarly if cGMP was being assayed, then 0.1 ml of solution IIb was added to each tube. After the addition of 0.2 ml of solution III, the tubes were incubated at 30° C for 10 minutes, immersed in a dry ice-acetone bath for 12 seconds then placed in boiling water for 45 seconds to convert the cyclic nucleotide to the 5' nucleotide form. The ten minute incubation was a valid time period based on the work of Thompson and Appleman (1971). Using various contractions of substrate, typical Michaelis-Menten kinetics were observed for the hydrolysis of both cAMP and cGMP. After cooling to 4° C, 0.1 ml of solution IV was added to each tube and mixed. After incubation for ten minutes at 30° C which reduced the nucleotide to the nucleoside form, tubes were immersed in an ice bath to stop the reaction. To each tube, 1.0 ml of ion exchange resin pH 5 (Bio Rad Lab. AG1-X2, 200-400 mesh) was added and vortexed. After incubation for 15 minutes at 4° C, tubes were centrifuged for ten minutes at 4° C in an IEC centrifuge. One half milliliter of the supernatant was removed and added to 10 ml of scintillation fluid consisting of naphthalene, 125 g; PPO, 7.5 g; POPOP, 0.382 g in one liter of dioxane. Radioactivity was counted in a Beckman LS230 liquid scintillation system. Phosphodiesterase activity expressed in picomoles of cyclic nucleotide hydrolyzed in ten minutes was calculated from the following equation, converting counts per minute to disintergrations per minute (efficiency was about 16% for tritium):

Table I. Buffers used in the assay of phosphodiesterase.

| Solution | Components |
|---|---|
| I. | Tris-HCl 40 mM pH 8.0 containing 2-mercaptoethanol 3.75 mM. This was made daily |
| IIa. | 0.02 ml of 1M $MgCl_2$<br>0.87 ml of Solution I<br>3 uCi of (8-$^3$H cAMP (Schwarz/Mann) 20 Ci/mM in 0.01 ml. |
| IIb. | 0.02 ml of 1 M $MgCl_2$<br>0.97 ml of Solution I<br>3.68 uCi of (8-$^3$H) cGMP (Schwarz/Mann) 8.8 Ci/mM in 0.01 ml. |
| III. | Enzyme preparation in phosphate buffer at 4° C. |
| IV. | Ophiaphagus hannah snake venom 1 mg/ml in glass distilled water. |

$$\text{Activity} = \frac{\text{sample dpm - blank}}{\text{total dpm - blank}}$$

where $6.66 \times 10^6$ dpm = 0.15 picomoles of cAMP and $8.1696 \times 10^6$ dpm is equal to 0.418 picomoles of cGMP.

# CHAPTER III

# RESULTS

## I. General Results

### Generation Time from Logarithmic Phase Cultures

In order to obtain information on the normal cell cycle, generation times were determined at 28.5° C. The generation time of Tetrahymena pyriformis GL-C was computed from a representative study of three experiments and estimated to be 3.6 hours (Table II) when grown at 28.5° C. These results are shown in Figure 2. Data displayed in Table II show the results from all experiments giving the initial cell concentrations and final cell numbers per milliliter in addition to the calculated generation time for that particular culture. The generation times expressed were calculated from a semilogarithmic regression analysis performed on each experiment. The average generation time for all cultures was approximately 3.6 hours with a standard error of the mean calculated to be 0.009 hours.

### Synchronization of Cell Division After Heat Shocks

In order to determine when cell division began after the last heat shock, changes in cell numbers at various times were derived by electronic cell counting in both control and X-irradiated synchronized cultures. Results are shown in Figure 3 and indicate in control cultures, cell numbers begin to increase at 50 minutes after EHS (EHS + 50) with over 60% increase in cell numbers between EHS + 75 and EHS + 100, with another

Table II. Experimental results for calculation of generation time.

| Experiment | Initial cell number | Final cell number | Generation time |
|---|---|---|---|
| I. | 2240 | 108360 | 3.810 hrs. |
| II. | 1300 | 276680 | 3.546 hrs. |
| III. | 1980 | 330080 | 3.552 hrs. |
| Average ± S. E. | | | 3.630 ± 0.009 hrs. |

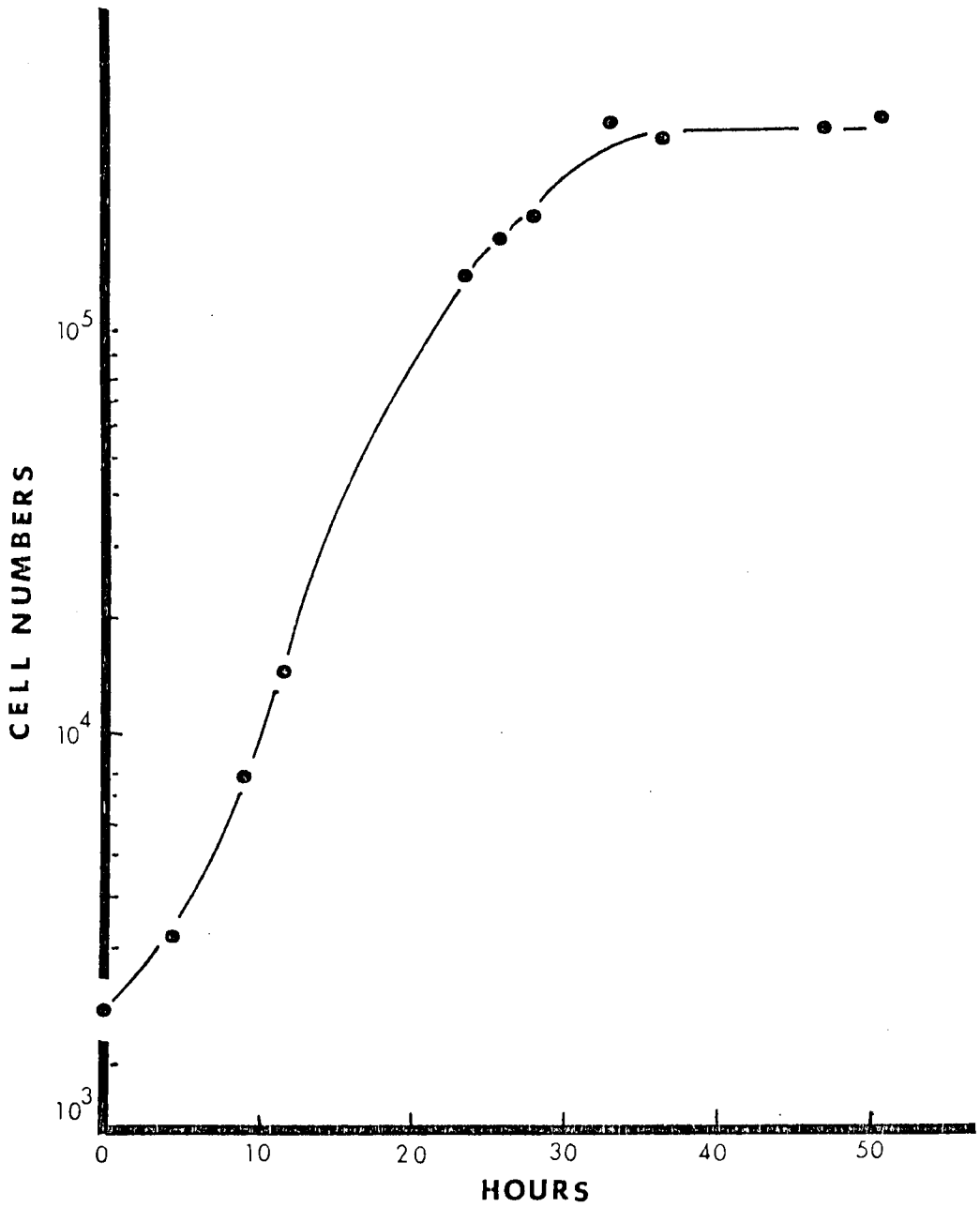



Figure 2. Representative growth curve of Tetrahymena pyriformis GL-C grown at 28.5° C.

