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DAVID H. WORTHINGTON for the DOCTOR OF PHILOSOPHY
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Title: HYDROXYUREA: PHYSIOLOGICAL EFFECTS ON
TETRAHYMENA PYRIFORMIS AND USE IN CELL CYCLE
ANALYSIS

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D. S. ~~Na~~htwey

Hydroxyurea (10mM) blocks the exponential growth of Tetrahymena pyriformis (GL-I) populations by arresting progress through the cell cycle once the cells enter S-phase. Autoradiographic analysis reveals that HU reduces the uptake of tritiated thymidine ($^3\text{H-TdR}$) into macronuclear DNA in S-phase cells to about 1/5 of that in untreated S-phase cells. These slowly synthesizing cells do not divide (even after 10 days) until the HU is removed. The HU-blocked cells are also rescued from S-phase arrest by adding 0.5mM each of all four DNA-precursor deoxyribonucleosides (XdRs) thus indicating the primary effect of HU is the inhibition of the reduction of ribonucleosides. When the HU-block is removed by either washing the HU from the medium or by rescue with XdRs an excessive delay in recovering the capacity to divide is specifically induced in cells which were in S-phase

at time of addition of HU. This delay may be due to the time needed to repair damage to the DNA which was induced specifically during S-phase.

Fluoromicroscopic examination of euchryesine-stained cells exposed for a minimum of two hours starting in S-phase show a radical morphological change in the macronucleus. This phenomenon, termed the "halo effect," is characterized by the formation of apparently membrane-associated chromatin aggregates surrounding a constricted chromatin mass. Halo-induction by HU is S-phase specific and upon removal of the HU-block the halo remains until the first recovery division. The halo effect is interpreted as being a morphological indication of the cell's repair response to the damaging effects of HU upon macronuclear DNA.

Observations of division of individual cells in microdrops, plus autoradiographic studies using ^3H -TdR and standard cell cycle analysis techniques, reveal that HU blocks all cells in the initial 92% of S-phase but does not affect cells in the remaining 8% of S-phase or in G_2 and division. Thus the fraction of the population of cells that is in G_2 can be approximately determined (within 6 minutes) by the fraction of the population able to divide in the presence of HU. This fraction can be related to the approximate duration of G_2 after mathematically compensating for the age gradient. Thus G_2 analysis of T. pyriformis (GL-I) is accomplished easily, quickly, and

inexpensively with the use of HU. The possibilities of utilizing S-phase specific recovery delay and halo induction for simple means of determining the durations of G_1 and S-phase respectively are also discussed.

The addition of all four XdRs (0.5mM) to exponentially growing cells in rich organic complete medium without HU results in the generation time of these cells being decreased by 20%. Cell cycle analysis with phosphorus-33 (^{33}P) and experiments involving cell phase sensitivity to the presence of excess XdRs show that the excess XdRs are taken up and incorporated or pooled entirely during the initial 50% of S-phase. This acceleration of early S-phase completely accounts for the abbreviated generation time seen under these conditions.

When HU is removed after 10 hours of treatment the cells undergo a period of recovery characterized by an increase in the duration of DNA synthesis and in the amount of DNA synthesized before they divide and enter a cell cycle abbreviated at the expense of G_1 . These results are related to a model for control of the initiation of DNA replication and cell division based upon the ratio of macronuclear DNA content to cytoplasmic mass.

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Tetrahymena pyriformis and Use in
Cell Cycle Analysis

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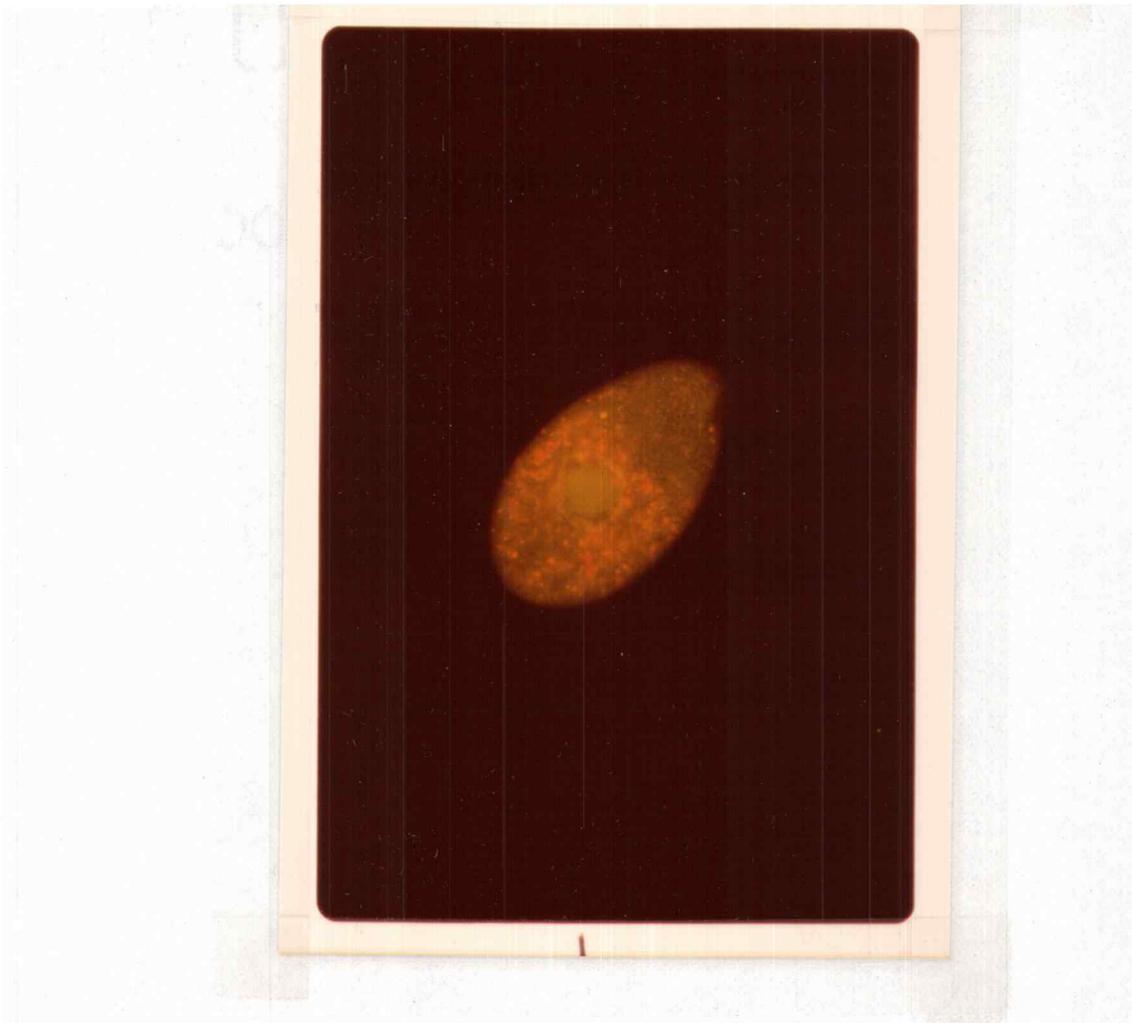
Director of Genetics Institute

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Dean of Graduate School

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Typed by Susie Kozlik for David H. Worthington



Frontispiece. Fluorescence photomicrograph of euchrysin stained Tetrahymena pyriformis (GL-1) cell expressing the halo effect (X950). This cell had been treated with HU for 4 hours.

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LIST OF ABBREVIATIONS

D	- division phase
DI	- division index
DNA	- deoxyribonucleic acid
ECB	- extra-nuclear chromatin body
G ₁	- "gap-1", the pre-DNA-synthesis phase
G ₂	- "gap-2", the post-DNA-synthesis phase
GT	- generation time
³ H-BUdR	- tritiated bromo-uridine
³ H-TdR	- tritiated thymidine
HU	- hydroxyurea
LGP	- logarithmic growth phase
ml	- milliliter
mM	- millimole
N/C	- ratio of nuclear DNA content to cytoplasmic mass
³² P	- phosphorus-32
³³ P	- phosphorus-33
RNA	- ribonucleic acid
S	- DNA-synthesis phase
T*	- time of addition of radionuclide
T _d	- time of 50% cell separation
t _D	- duration of division
t _{G₁}	- duration of G ₁
t _{G₂}	- duration of G ₂
t _{post-HU}	- duration of post-HU period
t _S	- duration of DNA-synthesis period
XdRs	- deoxyribonucleosides
μCi	- micro-curies

HYDROXYUREA: PHYSIOLOGICAL EFFECTS ON
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CELL CYCLE ANALYSIS

INTRODUCTION

The Problem

Cell growth is associated with a series of biochemical, physiological, and morphological events that occur from the time of termination of one cell division to the time of termination of the next cell division. This growth cycle from division to division is conveniently termed the cell cycle. In the quest for understanding the dynamics of cell growth it is desirable to identify and characterize major events that take place during the cell cycle. The temporal sequencing of biological phenomena involved in cellular growth, that is, the biochemical, physiological, and morphological dissection of the cell cycle, is known as cell cycle analysis.

This study was primarily concerned with the effects of a specific metabolic inhibiting agent, hydroxyurea (HU), and the application of some of these effects in analysing the cell cycle. Hydroxyurea, a known deoxyribonucleic acid (DNA) synthesis inhibitor, was revealed in this study, to arrest all cells from further progress through the cell cycle once they had entered the DNA-synthesis phase (S-phase). The specificity of HU's action on S-phase cells was exploited for the development of new and simple methods of cell cycle analysis.

An understanding of the mode of action of any given physiological stimulant or inhibitor of cell growth prepares the way for a better understanding of the dynamics of cellular growth itself. Thus, certain aspects of hydroxyurea's effects upon cellular physiology and metabolism, primarily DNA synthesis, were studied. The effects of hydroxyurea upon DNA synthesis were studied by means of 1) tritiated thymidine uptake, 2) the recovery characteristics of cell growth and reproductive capacity after HU treatment, 3) deoxyribonucleoside rescue from the HU block, and 4) the morphological characteristics of HU upon the cell nucleus. These studies paved the way for both the support of existing hypotheses and the formulation of new hypotheses concerning both the pharmacological action of HU and the regulation of cell growth and reproduction itself.

In addition, it was found that certain concentrations of all four DNA-precursor deoxyribonucleosides (XdRs) added to the growth medium act as a cell growth stimulants. Experiments were designed and conducted to determine which specific portion of the cell cycle expressed this growth acceleration response to the presence of these additional XdRs.

Review of Pertinent Literature

Cell Cycle Analysis

In 1953, Howard and Pelc demonstrated that the radionuclide phosphorus-32, which is incorporated into deoxyribonucleic acid, could be used to dissect the interphase portion of the cell cycle into a DNA-synthesis portion (S-phase), a pre-DNA-synthesis portion, and a post-DNA-synthesis portion. The pre- and post-DNA synthesis gaps are designated G_1 and G_2 respectively. Since 1953 there have been numerous studies employing a variety of radiotracer methods designed to analyse portions of the cell cycle. Radiotracer techniques, having the advantage of enabling the temporal monitoring of specific metabolic events within the cell, now serve as the chief mode of cell cycle analysis.

Autoradiography is one radiotracer technique commonly employed in cell cycle analysis. Autoradiography has the advantage of allowing for the differentiation of radioactively labeled and non-labeled cellular components. Thus, autoradiography enables the radiotracer to be localized to a specific region or structure within the cell. The primary disadvantages of autoradiography are that preparation is very laborious and time consuming and that quantitative studies result in a lower degree of accuracy than more refined radioactivity monitoring techniques, such as liquid scintillation detection. Most of these

radiotracer techniques for cell cycle analysis are reviewed, discussed and criticised by Feinendegen (1967) and also Nachtwey and Cameron (1968).

The DNA content of a cell can be measured by means of spectrophotometry. Feulgen stain complexes with nucleic acids and this Feulgen-DNA complex preferentially absorbs light of 5500 Å wavelength. After treating the cells with RNase or acid to remove the RNA the cells are stained with Feulgen. A monochromatic light is beamed through the nucleus of an individual cell and the amount of light absorbed, which is proportional to the amount of DNA present, is measured by means of a photocell. Limitations in accuracy usually relegate this technique to studies comparing the relative DNA content of pre- and post-mitotic cells, which differ by a factor of two. However, Mak (1965) has developed the two wavelength method of cytophotometry to a high enough level of accuracy to measure intermediate values of DNA content seen during the DNA-synthesis period, thus enabling cytospectrophotometric cell cycle analysis.

Mutagen induced chromosome aberrations may serve as markers indicating whether the cell was in the pre-synthetic (G_1) phase, DNA synthetic (S) phase, or post-synthetic (G_2) phase at the time of aberration induction (Davies and Evans, 1966). Aberrations induced in G_1 are replicated during the subsequent S-period and are expressed as chromosomal aberrations (expressed in both sister chromatids) by the

time the cell reaches mitotic metaphase. On the other hand, aberrations induced during G_2 are not replicated (S-phase is past) and are expressed as chromatid aberrations. Aberrations induced during the S-period result in both the chromosomal and chromatid classes of aberrations.

There are also naturally occurring morphological markers that characterize certain phases of the cell cycle. Mitosis and cytokinesis have long been morphologically characterized usually into four major stages; prophase, metaphase, anaphase, and telophase. In Tetrahymena it has been shown that, under controlled conditions, several morphological time markers appear prior to cytokinesis, including the oral morphogenesis of the posterior daughter cell (Frankel, 1962).

The use of pharmacological agents that specifically inhibit some metabolic pathway necessary for the continuation of the cell cycle forms the basis of a simple method of cell cycle analysis. A drug-induced metabolic block might result in the arrest of further progress through the cell cycle, thus establishing a physiological marker that defines a specific portion of the cell cycle (Puck, 1964; Peterson, et al., 1969). This is precisely how hydroxyurea was used in a major portion of this study.

HU-Induced Cell Cycle Block

Hydroxyurea, in concentrations from 3 to 10mM, selectively blocks mammalian cells in S-phase and at the G₁/S border from progressing further through the cell cycle (Yarbro, Kennedy and Barnum, 1965; Philips, et al., 1967; Adams, Roger and Lindsay, 1967; Bacchetti and Whitmore, 1969; Chan, et al., 1970) by inhibiting replicative DNA synthesis (Young and Hodas, 1964; Rosenkranz and Levy, 1965; Bose, Gothoskar and Ranadine, 1966; Rosenkranz, et al., 1966; Adams and Lindsay, 1967; Rosenkranz, et al., 1967; Smith, Boutwell and Potter, 1968; Suss and Maurer, 1968; Bacchetti and Whitmore, 1969; Coyle and Strauss, 1970; Horikawa et al., 1970; Wanka, Moors and Krijzer, 1972). Although HU inhibits DNA synthesis, it appears not to affect the synthesis of RNA's and proteins either in bacteria (Young and Hodas, 1964; Rosenkranz, et al., 1967; Robinson, 1971), mammalian cells (Sinclair, 1967; Bacchetti and Whitmore, 1969; Gelbard, et al., 1969; Chan, et al., 1970) or Tetrahymena pyriformis (Rudick and Cameron, 1972). It has also been shown that HU inhibits gene amplification but not gene transcription in the dipteran, Bradysia hygida (Sauvaia, et al., 1971).