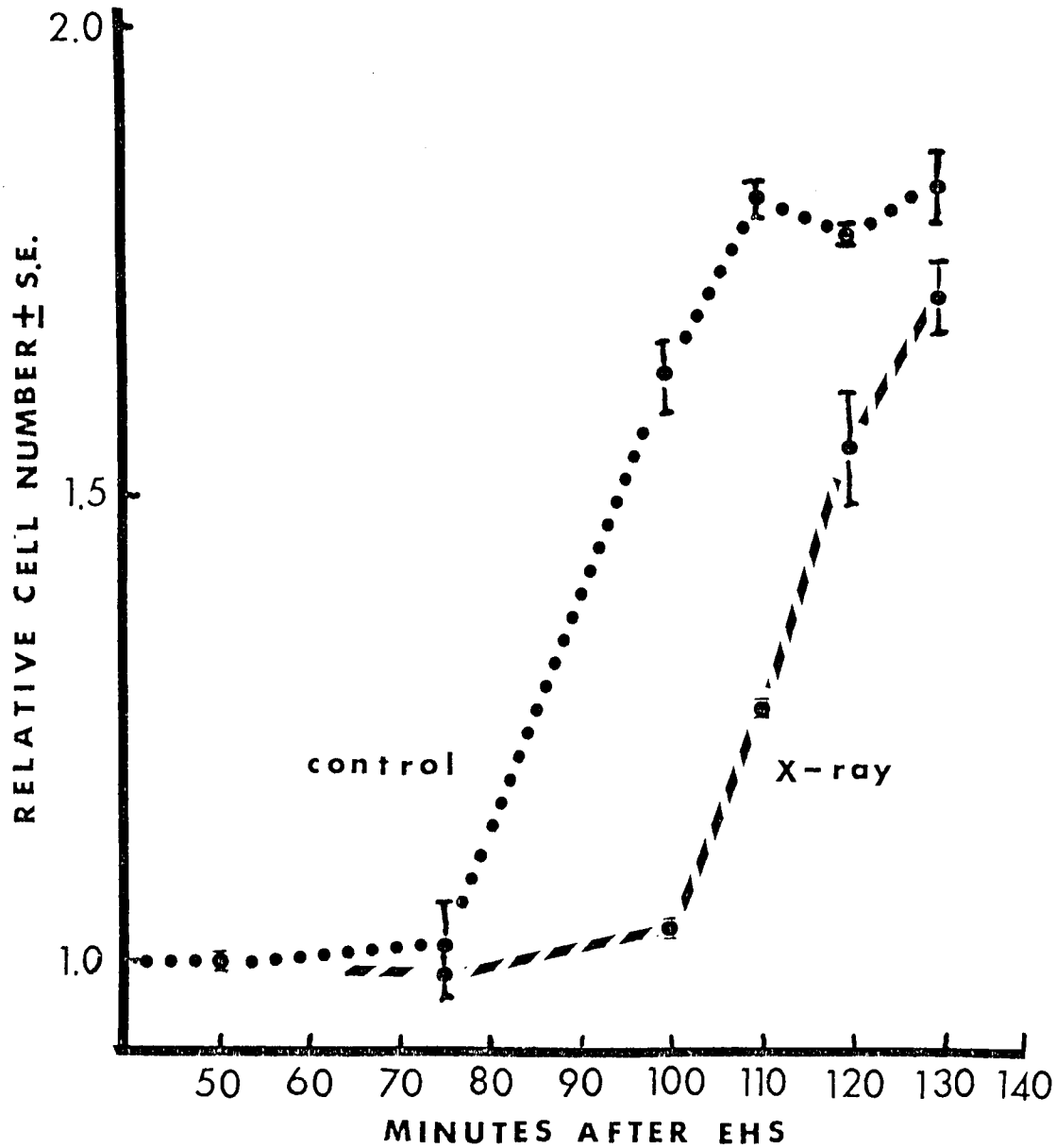



Figure 3. Synchronization of cell division after heat shocks in control and X-irradiated cultures.

11% increase in the following ten minutes (EHS + 110) at which time no further cell division occurs. On the other hand, irradiated cultures also grown at 28.5° C, showed an initial decrease in cell numbers. Cell numbers did increase 3% from EHS + 50 to EHS + 100. At that point there was a burst of cell division leading to an increase of 70% in cell numbers over that of EHS + 50.

X-ray-Induced Division Delay

After administration of X-rays, synchronized cultures were incubated at 28.5° C after EHS. Samples were removed by pipette every five minutes after EHS + 45 and fixed with two drops of Bouin's fixative. The percentage of cells in division was calculated by dividing the number of cells in division by the total number of cells. This number was plotted against time. Cells were classified as dividing if a division furrow was evident. A minimum of 200 cells from random microscopic fields were counted for each time point. Results are shown in Figure 4. The data as shown indicate that control cultures initiate cell division at about EHS + 60 and reach a peak of 81% synchrony 15 minutes later. The midpoint of the ascending and descending portions of the curves indicate that the majority of the cells divide within a span of 12 minutes. On the other hand, synchronous cultures which had been irradiated showed an X-ray induced division delay of about 35 minutes. That is to say, the onset of cell division began at about EHS + 90, reaching a peak of 51.5% synchrony 15 minutes later. However, the midpoint of the ascending and descending portions of the curves showed a 66.66% increase in time spent

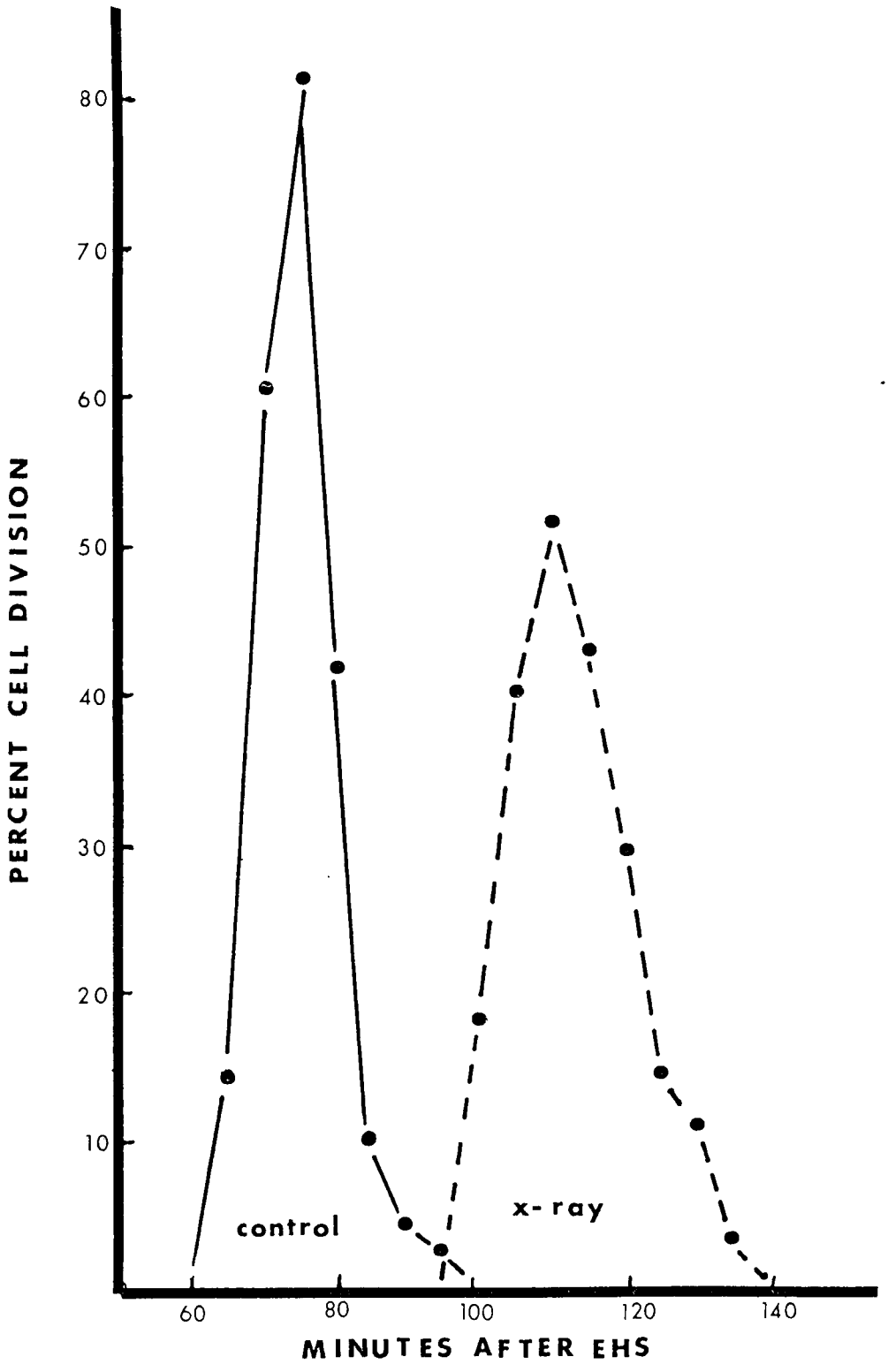



Figure 4. X-ray-induced division delay in heat-shock synchronized Tetrahymena pyriformis GL-C.

in cell division over that of the controls. A comparison of the results shown in Figure 3 and Figure 4 indicates that cell numbers began to increase about 15 minutes after cells began to divide and the large increase in cell number corresponds to the peak of cell synchrony, with the cessation of the increase in cell number occurring when the division index reached zero.

## II. Cyclic AMP Results

### Preparation of the cAMP Standard Curve

The standard curve was prepared by substituting 50 ul of known concentrations of cAMP in place of the reconstituted unknown samples. The assay was then performed in the identical manner as the unknowns as previously described in Chapter II, Section V. The results of the standard curve are shown in Table III, and plotted on a log-log scale as shown in Figure 5. The correlation coefficient for this plot was computed to be -0.998.