The most widely accepted view of the mode of action of HU in blocking replicative DNA synthesis is that it inhibits the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates (Frenkel and Arthur, 1967; Young, Schochetman and Karnofsky, 1967; Rosenkranz and Carr, 1970). This view has largely been supported by enzyme extraction experiments with both bacteria and mammalian cells which reveal a decrease in the in vitro conversion of cytosine riboside diphosphate and guanosine riboside diphosphate to their respective deoxy-forms in the presence of HU (Young and Hodas, 1964; Frenkel, Skinner and Smiley, 1964; Turner, Abrams and Lieberman, 1966; Adams, Abrams and Lieberman, 1966; Adams and Lindsay, 1967). In addition, it has been observed that HU-induced inhibition of DNA synthesis can be reversed by the addition of deoxy-ribonucleosides but not ribonucleosides (Adams and Lindsay, 1967; Weiss and Tolmach, 1967; Topping, 1968; Jasty and Chang, 1970; Rosenkranz and Carr, 1970). Echinoderms, which possess significantly large quantities of pooled deoxyribonucleotides in ovum cells (Nemer, 1962) have been found to be exempt from HU-induced inhibition of DNA synthesis for the first four or five cell generations of embryogenesis (Young, et al., 1967). Hydroxyurea does not appear to affect the action of either the kinase enzymes or DNA polymerase (Young and Hodas, 1964; Turner, et al., 1966; Adams and Lindsay, 1967; Rosenkranz, Jacobs and Carr 1968). In vitro studies with the

partially purified ribonucleoside reductase enzyme indicate that HU inhibits the reactivity of this enzyme by altering its stereochemistry (Young et al., 1967; Moore, 1969).

Of additional interest are the observations that HU does not inhibit the unscheduled DNA synthesis involved in excision-repair of damaged DNA (Painter and Cleaver, 1967; Sinclair, 1968; Cleaver, 1969; Coyle and Strauss, 1970; Horikawa, et al., 1970; Sawada and Okado, 1970; Liebermann, et al., 1971). In support of these results are the observations that HU treatment either before or after X-irradiation has no effect on survival during G_1 , G_2 and mitosis, but, if the radiation and HU treatment are applied during S-phase, survival is decreased (Weiss and Tolmach, 1967; Sinclair, 1968; Wolff, 1972). The possibility of a synergistic effect originating from a direct interaction between the radiation and the HU, such as the formation of free radicals from HU that induce additional lesions in the DNA, has been eliminated by the observation of Sinclair (1968) that the presence of HU during the irradiation itself has no effect on survival during S-phase. Prempre and Merz (1969) have found, using a split dose of X-irradiation, that HU does inhibit the repair of chromosome breaks during G_1 in phytohemagglutinin stimulated human lymphocyte cells. This apparent contradiction is further analysed in the Discussion section of this thesis.

In agreement with the observations that HU inhibits S-phase DNA synthesis (replicative DNA synthesis) but does not inhibit excision-repair DNA synthesis (unscheduled DNA synthesis) are the results of several studies involving ^3H -TdR and ^3H -BUdR incorporation into DNA in the presence of HU followed by strand separation in an alkylane CsCl gradient and liquid scintillation monitoring of the radioactive DNA (Painter and Cleaver, 1967; Cleaver, 1969; Cleaver, 1970; Lehmann, 1972). The results of these studies indicate that the semi-conservative DNA synthesis involved in the replication of DNA is essentially blocked in the presence of HU while the nonsemi-conservative mode of DNA synthesis associated with unscheduled DNA synthesis is not inhibited.

HU's Direct Effect Upon the DNA

It has been observed for some time that the HU treatment, in addition to causing cell cycle arrest by blocking replicative DNA synthesis at the reductase level, also causes cell lethality. Recent investigations have shown that HU-induced cytotoxicity is a long term, taking several hours to be expressed, rather than an immediate effect as is the inhibition of DNA synthesis (Yu and Sinclair, 1968; Bacchetti and Whitmore, 1969; Coyle and Strauss, 1970; Connell, et al., 1971). In addition, the cytotoxic effect cannot be reversed by either removing the HU (Connell et al., 1971) or adding excess deoxyribonucleosides

(Coyle and Strauss, 1970). In mammalian systems HU has been found to be specifically toxic for cells in the S-phase only (Sinclair, 1965; Philips, et al., 1967; Bacchetti and Whitmore, 1969; Yu and Sinclair, 1968; Byfield, Lee and Bennett, 1972). In connection with this is the observation that HU induces chromosome aberrations when it is added during S-phase but has no effect on chromosomes when added during the G₁, G₂ or M-phases of the cell cycle (Yu and Sinclair, 1968; Karon and Benedict, 1972). Thus, S-phase is the portion of the cell cycle that is sensitive to HU in respect to cell cycle arrest, the inhibition of DNA synthesis, the induction of cell death, and the induction of chromosome aberrations. However, different mechanisms are likely involved in the different effects.

The cytotoxicity which comes after continuous exposure to HU during S-phase is a result of direct action of HU upon the DNA (Rosenkranz, 1966; Bacchetti and Whitmore, 1969; Coyle and Strauss, 1970; Rosenkranz and Carr, 1970; Massafi, Carr and Rosenkranz, 1972). Several studies indicate that prolonged exposure to HU results in a pronounced degradation of the DNA in both in vivo and in vitro experimental situations (Bendich, et al., 1963; Fishbein and Carbone, 1963; Rosenkranz, 1966; Rosenkranz, Jacobs and Carr, 1968; Coyle and Strauss, 1970; Jacobs and Rosenkranz, 1970; Rosenkranz, 1970). The results of several investigations indicate that the degradation of DNA in the presence of HU during S-phase is due to the induction of

single strand breaks leading to the accumulation of double strand breaks in the DNA (Rosenkranz, 1966; Rosenkranz, Jacobs and Carr, 1968; Rosenkranz and Rosenkranz, 1969; Coyle and Strauss, 1970; Jacobs and Rosenkranz, 1970). In agreement with this conclusion is the observation that HU induces chromosome aberrations only of the chromatidal type (Yu and Sinclair, 1968; Karon and Benedict, 1972). (Chromatidal aberrations are known to be induced only during the DNA synthesis and the post-DNA synthesis periods.) As pointed out in the preceding paragraph, HU does not induce aberrations in G_2 cells; therefore, these chromatidal aberrations are induced in the replicating DNA only.

In bacteria it has been demonstrated by techniques employing the use of CsCl and sucrose density gradients and heat denaturation that a unique DNA which appears to consist of single stranded fragments possibly rich in thymine (T) and adenine (A) bases arises from newly synthesized DNA in the presence of HU (Rosenkranz, 1966; Rosenkranz, Jacobs and Carr, 1968). Similarly, in mammalian cells (HEp-2) it has been shown that only small DNA fragments are synthesized in the presence of HU (Coyle and Strauss, 1970). Upon removal of the HU within 24 hours these fragments are rapidly converted to the large native DNA but after 24 hours incubation in HU an increasing percentage of the small DNA fails to convert to the large double stranded fraction and fragmentation of the native DNA begins.

Although the exact mechanism of HU's direct action on DNA is still unclear, a perusal of some of the past proposed models is worthwhile. Bendich, et al. (1963) proposed that HU breaks down resulting in the formation of nitroxyl radicals (HON°) which become dimerized to hyponitrous acid, $\text{HON}^\circ + \text{HON}^\circ \rightarrow \text{HON}=\text{NOH}$. This oxidizing agent then produces main chain breaks in the DNA. Fishbein and Carbone (1963) suggested another possible derivative of HU, hydroxylamine, may directly attack the DNA inducing main chain breaks. Hydroxylamine, however, is known to inhibit RNA and protein synthesis (Bose, et al., 1966), which is contrary to the findings for HU. In addition, hydroxylamine does not produce chromosome aberrations with as much efficiency and with the same temporal consistency as does HU (Oppenheim and Fishbein, 1965).

More recent work from the laboratory of Herbert S. Rosenkranz has concentrated on the effects of an oxidation product of HU, N-carbamoyloxyurea. Freshly prepared solutions of purified HU do not contain N-carbamoyloxyurea and do not degrade DNA in vitro, while solutions of HU which are allowed to stand for several hours at room temperature contain N-carbamoyloxyurea and these solutions do cause the in vitro degradation of DNA (Rosenkranz and Rosenkranz, 1969; Rosenkranz, 1970; Jacobs and Rosenkranz, 1970). Treatment with N-carbamoyloxyurea causes the degradation of purified DNA to small fragments by the induction of single strand breaks (Rosenkranz and

Rosenkranz, 1969; Jacobs and Rosenkranz, 1970). In addition, it is believed that HU, or N-carbamoyloxymurea, modifies cytosine in the DNA (Jacobs and Rosenkranz, 1970). It must be emphasized, however, that the biochemical environment of these in vitro studies is in no way similar to that of the in vivo DNA replicating (S-phase) cells in which HU is observed to induce single strand breaks. The exact mechanism of HU's interaction with replicating DNA in vivo remains unknown, but in light of the results of this study, along with some other recent studies, a model is proposed in the Discussion section.

Deoxyribonucleoside Uptake and Pool Formation in Tetrahymena

The rate of uptake of exogenous DNA precursors and the availability of pooled DNA precursors may be of significant importance in influencing both the time when DNA replication is initiated and the rate of DNA synthesis once it has been initiated. Using autoradiographic techniques, Stone, Miller and Prescott (1965) showed that the uptake of thymidine into an acid soluble pool occurred only during S-phase (and possibly early G_2) in Tetrahymena pyriformis. Similar results have been noted in mouse L cells where it was also demonstrated that the thymidine (TdR) is phosphorylated to thymidine triphosphate (TTP) which is the major constituent of the DNA precursor pool (Adams, 1969a, b). This pool, which is primarily formed during S-phase, is retained through G_2 , division, and G_1 before its

nucleotides are incorporated into the DNA during the next S-phase (Stone, Miller and Prescott, 1965; Adams 1969a, b). Adams (1969a) suggests that the TTP pool is largest outside of the S-period and that its maximum size regulates uptake of TdR by inhibiting the thymidine kinase enzyme thus preventing the phosphorylation and entry of exogenous TdR. The nucleotide pool size may, therefore, govern the uptake of nucleosides. Indeed, in Tetrahymna pyriformis both the thymidine synthetase and thymidine kinase enzymes are probably only synthesized during S-phase (Prescott and Stone, 1967).

MATERIALS AND METHODS

The Organism

Classification, Morphology, and Genetics

The organism studied was an amiconucleate strain (GL-I) of the ciliated protozoan, Tetrahymena pyriformis (Corliss, 1954) which had been earlier described as Leucophrys pyriformis (Ehrenberg, 1830) and as Tetrahymena geleii (Furgason, 1940). According to the latest classification of the phylum Protozoa (Honigberg, et al., 1964), T. pyriformis is in the suborder Tetrahymenina, order Hymenostomatida, subclass Holotrichia, class Ciliatea, and subphylum Ciliophora. T. pyriformis has a world wide distribution and, in its natural fresh water habitat, is a microphage (bacterial-feeder). The GL-I strain (Frankel, 1965) is a subculture from the original GL stock which has been maintained axenically in the laboratory of Andre Lwoff in Paris since 1925.

T. pyriformis GL-I ranges in shape from pyriform to ovoid, is about 30 microns wide and 50 microns long, and has an ovoid to spherical macronucleus that measures from 12 to 20 microns in diameter. The macronucleus possesses a DNA-ploidy level of approximately 40 to 84n (Nilsson, 1970). The polyploidy characteristic of this species allows for a remarkable degree of genetic stability and uniformity

even though it divides amitotically. The amitotic division occurs by the "pinching off" of the macronucleus immediately prior to cytokinesis (Brunk, 1967). The formation of a cleavage furrow during cytokinesis allows the division period to be morphologically distinguishable from other portions of the cell cycle.

Studies of DNA metabolism and the effects of drugs upon DNA metabolism and cell growth in a eucaryotic cell system is promoted by using a cell line that is 1) relatively inexpensive to maintain and easy to handle, 2) has a relatively short cell generation time, 3) is genetically stable, 4) has a large well-defined nucleus that incorporates tritiated thymidine ($^3\text{H-TdR}$) during the DNA synthesis period (S-phase) and 5) expresses age specific morphological heterogeneity which can be easily used as a marker for dissecting the cell cycle. In addition to offering these advantages, T. pyriformis has been the focus of extensive studies in the past resulting in the accumulation of a wealth of information regarding cell cycle analysis and DNA metabolism. Thus T. pyriformis GL-I was the organism of choice for this study.

Culturing the Organism

The Tetrahymena were maintained axenically in the logarithmic growth phase (LGP) at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in Tyess nutrient growth medium. Tyess nutrient medium is a "complete" medium consisting of 0.25%

tryptone (Difco Laboratories, Detroit), 0.5% yeast extract (Anheuser-Busch Inc., St. Louis), 0.5% soluble starch (Difco Laboratories), and 0.5% concentrated Osterhout's balanced salt solution (BSS). The concentrated (200 X) Osterhout's BSS is an aqueous (glass distilled water) solution of the following salts (expressed in grams per liter) NaCl, 20.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.64; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.2; KCl, 4.6; CaCl_2 , 0.2. All of the salts were obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

The cells were cultured in 5 ml aliquots of medium contained in 15 ml disposable glass tubes capped by metal closures. Maintenance of LPG cells was assured by a daily bacterial loop transfer, using aseptic technique, of LPG cells into fresh medium.

Handling the Organism

Some experiments required the observation of individual LPG cells through one or more cell generations. This was easily facilitated by employing the "microdrop culture" technique of Nachtwey and Dickinson (1965). Small drops (approximately 1 to 3 ml) of medium were prepared by tapping a 2 mm diameter capillary tube or Pasteur pipette containing nutrient medium on the bottom of a 10 x 35 mm tissue culture dish (Falcon Plastics, Los Angeles) and covering the drops with paraffin oil, Saybolt viscosity at 100°F = 340-355 (Matheson, Coleman and Bell, Los Angeles) to prevent evaporation.

The cells were then pipetted individually into each droplet of culture medium thus enabling, with the aid of a dissecting microscope, the observation of individual cells for several generations.

Most of the experiments conducted in this study involved picking individual cells from exponentially growing populations and either isolating them into microdrops as described above in the previous section or "spotting" them onto microslides in preparation for autoradiography. The micromanipulation of the cells was accomplished by using micropipettes which were constructed in the laboratory. Pasteur pipets were converted into micropipets by heating the extended tip over a small flame jet from a 17g syringe needle until the glass began to melt and then swiftly pulling the tip away from the body of the pipet thus extending the remaining tip to a microscopically fine point. The micropipet was then completed by using jewelers forceps to clip off the distal portion of the tip to leave an opening just large enough (approximately 40 to 60 microns in diameter) to allow a Tetrahymena to pass through without causing damage to it. The intake and expelling of individual cells from the micropipet was controlled by mouth pressure via three feet of eighth inch rubber tubing connecting the pipet to a plastic mouth piece.

Some experiments, such as those involving the determination of generation time from the relative increase in cell number over a period of time, require the picking by micropipet of a random cell

sample from an exponentially growing population. The population in logarithmic growth phase should be mixed by simple agitation prior to picking the random sample. Though the population may be well mixed prior to pipetting, the actual picking of cells may involve some bias which is beyond the control of the investigator thus resulting in a non-random sample. Even if no bias in cell picking exists, there is a chance, which can be totally attributed to the laws of probability, of isolating a non-random sample. This may result in some degree of synchrony during the initial period of growth for such a population. After a generation or two, however, any synchrony that may have originally existed in the cell sample is usually lost. The differential growth rate, that is, the lognormal distribution of generation times for cells undergoing exponential growth, serves to rapidly attenuate any small degree of synchrony which has artifactually been introduced (Nachtwey and Cameron, 1968).

Hydroxyurea Treatment

A large number of experiments involved exposing the cells to hydroxyurea (HU) at a final concentration of 10mM. The HU (Mann Research Laboratories, New York) was added to glass distilled water to make a stock concentration of 200mM. The stock solution was transferred, using a disposable lcc syringe (Becton, Dickinson & Co., Rutherford, N. J.), to the medium in the correct amount to make the

desired final working concentration. HU could be added directly to an exponentially growing culture or be added to some medium into which the cells would be transferred. In those experiments involving a pulse exposure to HU, the drug was removed from the medium by a series of centrifuge washes resulting in a 10,000 fold dilution of the original concentration of HU. The HU could also be removed by passing the cells through several drops of HU-less medium by means of a micropipette.