### Levels of cAMP in Syncronous Cultures

The levels of cyclic AMP were determined from a minimum of three experiments using control and irradiated cultures of Tetrahymena as previously described. Results in Table IV indicate that in control cultures, cAMP increased before the cell numbers increased (Figure 6). The rise in cAMP also corresponds to the peak of the division index. At the point where cell numbers begin to increase at an accelerated rate (EHS + 75), cyclic AMP levels begin to decrease. However, this

Table III. Amounts of $^{3}H$- cAMP used in computing the standard curve.

| Picomoles of $^{3}H$-cAMP | Average $^{3}H$ cpm ± S.E. |
|---|---|
| 0 | 29 ± 2 |
| 1 | 676 ± 33 |
| 2 | 472 ± 37 |
| 4 | 352 ± 43 |
| 8 | 264 ± 27 |

Correlation coefficient = -0.998

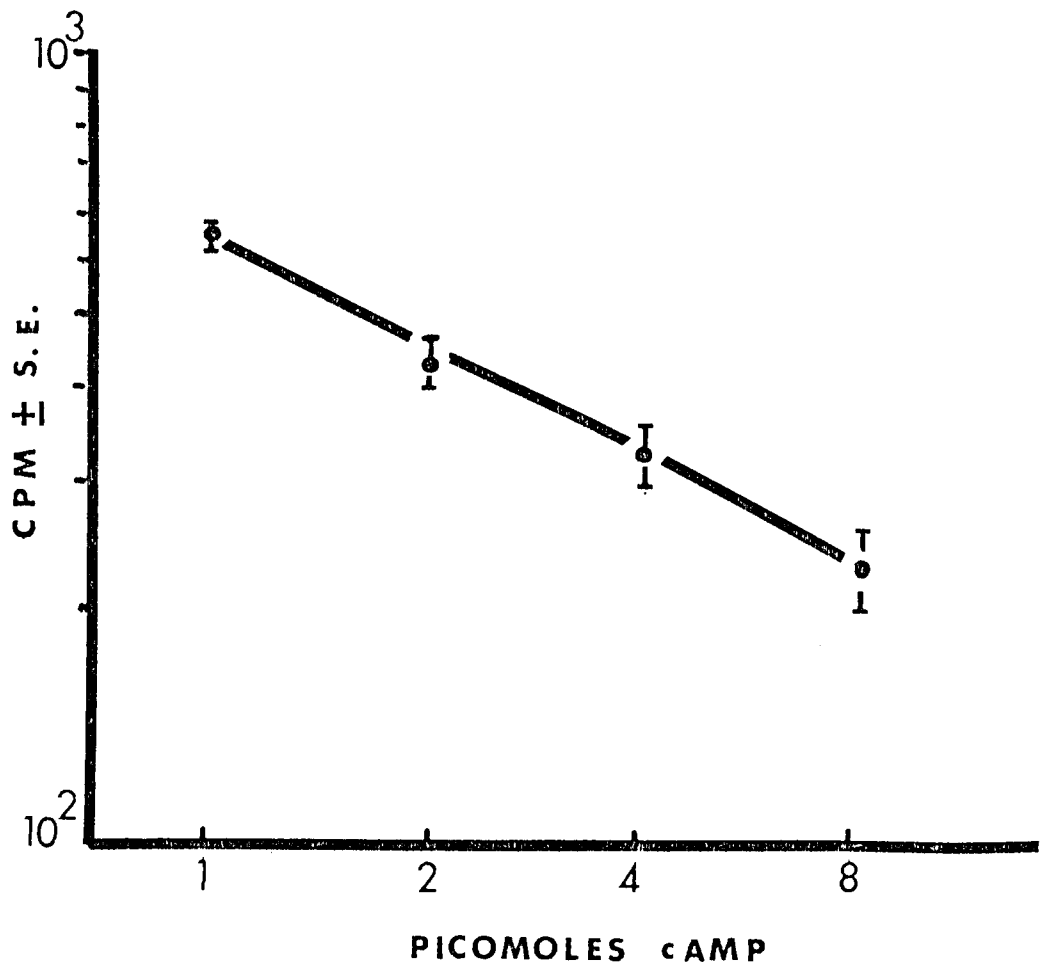



Figure 5. Cyclic AMP standard curve. The curve was prepared by substituting a known concentration of cAMP for sample unknowns. The assay was performed as described in Chapter II, Section V. Tritium counts represent that amount bound to the binding protein which did not pass through the filters.

Table IV. Levels of cAMP in experimental cultures.

| Minutes after EHS | pM/$10^6$ cells ± S.E. | |
|---|---|---|
| | Control | Irradiated |
| 50 | 4.274 ± .338 | 1.503 ± .23 |
| 75 | 4.597 ± .403 | 2.141 ± .2 |
| 100 | 3.426 ± .4 | 1.56 ± .23 |
| 110 | 2.95 ± .316 | 1.412 ± .13 |
| 120 | 2.276 ± .168 | 1.155 ± .26 |
| 130 | 2.339 ± .185 | 1.293 ± .28 |

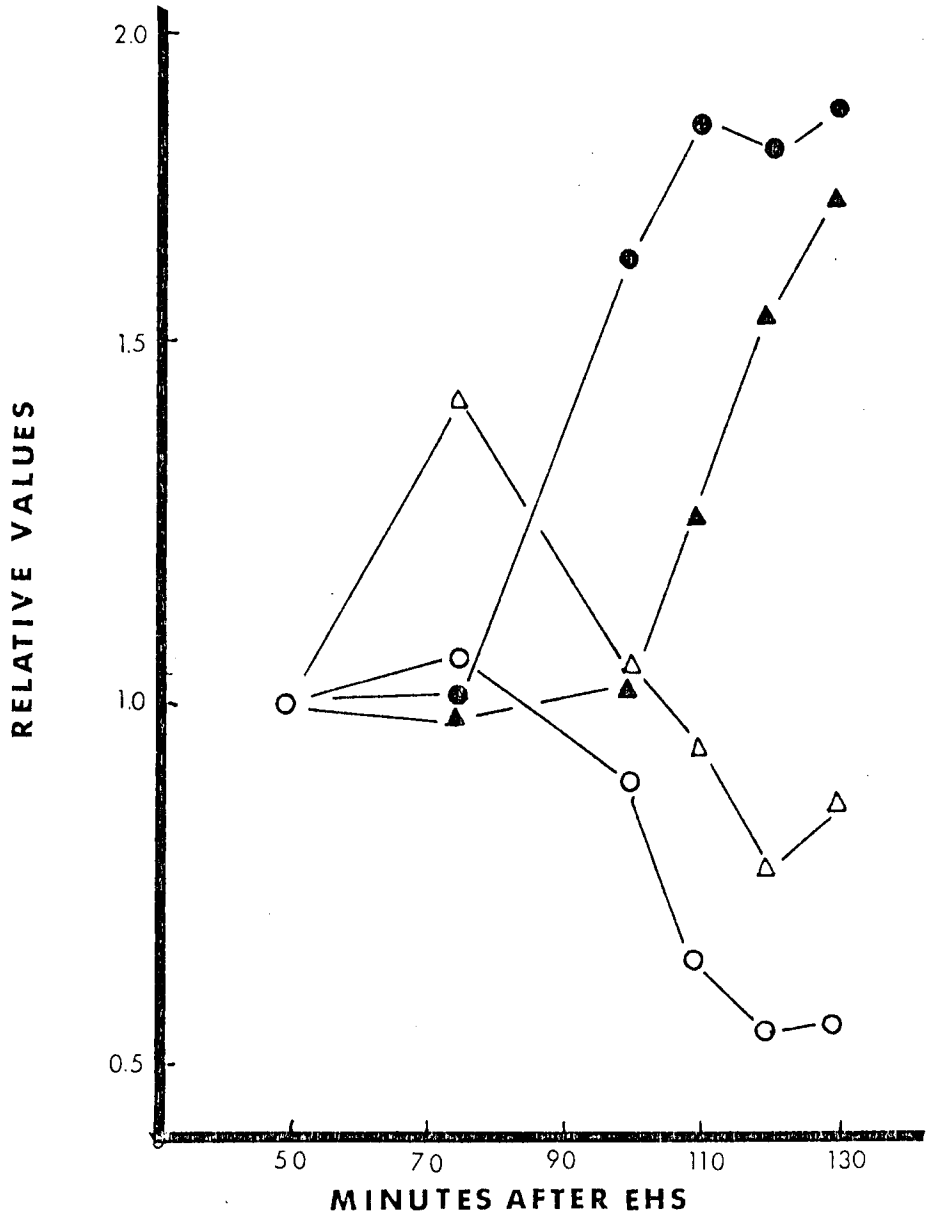



Figure 6. Relative changes in cAMP levels in control and irradiated synchronized cultures as compared to changes in cell numbers. (Control cultures cAMP—O—O—; control cell numbers—●—●—; irradiated cultures cAMP—Δ—Δ—; irradiated culture cell numbers—▲—▲—).

decreasing trend in cAMP reverses and then begins to rise when cells were not dividing. On the other hand, in irradiated synchronous cultures of Tetrahymena, the cyclic AMP levels showed a 42% increase from EHS + 50 to EHS + 75 at which time the levels began to decrease. It must be noted that even though the levels of cAMP were decreasing, neither the division index nor the cell numbers showed a change. The division index increases at about EHS + 90 and the increase in cell numbers occurs at about EHS + 100.