XdR Treatment

Similarly, some experiments involved either terminal or pulse exposure to the four DNA-precursor deoxyribonucleosides (XdRs), thus requiring the preparation of a stock solution. A stock solution of 40mM each of cytosine deoxyriboside (Sigma, St. Louis, Mo.), thymidine (Mann Research Laboratories, New York), adenine deoxyriboside (Sigma), and guanine deoxyriboside (Sigma) in Tyess complete medium was prepared and sterilized by filtration through a 0.4 micron diameter pore filter (Millipore Corporation, Redford, Mass.). The stock solution could then be added to a cell culture in an amount that ended in the desired final concentration of XdRs. Pulse XdRs experiments involved a centrifuge wash or transfer wash as previously described in this section. The specific experimental procedures are described in detail in the Results section but the

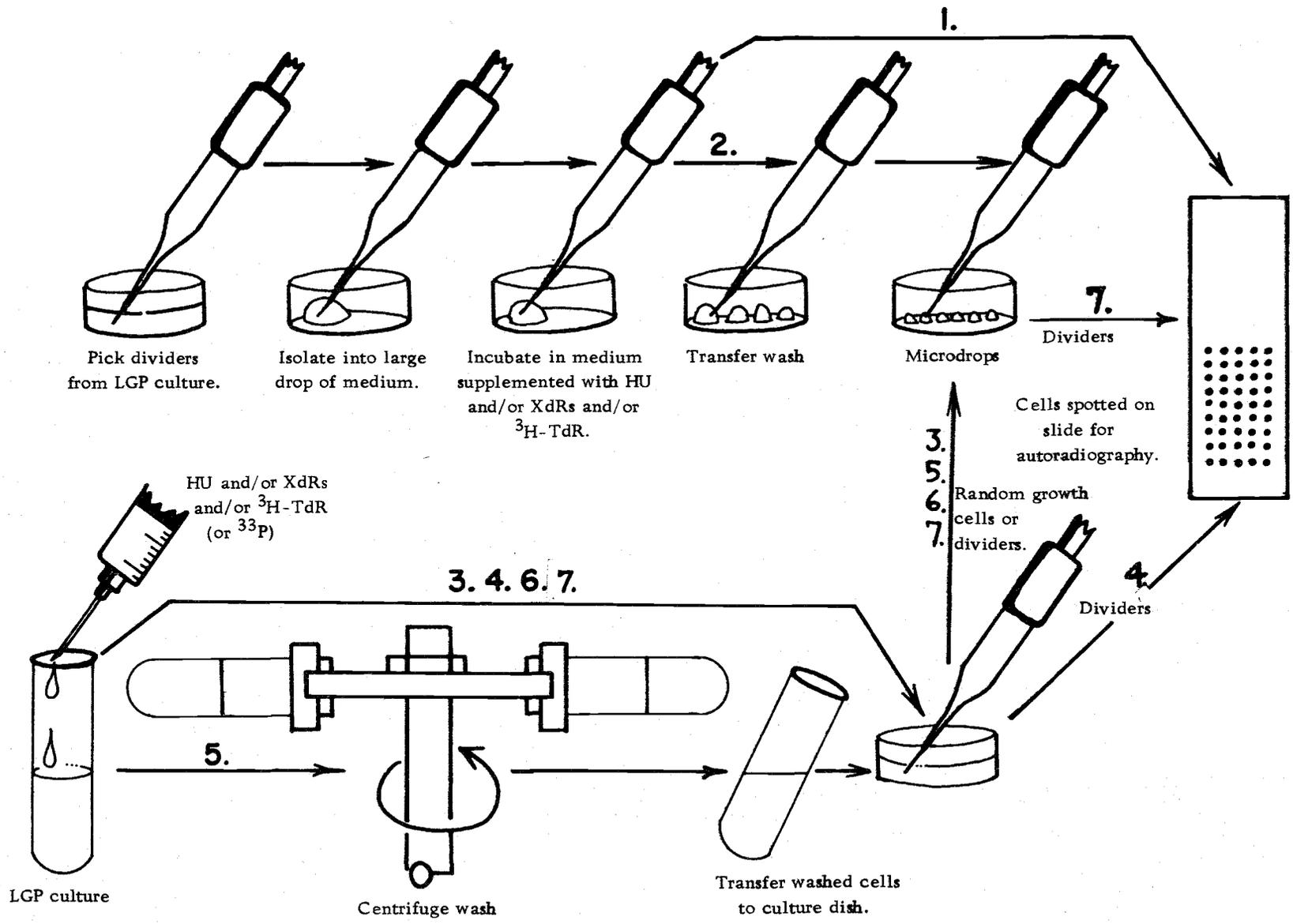
diagram in Figure 1 illustrates the general procedures for several types of experiments involving both the use of HU and XdRs.

Autoradiography

The use of tritium as a radioactive tracer in autoradiographical studies is highly advantageous. The low energy ($E_{\text{max}} = 0.0186 \text{ Mev}$) and short range ($0.9 \mu\text{m}$ in water) of the beta emission allows the attainment of very good resolution, from 0.6 to 1.0μ , in light microscopic autoradiography (Feinendegen, 1967). Virtually any organic compound can be labeled with tritium because hydrogen is such a common elemental component of these molecules. The long half-life of tritium (12.3 years) is a definite advantage in designing and conducting experiments that involve its use as compared, for example, to the 14 day half-life of phosphorus-32.

The deoxynucleoside, thymidine, is a precursor specific to DNA and is not incorporated into RNA. The implementation of radioactively labeled thymidine offers convenient access to information regarding the time of DNA synthesis during the cell cycle. The use of thymidine-methyl- ^3H (Tritiated thymidine) involves no measurable isotope effects and is, therefore, not metabolically distinguishable from non-labeled thymidine (Feinendegen, 1967). It should be noted, however, that lengthy storage of tritiated thymidine may result in its

Figure 1. A flow chart illustrating the basic experimental designs employed in this study. 1. The experimental sequence followed in the continuous labeling (^3H -TdR method of G_1 phase analysis). 2. This sequence (pulse exposure method) followed when analysing the portion of the cell cycle that responds, by a modification of the generation time, to a pulse exposure of an agent such as HU or the XdRs. 3. The HU method of G_2 analysis culminating in the micro-drop culturing of a random cell sample in HU-supplemented medium for the purpose of determining the accumulated fraction of dividing cells. 4. Standard continuous labeling method of G_2 analysis. 5. HU or XdR pulse experiments similar to pathway - 2 except with a centrifuge wash. 6. Experiments involving the rescue of cells from the HU block by the addition of XdRs or the determination of the effects of XdRs exposure upon the generation time. 7. Procedure for determining the fraction of cells beyond the HU-block which are still synthesizing DNA. All these procedures are described in detail in the Results section.



extensive decomposition by self-radiolysis (Wand, Zeuthen and Evans 1967). In this study extensive use was made of thymidine labeled with tritium ($^3\text{H-TdR}$).

Thymidine-methyl- ^3H (New England Nuclear, Boston, Mass.) with a specific activity of 6.7 Ci/mM was used at final concentrations of either 5 or 10 $\mu\text{Ci/ml}$ in all experiments employing autoradiography. A five to seven day exposure time was found to be optimum for autoradiography.

Phosphorus-33 is also a low energy ($E_{\text{max}} = 0.25 \text{ Mev}$) beta particle emitter and is therefore useful as a radiotracer in studies employing autoradiography (Robinson, 1969). Phosphorus-33 in the form of monopotassium phosphate ($\text{KH}_2^{33}\text{PO}_4$) in 1 ml aqueous solution with a radioactivity of 0.5 mCi was purchased from New England Nuclear, Inc., Boston, Mass. The radioactive half life ($T_{1/2}$) of ^{33}P is only 25 days so it was necessary to calculate the total activity of the shipment for each day and to utilize the radionuclide as soon after its arrival as possible. The mathematical equation used to calculate total activity is

$$\ln \frac{A_0}{A_t} = \frac{0.693(t)}{T_{1/2}}$$

therefore,

$$\ln A_t = \ln A_0 - \frac{0.693(t)}{T_{1/2}}$$

where \ln = natural log; A_0 = the initial radioactivity (mCi) at time zero; A_t = the radioactivity remaining after an elapsed period of time; t = elapsed period of time (days) from time zero; $T_{1/2}$ = radio-decay half-life (days). The ^{33}P was used at a radioactive concentration of 20 Ci/ml in most of the experiments and several "test slides" were always prepared so that the optimum exposure time could be determined for each specific experiment. (This usually turned out to be 9 or 10 days.)

Experiments involving autoradiographic analysis required that the cells be individually placed in rows upon clean microscope slides. This individual "spotting" of the cells was accomplished by using the micropipet to transfer individual cells in tiny drops to the slide and then quickly sucking up the excess medium around each cell so that it air dried and became affixed to the slide with a minimum of physical distortion.

The acid soluble radioactive compounds were removed from the cells in the preparation for autoradiography by fixing the cells in 1:3 glacial acetic acid-ethyl alcohol at 10° overnight.. In addition the cells were sometimes treated with 5% trichloroacetic acid (TCA) at 5° for one hour.

Phosphorus, unlike thymidine, is not specifically incorporated into DNA alone but is taken up by RNA as well. In order to monitor the temporal parameter of DNA synthesis it was thus necessary to

remove all the RNA from the cell before autoradiography. This was accomplished by digesting away the RNA with a working solution of 1 mg RNase (Worthington Biochemical Corp., Freehold, New Jersey)/ml distilled water. This working concentration was buffered to a pH of 6.8 with a small amount of 0.5N NaOH. The cell bearing slides were then placed in clean Coplin jars with RNase solution for two hours at room temperature followed by washing with four changes of distilled water, 5 minutes each, and air drying (Gude, 1968). The slides were then ready for emulsion dipping.

Autoradiographs of the cells were prepared using the liquid emulsion dipping technique (Yunis, 1965). The emulsion, K-5 (Ilford Limited, Essex, England), was mixed to a 1:1 final concentration with distilled water. This dilution with water results in the final application of a thinner and more uniform sheet of photographic emulsion over the slide. K-5 emulsion has an average silver grain diameter of 0.20 microns, is sensitive to all charged particles of any energy, has minimal inherent background or problems with latent image fading and is relatively inexpensive.

The microscope slides carrying the labeled cells were pre-warmed, dipped into the emulsion, dried, and placed in the dark for exposure to take place. The procedure is outlined in step by step detail below.

1. Place the combination water bath-slide warmer in the darkroom and allow the water temperature to reach 40 to 42°C.
2. Mark on the dipping vial with a black permanent felt pin the final level of emulsion mixture desired. This amount should be sufficient to cover three quarters of a slide. The dipping vial is precalibrated by determining the total volume of the marked-off portion and then the half volume level is marked-off in order to facilitate the preparation of a 1:1 water-emulsion mixture.
3. Add water into the dipping vial to the half volume level.
4. Turn out all the darkroom lights except a Number 1 (or No. 2) Wratten red safelight and then add stock emulsion to the final level of the dipping vial to make a 1:1 emulsion to water concentration. (The refrigerated stock emulsion is in gel form and resembles short pieces of spaghetti.)
Place the dipping vial into a small beaker in the water bath and wait 15 to 20 minutes for the emulsion to reach 40 to 42°C.
5. With a clean glass rod, gently stir the emulsion-water mixture in preparation for dipping. Place 5 to 10 radioactive cell bearing slides around the rim (slide warmer portion) of the water bath and allow a couple

of minutes to pass. Dip the prewarmed slides, one at a time, into the emulsion mixture for no longer than 5 seconds and place in a test-tube rack for drying (Figure M-5).

6. Allow the dipped slides to dry in the darkroom for 30 to 45 minutes and then place them into a light-tight plastic slide-box with a small open vial of CaSO_4 desiccant (W. A. Hammond Drierite Co., Xenia, Ohio) at one end of the box. Seal the box shut with black electrician's tape and place inside a refrigerator at 5 to 10°C for exposure.
7. Correct exposure time is determined by developing a slide every day until the desired density of black silver grains is obtained.
8. After an adequate duration of exposure to the beta emission the slides are then developed as follows:
 - (1) Kodak D-19, 17-18°C 3 min.
 - (2) Distilled H_2O , 17-18°C 30 sec.
 - (3) Fixative, 17-18°C 10 min.
 - (4) Distilled H_2O #1, 17-18°C 10 min.
 - (5) Distilled H_2O #2, 17-18°C 20 min.
 - (6) Dry slides

The slides were then usually stained in Giemsa, pH6.5 for 10 to 30 minutes and rinsed in tap water. The autoradiographs were qualitatively analysed for the existence of nuclear label. The nucleus

was considered labeled if over twice the number of exposed silver grains were superimposed over it as over an equivalent area of the cytoplasm.

Radionuclides and Labeled Compounds

Tritium (^3H) and Tritiated Thymidine ($^3\text{H-TdR}$)

Due to the low-energy of tritium's beta emission, irradiation from an external ^3H source is not a safety hazard. The irradiation danger from an internal ^3H source varies depending upon the nature of the carrier molecule involved. Tritiated thymidine ($^3\text{H-TdR}$) has been calculated to be 1,000 to 10,000 times more dangerous than tritiated water when ingested (Drew and Painter, 1959; Feinendegen, 1967). Thymidine may carry the ^3H directly into the DNA where the biological turnover rate is extremely low and the damage sensitivity of the primary target, DNA, is critically high. Tritium's low energy beta emission and long half-life result in localized energy dissipation that, in turn, results in maximum interaction with the DNA target molecule. This maximum interaction of the beta particle with DNA causes significant damage which may accumulate over the years in the DNA. In addition to causing cell lethality and mitotic delay, irradiation which is localized primarily in the nucleus will induce molecular and chromosomal aberrations that may result in delayed

manifestations such as mutations and tumor initiations that are of primary concern to the fate of the entire organism.

Phosphorus-33

Phosphorus is also incorporated into DNA, although not specifically like thymidine. Phosphorus-33 (^{33}P) was used during part of this study as radiotracer to determine the time of DNA synthesis. The maximum energy of the ^{33}P beta particle emission is 0.25 mev, some 175 fold greater than tritium's beta emission energy, however, it is still considered a low energy beta emitter as compared to others. Similar to ^3H , the main hazard in handling ^{33}P is the possibility of ingesting or inhaling the radionuclide. A major portion of ingested phosphorus becomes fixed in the bone tissues and has a very low turnover rate. The localized irradiation from bone-incorporated ^{33}P may induce bone cancer as has been demonstrated to occur with phosphorus-32, (Emax = 1.7 mev)(Feinendegen, 1967). Phosphorus-33 has one major safety asset over ^3H -TdR; its 25 day radioactive half-life allows it to decay rapidly enough so that in a few months it is no longer hazardous.

Precautions

In order to prevent accidents with ^{33}P and ^3H -TdR some standard procedures were followed. The laboratory and all radionuclide

shipments were routinely monitored for radioactive contamination. Disposable gloves were worn when handling radioactive solutions. Pulse experiments involving the centrifuge-wash removal of radioactive material from the culture required that the centrifuge be covered to prevent the formation of a radioactive aerosol from centrifuge-induced agitation of the solution. All radioactive waste materials were disposed of by putting them into radioactive waste receptacles specifically for liquids or for solids. Extra precautions were exercised to prevent ingestion of radioactive material during experiments which involved the mouth micropipetting of radioactive cells and medium: Moisture traps containing cotton and CaSO_4 were situated near both ends of the rubber tubing of the mouth micropipet apparatus.

Fluorescence Microscopy

Fluorescence microscopy was employed in order to observe the morphological effects of HU in living Tetrahymena. The cells were vitally stained with euchrysin, a purified aminoacridine dye, and observed under a fluorescence microscope. With this stain the double helical nucleic acids of the macronucleus stain bright green, single stranded nucleic acids tend to stain more yellow and lysosomes stain bright red (Allison and Young, 1969). A stock solution of euchrysin (#4851 K + K Laboratories, Plainview, New York) was made up at a

concentration of 1mg/ml in a brown bottle and diluted with the cells in Tyess to a final concentration of 0.05mg/ml. The photodynamic properties of the dye required the avoidance of light while preparing the slides. This was accomplished by using minimal yellow light provided by a General Electric "Buglight."