## III. Cyclic GMP Results

### Preparation of cGMP Standard Curve

The standard curve was determined by the substitution of known concentrations of cGMP in place of the unknown samples. The remaining protocol was as previously described in Chapter II, Section V. The results of the standard curve (See Table V) were plotted as percent antibody bound versus levels of cGMP on a log-log scale as shown in Figure 7. The correlation coefficient determined by logarithmic regression analysis was computed to be -0.971.

### Levels of cGMP in Synchronous Cultures

Cyclic GMP levels in heat shocked synchronized cultures of Tetrahymena were determined from a minimum of three experiments. Results shown in Table VI and Figure 8 indicate that control culture levels of cGMP showed an increase of over 100% between EHS + 50 and EHS + 75, the point where the division index was at its maximum and corresponding to

Table V. Amounts of $^{125}$I-cGMP used in the standard curve.

| Picomoles of $^{125}$I-cGMP | Average cpm | Percent bound |
|---|---|---|
| Trace[a] | 1787 | 100 |
| 0.5 | 1563 | 87.47 |
| 1.0 | 1314 | 73.53 |
| 2.0 | 1070 | 59.88 |
| 4.0 | 844 | 47.23 |
| 10.0 | 443 | 24.79 |

Correlation coefficient = -0.971

[a]Trace binding is binding in the absence of a standard.

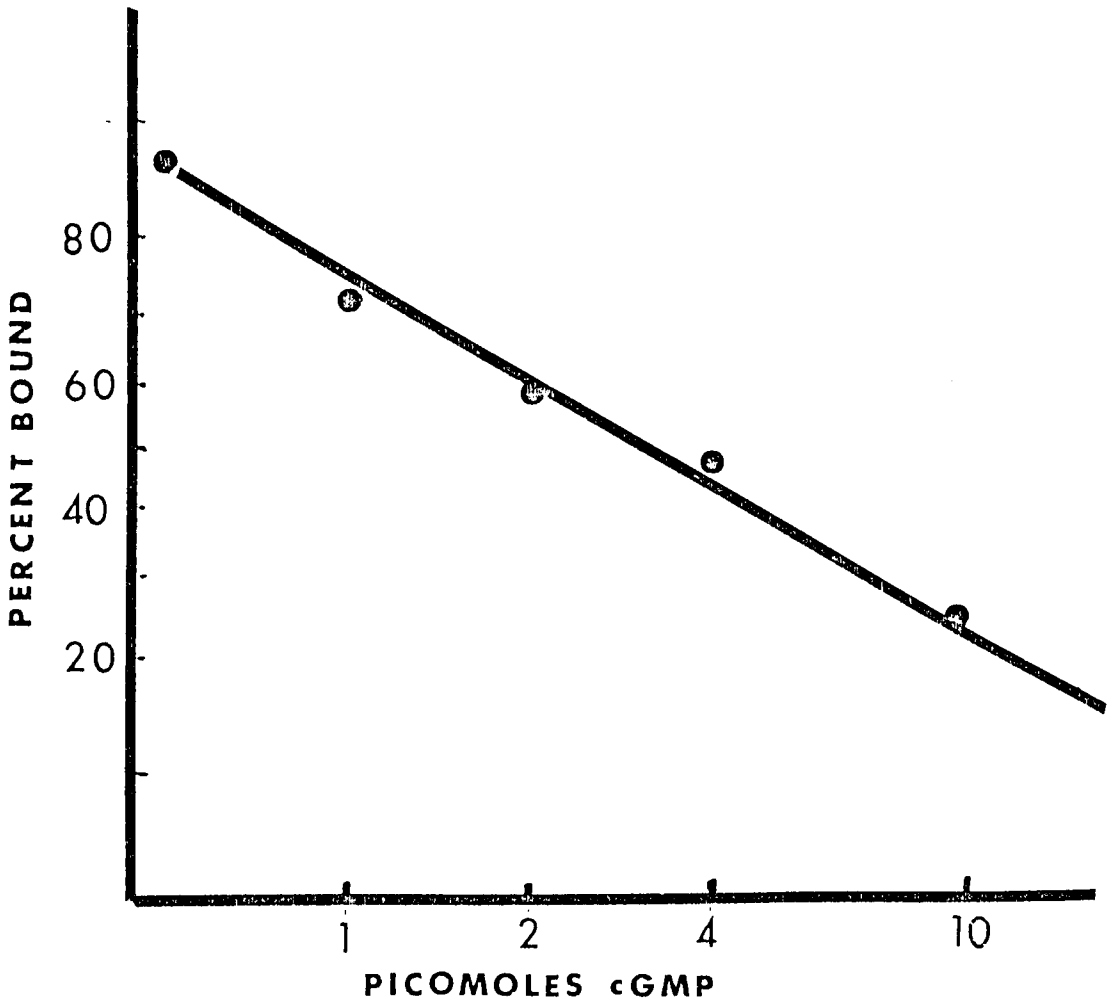



Figure 7. Cyclic GMP standard curve. The standard concentration of cGMP, 200 ul, were substituted for sample unknowns. The remaining protocol was performed as reported in Chapter II. Percent bound represents the amount of labeled antibody bound to the antigen.

Table VI. Levels of cGMP in experimental cultures.

| Minutes after EHS | pM/$10^6$ cells ± S.E. Control | Irradiated |
|---|---|---|
| 50 | 3.184 ± .483 | 10.92 ± 1.046 |
| 75 | 3.889 ± .27 | 5.089 ± .518 |
| 100 | 324.8 ± 16.57 | 7.397 ± .432 |
| 110 | 197.63 ± 1.97 | 173.4 ± 25.981 |
| 120 | 225.7 ± 39.202 | 952.8 ± 23.902 |
| 130 | 184.2 ± 2.343 | 1932 ± 165.7 |