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 366 nanometers. A dark field condenser lens was utilized along with a yellow barrier filter to achieve optimum clarity and contrast for visualizing the fluorescent cells.

Photomicrographs were made with a Nikon AFM microscope-camera assembly using high speed color film (Anscochrome 500, ASA = 500) or black and white (Kodak Tri X Pan, ASA = 400). It was necessary to make the exposure times as short as possible because the cells were alive and moving and because the dye caused them to be photodynamically sensitive to the light source (which caused photolysis of the cells after relatively short periods of exposure). The exposure times ranged from 2-5 seconds.

Data Analysis

The treatment of the data in the present study was, for the most part, simple and straight forward. The only special treatment involved pooling the results of several experiments, which varied in sample sizes and sampling times, into one composite presentation. In this case the data were "weighted" (or normalized) before pooling in order to account for their parametric variations. Weighting and pooling of data were done in two ways: By either the point cluster method or the interpolation method.

Point Cluster Weighting and Pooling

Consider an instance when the data of three experiments to find generation times are combined and presented as one curve. For example, the results of one experiment show that 1 out of 28 cells had divided 185 minutes after their previous time of division. Another shows 4 out of 24 cells had divided after 180 minutes and the third experiment shows that 1 out of 23 cells had divided after 182 minutes. Weighting and pooling by the point cluster method allows these data to be combined and expressed as a single data point even though they differ in sample size and time of observation. The time of division is weighted as follows:

$$\text{weighted time} = \frac{\sum_{i=1}^{i=dp} (n \times \text{sample time})i}{\sum_{i=1}^{i=dp} n_i}$$

$$\text{weighted time} = \frac{\Sigma \text{ products}}{N}$$

where i = number of an individual data point, dp = total number of data points, Σ = sum of, n = sample size of each experiment, and N = sum of the sample sizes. For example,

<u>n</u>	<u>sample time</u>	<u>n x sample time</u>
28	185	5180
23	182	4490
24	180	4320
<hr/>		<hr/>
N = 75		$\Sigma \text{ products} = 13690$

$$\text{weighted time} = \frac{\Sigma \text{ products}}{N} = \frac{13690}{75} = 182.3 \text{ minutes}$$

The weighted percentage of cells that had divided at the weighted time was calculated by pooling the number of cells that had divided and dividing by the total number of cells observed in all samples and multiplying by 100:

$$\frac{\sum_{i=1}^{i=dp} n_i d_i / n_i}{N} = \frac{\sum_{i=1}^{i=dp} d_i}{N}$$

where d_i = number of divided cells for each experiment, n_i = sample size for each experiment, N = sum of the sample sizes. Substituting

the actual values, the pooled percent dividers is:

$$\frac{\sum_{i=1}^{i=dp} d_i}{N} = \frac{(1 + 1 + 4)}{28 + 23 + 24} = 6/75 (100\%) = 8\%$$

Therefore, 8% of the cells had divided by 182.3 minutes.

Interpolation Weighting and Pooling

When the data points did not conveniently fall in clusters, pooling was achieved by the interpolation method. In this method, interpolated data points for one experiment are pooled with actual data points from another experiment. In some cases, the interpolation was performed graphically; in others it was performed mathematically by the following expression:

$$\text{weighted \% dividers at time } t_T = \left[\frac{(t_T - t_1)(d_2 - d_1)}{(t_2 - t_1)} + d_1 \right] (100\%)$$

$$t_T = (d_e/n)(100\%)$$

where t_T = a selected time point corresponding to an actual data point from another experiment, d_e = number of dividers expected, n = number of cells in sample, d_1 and d_2 = the number of dividers observed at times t_1 and t_2 .

To obtain the weighted and pooled percentage dividers at time, t_T , the interpolated data point was weighted and pooled with data points from other experiments by means of the following equation:

$$\frac{\sum_{i=1}^{i=dp} n_i d_i / n_i}{N} (100\%) = \frac{\sum_{i=1}^{i=dp} d_i}{N} (100\%)$$

where d is the number of dividers, either actual or expected (interpolated), at time t_T .

Example:

Exp't. 1

$$t_1 = 175, (d/n)_1 = 1/24$$

$$t_2 = 185, (d/n)_2 = 6/24$$

Exp't. 2

$$t_1 = 180, (d/n)_1 = 1/23$$

$$t_2 = 190, (d/n)_2 = 8/23$$

$$t_1 = 181, (d/n)_1 = 4/28$$

$$t_2 = 186, (d/n)_2 = 10/28$$

For a selected time, $t_T = 185$ minutes the number of dividers (d) are calculated as follows:

$$\begin{aligned} d_{185_1} &= \frac{(t_T - t_1)(d_2 - d_1)}{(t_2 - t_1)} + d_1 \\ &= \frac{(185 - 175)(6 - 1)}{(185 - 175)} + 1 = 6 \text{ dividers (same as actual data point)} \end{aligned}$$

$$d_{185_2} = \frac{(185 - 180)(8 - 1)}{(190 - 180)} + 1 = 4.5 \text{ dividers (interpolated)}$$

$$d_{185_3} = \frac{(185 - 181)(10 - 4)}{(186 - 181)} + 4 = 8.8 \text{ dividers (interpolated)}$$

$$\begin{aligned}\text{weighted \% dividers at 185 min} &= \frac{(4.5 + 6 + 8.8)100}{75} \\ &= \frac{1930}{75} = 25.7\%\end{aligned}$$

Therefore, by 185 minutes, 25.7% of the cells had divided.

RESULTS

Standard Cell Cycle Analysis with 3H-TdR

In order to test the effects of different agents upon the cell cycle and to investigate new methods of cell cycle analysis it is first necessary to obtain data from standard techniques of cell cycle analysis under standard environmental conditions. In this manner a set of standardized cell cycle data is established that can be subsequently made reference to as a generalized source of control data.

Generation Time

An accurate determination of the generation time (GT) takes precedence over any temporal dissection of the cell cycle. Experiments which are designed for determining the duration of a specific phase of the cell cycle must include plans for simultaneously determining the generation time of these cells. In this study the generation times were primarily determined by two methods.

The method used most frequently was the "exact method" of Nachtwey and Cameron (1968). The generation time of cells can be accurately determined by taking the median generation time from observations of a number of individual cells. This was achieved by picking about thirty dividing cells (hereinafter called dividers) with

a micropipet from a logarithmic growth phase (LGP) culture and isolating these into microdrops of medium under oil as described in the Methods and Materials section. The completion of cell division is marked by the separation of daughter cells. The generation time is defined as the time duration from the completion of the first division for 50% of the cells to the completion of the second division for 50% of the cells. (See Figure 2).

An alternative method for accurately determining generation time involved monitoring the increase in relative cell number over a period of time. An LGP culture was agitated until the cells were thought to be distributed randomly. Thirty to fifty cells were then randomly picked with a micropipet and individually isolated into microdrop cultures. Each of the 30 to 50 microdrops was observed and the total cell number recorded every hour until the cells had undergone 3 generations. The total cell counts for each counting interval were converted to relative values in proportion to the starting number of cells and plotted on semi-logarithmic graph paper. (See Figure 3). The population doubling time was designated the cell generation time and could be determined from any two points along the linear portion of the growth curve. (For a discussion concerning the cause and origin of non-linearity in the initial portion of the growth curve, refer to p. 19 of the Methods and Materials section). Table 1 lists average generation times obtained by both methods.

Figure 2. Results of an experiment using the exact method of determining generation time. Thirty dividers were isolated in microdrops under oil and observed from the time of 50% cell separation (time = 0 min.) until all the cells had divided again. This second 50% cell separation time (arrows) marks the completion of one cell cycle and is designated the generation time. The generation time is slightly greater than 200 minutes in this example.