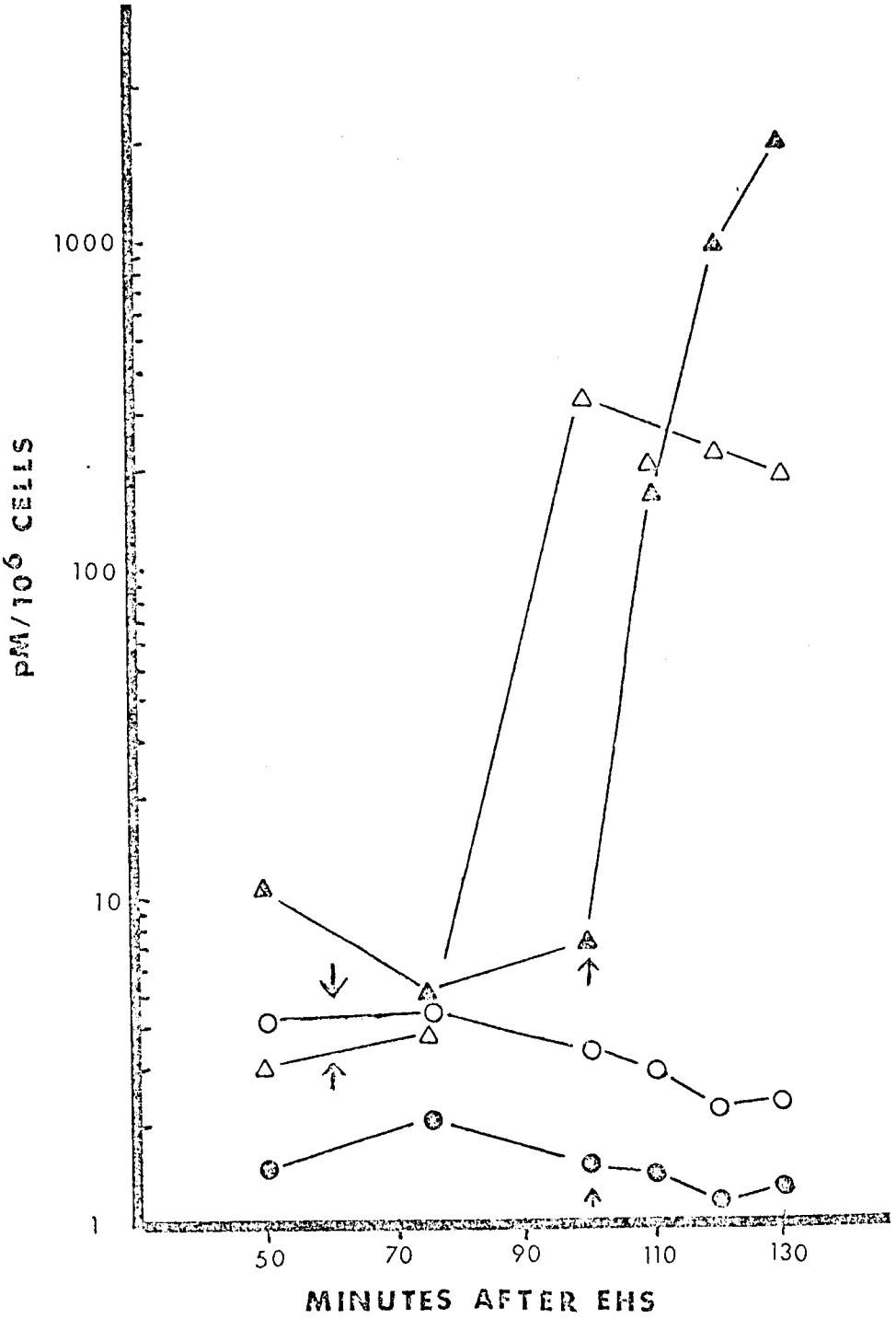



Figure 8. Absolute amounts of cAMP and cGMP in control culture and irradiated cultures. Arrows at EHS + 60 indicate onset of cell division in control cultures. Arrows at EHS + 100 indicate onset of cell division in irradiated cultures. Control cAMP—0—0—, irradiated cAMP—●—●—, control cGMP—Δ--Δ—, irradiated cGMP—▲—▲—.

the time when cell numbers began to rapidly increase. After EHS + 75, the cGMP levels decreased to a value of approximately 70% above the predivision state of the Tetrahymena where the levels appear to level off or slightly decrease.

Examination of cell cultures receiving 5000 R of ionizing radiation indicated that there was a decrease in cGMP until EHS + 75 (Figure 8), at which time cGMP levels began to increase. At EHS + 100, the point at which cell division was beginning in irradiated cultures, there was a rise in cGMP levels. From EHS + 100 to EHS + 110, when irradiated cultures showed the maximum value in division index, relative levels of cGMP increased over 15 times and continued to increase even after the division index dropped to zero at EHS + 130.

## IV. Phosphodiesterase Studies

Experiments were performed to measure the enzyme activity of cyclic nucleotide phosphodiesterase (PDase) in a crude homogenate to determine if there was a change in enzyme activity to account for the fluctuations observed in the levels of cyclic nucleotides in control and irradiated cultures of synchronized Tetrahymena pyriformis. Assays for PDase were carried out as described by Thompson and Appleman (1971) and outlined in Materials and Methods. All results are expressed as relative levels to the activity present at EHS + 50 and are displayed with its associated cyclic nucleotide levels.

Control Levels of cGMP PDase in Tetrahymena

Phosphodiesterase results were based on a minimum of three experiments. The results shown in Table VII and Figure 9 indicate that from EHS + 50 to EHS + 75, there was an initial increase in PDase activity of 16% which corresponds to a slight rise in cAMP levels and the peak of synchronous cell division. At EHS + 100, when the division index reached zero, PDase activity had decreased by 67% of that measured at EHS + 50. During this same period, cAMP levels had decreased by 11%. From EHS + 100 to EHS + 120, the PDase levels return to the predivision activity and levels of cAMP showed a decrease of 47%.

Cyclic AMP PDase Activity in X-irradiated Cultures

Results, based on at least three experiments, are shown in Table VII and Figure 10. The activity of Pdase showed a 37% increase in activity over the time period from EHS + 50 to EHS + 75 which corresponds to the peak of the division index in unirradiated cultures. However, at EHS + 100, when irradiated cells began to divide, the PDase activity had increased another 28% from the activity present at EHS + 75; whereas, the cAMP levels have dropped by 27% during the 25 minutes prior to cell division. At the peak of division synchrony in irradiated cell cultures the PDase activity decreased by 16% during the time from EHS + 100 while cAMP levels dropped approximately 10% during this same time. When the division index approached zero at EHS + 130, the PDase activity decreased by 9% from that as EHS + 50 and cAMP levels showed a net decrease of 14% from the EHS + 50 levels.

Table VII. Picomoles of $^{3}$H-cAMP hydrolyzed/$10^{6}$ cells.

| Minutes after EHS | Control[a] | X-ray[a] |
|---|---|---|
| 50 | 38.78 ± | 15.14 |
| 75 | 44.98 ± .77 | 20.74 ± 1.36 |
| 100 | 12.8 ± 3.1 | 52.99 ± 8.48 |
| 110 | 28.31 ± 1.55 | 44.21 ± 3.03 |
| 120 | 38.39 ± .39 | 11.05 ± 1.97 |
| 130 | 29.86 ± 2.71 | 13.78 ± .15 |

[a]Levels are expressed as picomoles ± S.E. hydrolyzed in ten minutes per million cells.

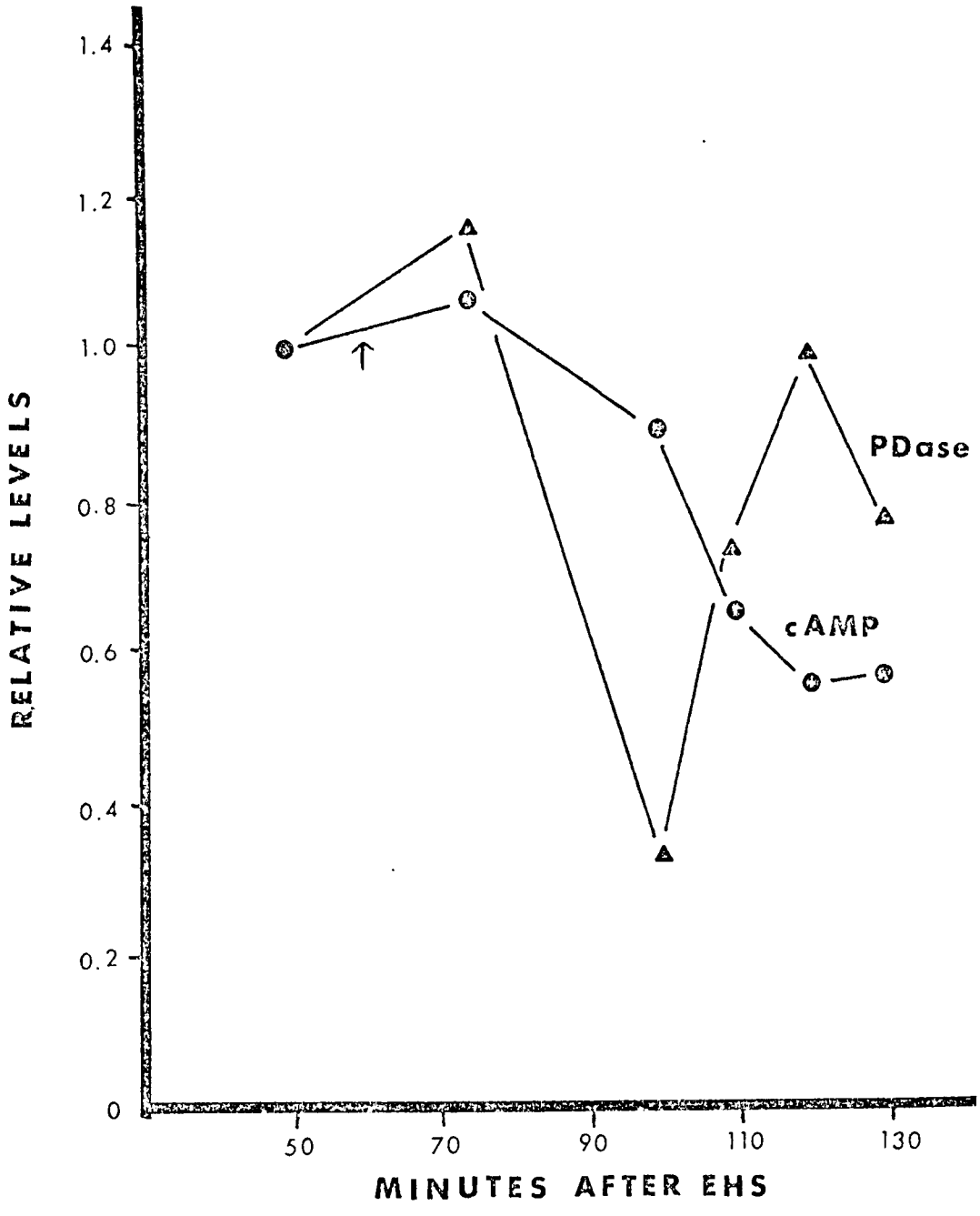



Figure 9. Relationship of cAMP and cAMP phosphodiesterase in control cultures of synchronized Tetrahymena. The PDase activity at EHS + 50 is equal to 38.78 ± 2.17 pM/$10^6$ cells. Activity is expressed as moles hydrolyzed in ten minutes. (Arrows indicate the onset of cell division.)

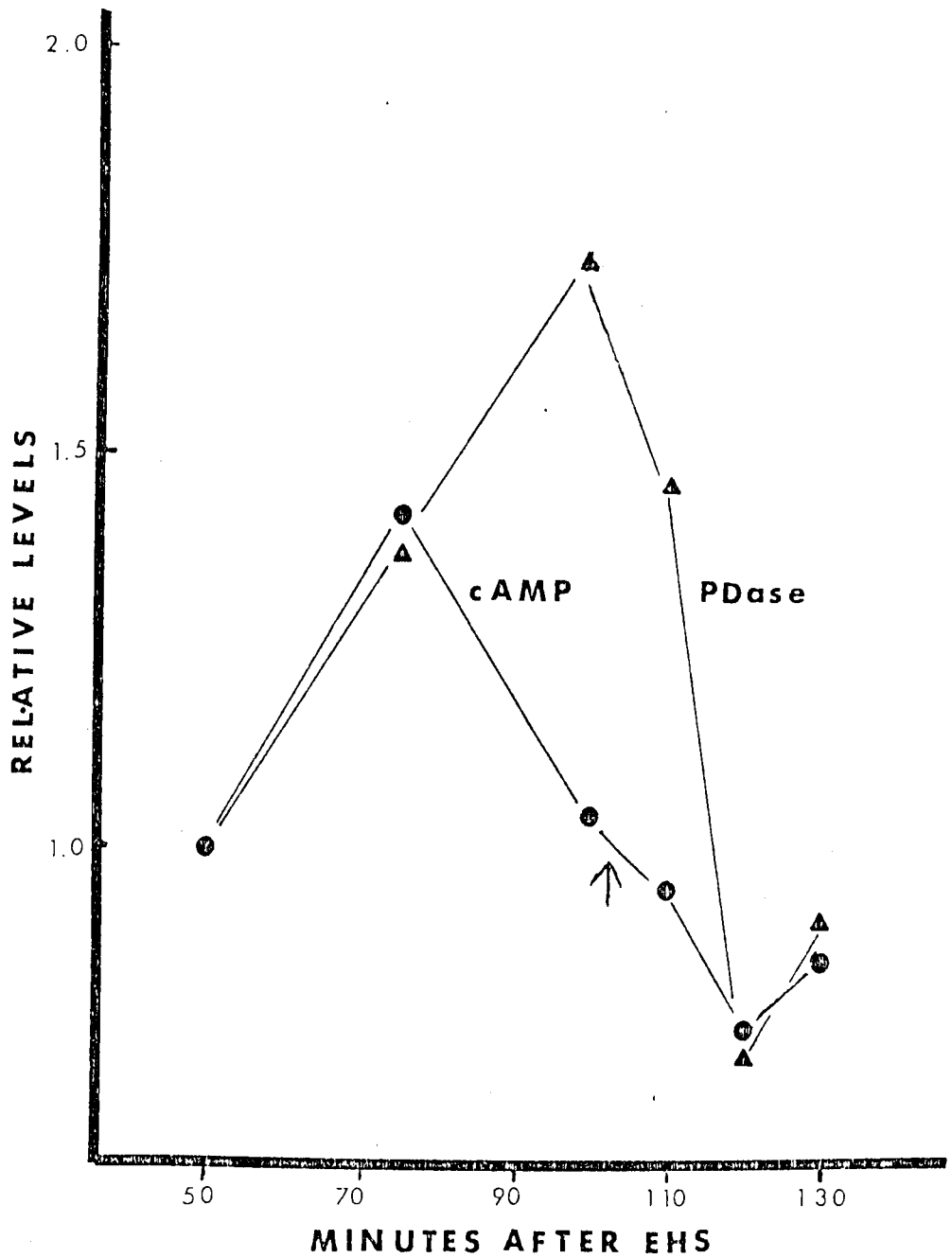



Figure 10. Relative levels of cAMP and cAMP phosphodiesterase in irradiated cultures of synchronized Tetrahymena. The PDase activity at EHS + 50 is equal to 15.14 ± 5.16 pM hydrolyzed in ten minutes per million cells. (Arrows indicate the onset of cell division.)

Activity of cGMP PDase in Control Cultures

Results were based on at least three experiments are shown in Table VIII and Figure 11. The activity of PDase decreased by 27% from EHS + 50 to EHS + 75 corresponding to the peak of cell division in unirradiated controls. During this same time period cGMP levels increased 21%. When the cell division index reached zero at EHS + 100, the PDase activity had decreased by 54% while the cGMP level showed an increase of approximately 100% that of predivision levels. From the time EHS + 100 to EHS + 130, the activity of PDase remained relatively constant, approximately 45% less than the EHS + 50 levels; however, the levels of cGMP during this same elapsed time decrease over 43%.

Cyclic GMP PDase Activity in X-irradiated Cultures of Tetrahymena

Results, based on a minimum of three separate experiments, are shown in Table VIII and Figure 12. At EHS + 100, five minutes before the peak of the division index in the irradiated cultures, the PDase activity showed an increase of 48% over that seen at EHS + 50. Likewise, the levels of cGMP decreased by 32%. At EHS + 110, after the peak of the division index has reached a maximum, PDase began to decrease and the cGMP levels commenced to rise. When the division index approached zero at EHS + 130, the PDase activity plateaued showing approximately a 38% increase in activity present at EHS + 50 and the cGMP levels increased over 175% of the amount present at EHS + 50.

Table VIII. Picomoles of $^{3}$H-cGMP hydrolyzed/$10^{6}$ cells.

| Minutes after EHS | Control[a] | X-ray[a] |
|---|---|---|
| 50 | 6.63 | 8.89 |
| 75 | 4.48 ± .28 | 12.45 ± .62 |
| 100 | 3.05 ± .2 | 13.15 ± .44 |
| 110 | 2.78 ± .13 | 15.38 ± .71 |
| 120 | 3.25 ± .13 | 12.09 ± .8 |
| 130 | 2.65 ± .07 | 12.53 ± .8 |

[a]Levels are expressed as picomoles ± S.E. hydrolyzed in ten minutes per million cells.

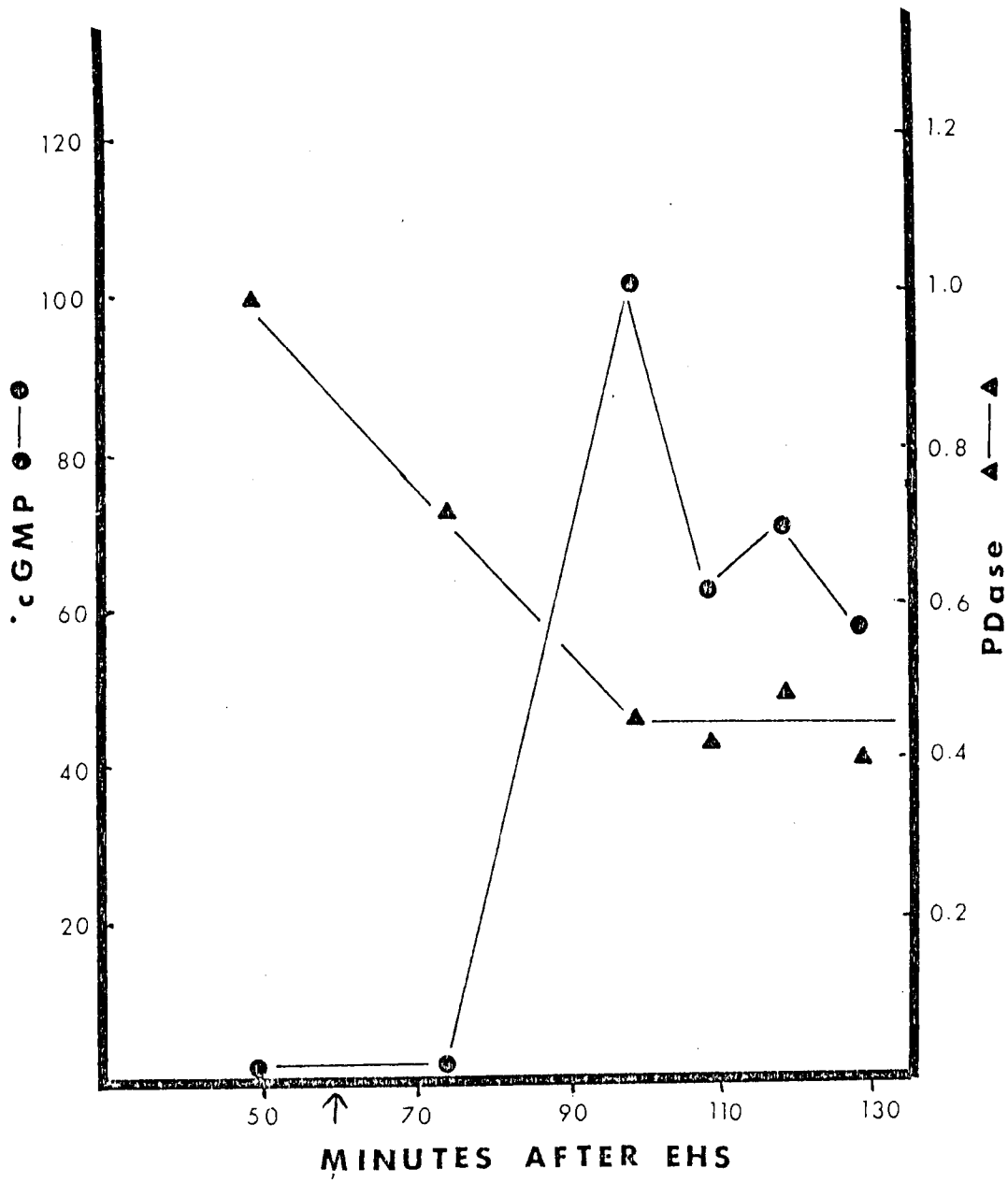
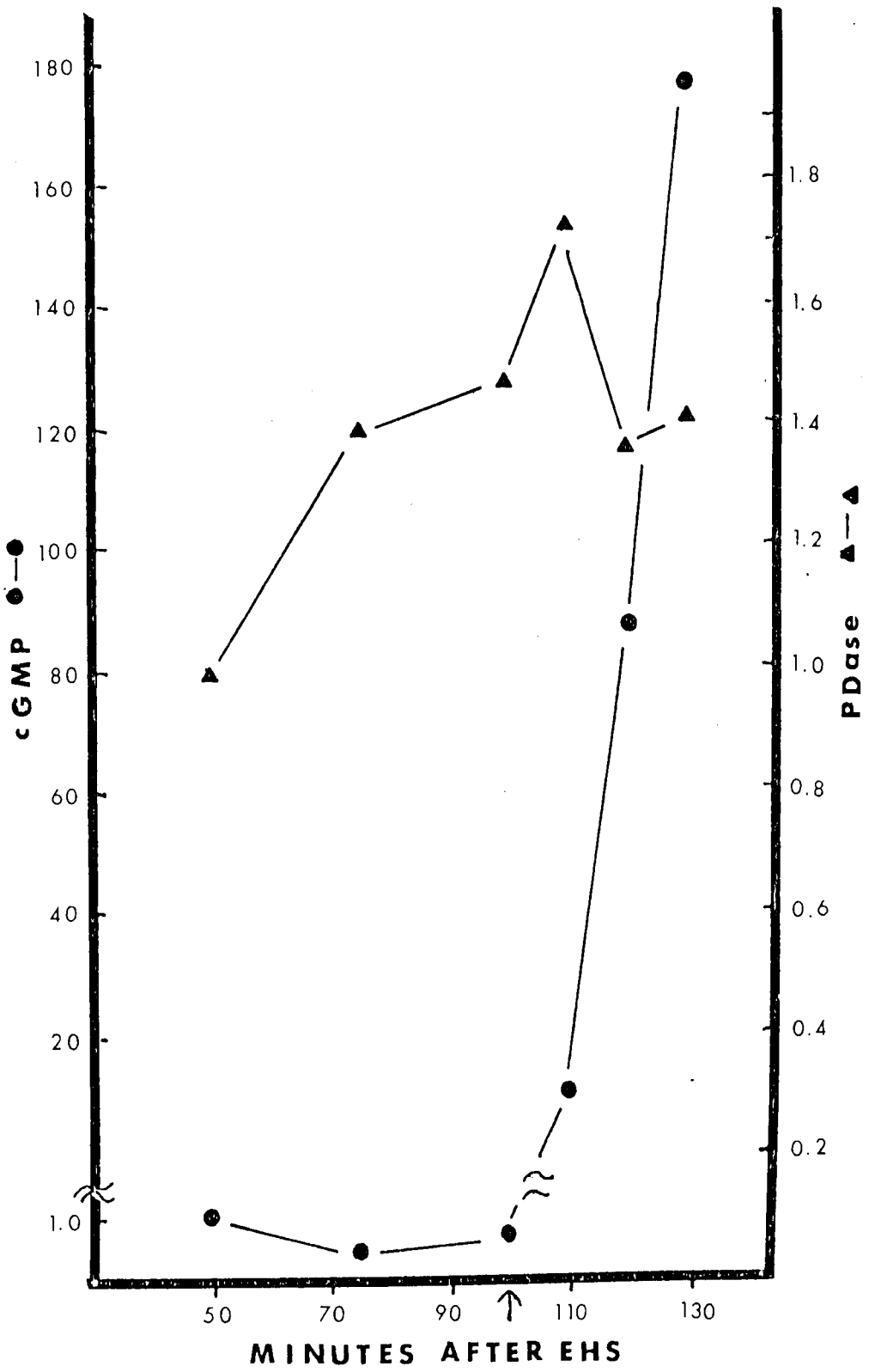



Figure 11. Changes in cGMP and cGMP phosphodiesterase in synchronized cultures of Tetrahymena. The PDase activity at EHS + 50 is equal to 6.63 ± 1.32 hydrolyzed in ten minutes per million cells. (Arrows indicate the onset of cell division.)




(EHS + 50 value is equal to 8.89 ± .39 pM/$10^6$ cells).

Figure 12. Relative changes in the levels of cGMP and cGMP phosphodiesterase in irradiated synchronized cultures of Tetrahymena. (Arrows indicate the onset of cell division.)

# CHAPTER IV

## DISCUSSION

This investigation was pursued in order to gain a clearer understanding of the division process in heat-shocked synchronized Tetrahymena pyriformis GL-C by measuring the levels of both cAMP and cGMP in relation to normal synchronized cell division and to radiation-induced delays in cell division.

Experiments in this present investigation indicated that in radiation-induced division-delay although the amount of ionizing radiation was 1% of the calculated $LD_{50}$ for this organism, that there was a definite increase in time before initiation of the first synchronous division with an associated decrease in the percent of synchrony. Frankel (1964) stated that before cell division occurs in Tetrahymena, oral morphogenesis must be completed. Therefore, the possibility exists that 5 Kr interferes with some morphological steps necessary for the formation of the oral apparatus (Hodge and Nachtway, 1972). In order for X-rays to produce such a morphological effect, one or perhaps both of two processes could occur. Either the X-rays are absorbed directly by certain molecules involved in oral morphogenesis such as mRNA as suggested by Paul and Zimmerman (1973) or the X-rays could be absorbed by cysteine-rich proteins which seemingly play a role in cell division (Clawson, 1974). For instance, sulfydryls are known to act as free radical scavengers and proteins rich in sulfhydryl content could thus be altered by ionizing radiation (Okada, 1970). Once synthesis of mRNA associated with cell

division has occurred it would seem unlikely that direct damage to the DNA molecule itself would lead to a division delay. While X-rays are nonselective in targets producing damage, DNA and RNA are more sensitive than proteins (Dertinger and Jung, 1970).

In studies of radiation-induced division delay, it has been suggested that Tetrahymena is radioresistant because it is a highly polyploid organism with multiple copies of its genome (Whitson, 1972). Tetrahymena DNA demaged by low doses of radiation can be repaired quickly and efficiently (Whitson, 1968). Paul and Zimmerman (1973) proposed that X-rays depress the synthesis of mRNA and the necessary synthesis of protein involved in cell division. Moreover, Yatvin and coworkers (1976) found that in irradiated Tetrahymena, polysomes were dissociated and that there was a subsequent decrease in amino acid incorporation. On the other hand, the formation of radiation-induced by-products appears not to be important to cellular damage, because Roth and Buccino (1965) showed that catalase rapidly destroys hydrogen peroxide and offers radioprotection to cells exposed to ionizing radiation up to 500 Kr.

Some researchers observed that cell division is indirectly related to fluctuating levels of cAMP (Abell and Monahan, 1973; MacManus and Whitfield, 1971) and cGMP (Illiana et al., 1973; Hadden et al., 1972). Studies on cyclic AMP indicated that both control and irradiated cultures of Tetrahymena showed similar patterns in the levels of cAMP. Cyclic AMP increased in synchronized cells up to a time of EHS + 75 after which levels of this cyclic nucleotide began to decrease in both dividing cells

and radiation-induced delayed cells. The rise in cAMP levels until EHS + 75 agrees with the results of Dickinson and coworkers (1976) using a different method of synchrony. They found that cAMP levels are the highest prior to the onset of cell division in Tetrahymena and drops at the peak of the division index in the unirradiated controls and drops markedly during cell division. However, since cells which were irradiated and not dividing showed a greater increase in the levels of cAMP, it seems unlikely that the drop in cAMP was in some way the signal for cell division. This does not rule out that the decrease in cAMP levels at this point is essential in some process closely associated with development of the oral apparatus.

Data on the morphogenetic influence of cAMP in primitive eucaryotes are sparce and conflicting. For instance, it was shown by Nestle and Sussman (1972) that high levels of cAMP blocked morphological development in the slime mold Dictyostelium. In this study it was reported that externally applied cAMP interfered with the normal formation of fruiting bodies thus preventing reproduction in this organism. On the other hand, Bonner (1970) showed that cAMP was the substance responsible for cell aggregation in this slime mold.

Abell and Monahan (1973) in a review on the role of cAMP in mamalian cell division showed that a high level of cAMP lead to an inhibition of cell division in certain mammalian cell lines, both normal and transformed. The possible role of cAMP in cell division in mammalian cells has been the subject of many investigations. For instance, Sheppard (1972) showed that contact-inhibited cells in which no further

division occurred had higher levels of cAMP than transformed cells. Otten and coworkers (1972) showed that transformed cells could revert to characteristics of nontransformed cells with the addition of dibutyryl cAMP, an analog of cAMP. Also, it was shown by Froehlich and Rachmeler (1974) that in contact-inhibited cultures of human fibroblasts, cell division could be induced by treatment of the cultures with proteases and this stimulation of cell division was accompanied by a decrease in cAMP levels.

My results with synchronized Tetrahymena indicate an immediate drop in cAMP might not signal cell division, studies on the changes in cGMP levels indicated a more direct relationship (Figure 8, page 34). Results on changing levels of cGMP indicated that at the onset of cell division, there is a marked rise in the intracellular amounts of this cyclic nucleotide. This increase was only observed in control as well as irradiated cultures when cells began to divide. It seems to me, therefore, that the rise in cGMP levels and not the initial rise followed by a sudden decrease in cAMP levels which signals the onset of cell division. This change in cGMP thus acts as a cell division indicator and/or a mitotic stimulator. This kind of result is in agreement with studies by Hadden and coworkers (1972) on lymphocyte mitogens in which they observed a great increase in intracellular levels of cGMP after treatment with the mitogens. On the other hand, intracellular levels of cAMP showed little if any change after treatment. Further support of these results comes from work by Goldberg (1975) in which he stated that as a stimulator of cellular processes, cGMP had a more positive role

whereas increases in cAMP appeared to be antogonistic. He thus called it the yin-yang hypothesis (Goldberg et al., 1975).

The findings that cAMP levels decreased at the same times after EHS in both control and irradiated cultures while cGMP levels increased only at the onset of cell division indicated that alterations in cyclic nucleotide phosphodiesterase might play a role in affecting the cyclic nucleotide levels (Figure 8, page 34). Studies on the cAMP dependent PDase in control cultures indicated that the total activity of PDase began to decrease at EHS + 75 and this decrease halted at EHS + 100. It was also noted that cAMP levels decreased during this time period. These findings are not in agreement with those of Voichick and coworkers (1973). Using unsynchronized Tetrahymena, they observed that PDase levels were inversely related to levels of cAMP. However, in a mammalian transformed cell line, d'Armiento and coworkers (1972) described a system which had low levels of cAMP as well as low levels of PDase activity. These researchers also stated the PDase present in which cells had distinct differences in its affinity for cAMP. However, none of these results can explain the data obtained in the present study.

In synchronized Tetrahymena, there may be one or several reasonable explanations for the relationship of decreased activity in PDase which accompanies the drop in cAMP levels. Ramanathan and Chou (1973) described three distinct phosphodiesterase fractions in Tetrahymena, two of which hydrolyzed cAMP and each having a different affinity for the substrate. Hardeman (1971) reported that when phosphodiesterase isolated form mammalian sources was added to preparations of cAMP and cGMP, the cGMP

was hydrolyzed at a much faster rate than cAMP indicating the enzyme had a better affinity for cGMP than cAMP thus lowering the rate of cAMP hydrolysis. A lowering of the affinity of the enzyme for cAMP may also explain the drop in cAMP PDase activity in Tetrahymena, as well as a two enzyme system. At the time the cAMP PDase activity begins to drop, cGMP levels are beginning to increase. It may be the raising of the substrate concentration of cGMP which forces competitive inhibition of the enzyme at the catalytic site for hydrolysis of cAMP. It was also noted that PDase activity in cAMP experiments began to rise after the end of division in control cultures which was the time when cGMP levels began to drop. This drop in cGMP levels could be sufficient to raise the affinity of the enzyme for cAMP and increase the hydrolysis rate of this cyclic nucleotide.

Nonetheless, these theories do not explain the decrease in PDase activity associated with cAMP in the irradiated cultures. In this case, the enzyme activity decreased as the cAMP levels decreased. It is possible that as the cells progress toward synchronous division, allosteric regulation for each possible fraction of Tetrahymena PDase decreases the activity by lowering the Vmax of the reaction. It is also possible that the organism releases the enzyme into the media and thus lowering the intracellular activity. This phenomenon of PDase release into the media is seen in the slime mold Physarum as reported by Murray and coworkers (1971). Their reasoning was that enqyme release acted as a protective mechanism to allow for cell growth. On the other hand, it was possible the PDase activity in synchronized X-irradiated Tetrahymena was

low because the cAMP levels were low due to cAMP leakage across the cell membrane by some sort of transport process, as seen in other cell lines (Kelly et al., 1975). If this were the case, PDase activity would be lower since low substrate concentration would lower enzyme activity. Results on cGMP phosphodiesterase showed that in both control and irradiated cultures, the activity of PDase did not decrease until the cells entered division. The drop in PDase activity was also followed by a marked rise in the levels of cGMP. In the irradiated cultures, there was a burst of PDase activity when the first signs of division were seen. This was not seen in the control cultures. The explanation for this short rise in the cGMP PDase activity in the irradiated cultures relates to the possibilities that the cells have overcome the radiation-induced division delay and that the cells were then gearing up for cell division.

Results obtained only answer certain questions raised at the end of Chapter I only in irradiated nondividing cells. The onset of cell division in heat-shocked synchronized Tetrahymena pyriformis GL-C is signaled by a decrease in the levels of cAMP and by a rapid increase in the levels of cGMP in unirradiated cultures. However, in irradiated synchronized cells there was an increase in the levels of cAMP which began to decrease sometime before the initiation of cell division. This change is not explainable at this time other than that it may indicate that a decrease beyond a certain crucial level is required to initiate or signal the onset of cell division. The phosphodiesterase activity associated with the cyclic nucleotides appears to be functioning in a normal manner.

The present investigation attempted to answer some questions regarding cell division in Tetrahymena, especially the relationship of fluctuating levels of cAMP and cGMP. It was interesting to note that cAMP levels in control cultures decreased 2-fold during synchronized cell division. In the irradiated cultures, there was also a 2-fold decrease in the levels of cAMP. However, this drop did not coincide with cell division in the irradiated cultures. On the other hand, there was a 100-fold increase in cGMP levels during the process of cell division in the unirradiated cultures; whereas, in irradiated cultures, cGMP levels showed approximately a 500-fold increase over that seen in control nondividing cells.

Present studies of cAMP concur with current concepts in that dividing cells have lower levels of cAMP than nondividing cells. This study also concurs with the present knowledge that cGMP levels increase in dividing cells. At this time, one cannot say that the rise of cGMP signals the onset of cell division. However, if these opposing changes are related, it may indicate a common underlying mechanism controlling cell division in all types of eucaryotic cells.

# CHAPTER V

## SUMMARY

1. The content of cAMP in control cultures of synchronized Tetrahymena decreased 2-fold during the process of division (EHS + 75) and reached its lowest levels after cells had stopped dividing. Irradiated cultures which showed a delay in cell division of approximately 35 minutes longer than in control cultures, also showed a decrease in the level of cAMP at EHS + 75. However, at this time there was no cell division or increase in cell number in these cells.

2. The content of cGMP increased over 100-fold during the process of division in control cells beginning at EHS + 75 reaching a peak at EHS + 100. Increases in the levels of cGMP did not occur in irradiated cultures until cell division began at EHS + 100. At this time cGMP levels increased over 500 times that of the amounts seen in nondividing control cultures.

3. Assays for cyclic nucleotide phosphodiesterase indicated that the activity of this enzyme was not significantly altered by radiation. Cyclic AMP PDase activity decreased at EHS + 75 in control cultures (at the peak of cell division); whereas, in irradiated cultures, cAMP PDase began to decrease when cells were not dividing. Cyclic GMP PDase activity in control cultures decreased at EHS + 75 when cells began to divide. In irradiated cultures, the enzyme activity decreased when cells began to divide at EHS + 100.

4. All of these results indicate that the marked increase in intracellular levels of cGMP during cell division appears to be a stimulus or a signal for the onset of cell division. It is unclear at this time what molecular mechanism underlies this process. Not unlike other cell systems, cAMP levels also drop during cell division in Tetrahymena but the drop in cAMP is less than the corresponding rise in cGMP. It is not known at this time what alteration in cell function lead to the decrease in cAMP during cell division.

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## LIST OF REFERENCES


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# VITA

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