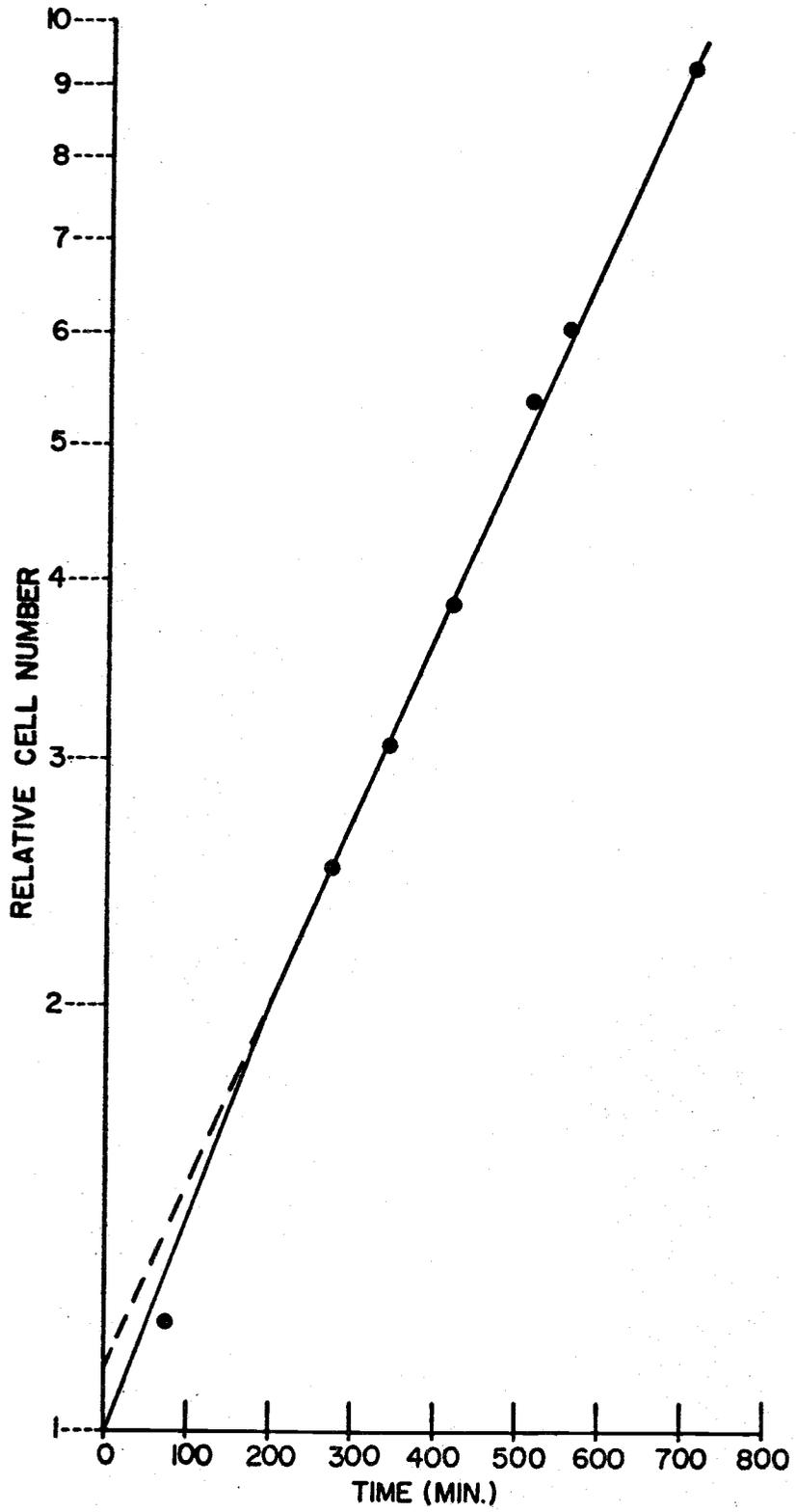


Figure 3. Results of an experiment using the increase in relative cell number for determining generation time. Thirty cells were randomly picked from a log growth phase culture and isolated into microdrop cultures. The increase in total cell number was recorded with time. The mean generation time was determined from the population doubling time along the linear portion of the growth curve. This is equal to 230 minutes in this example.

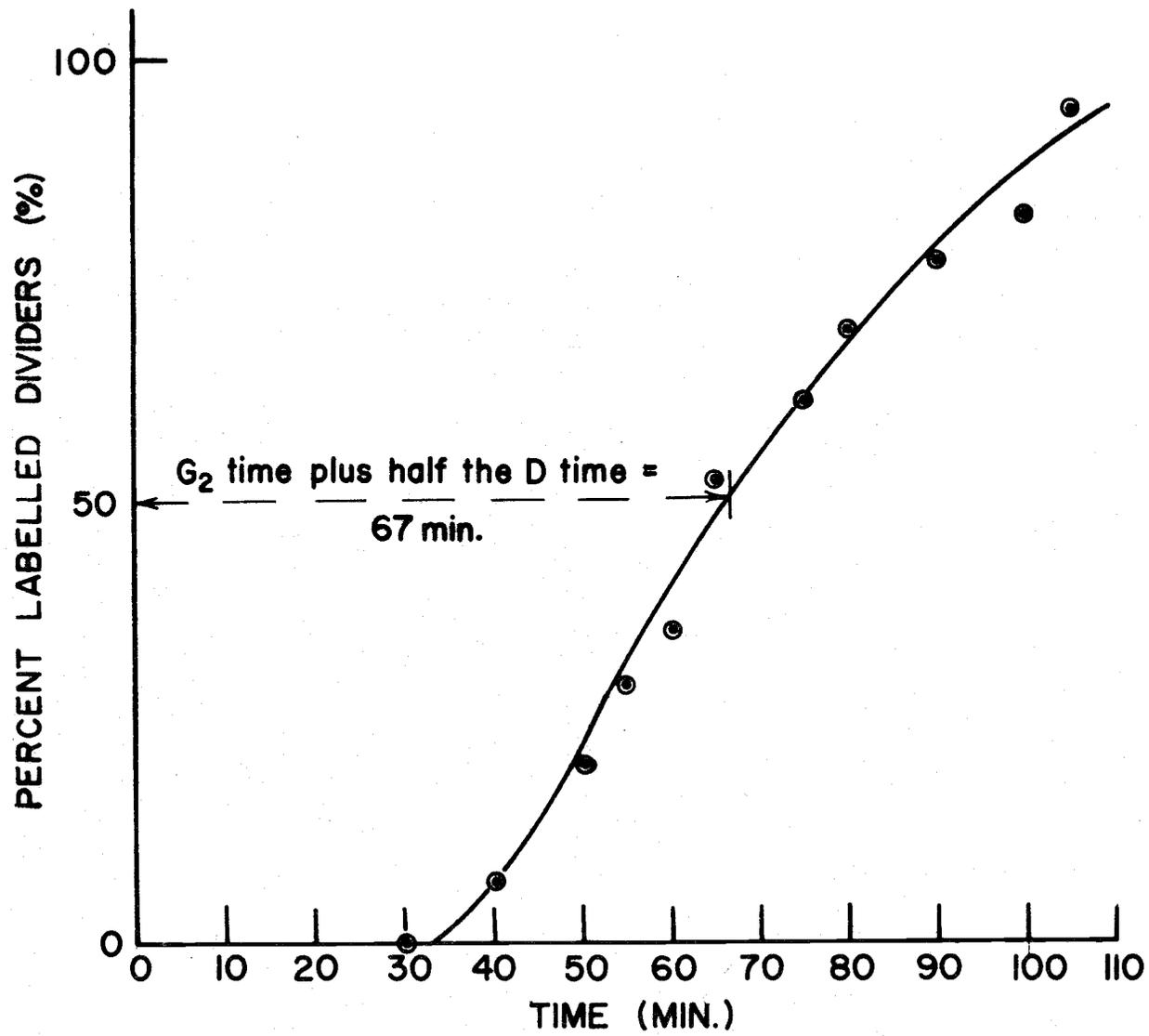


Table 1. Average generation times (GT) for 32 experiments.

Method	No. Experiments	No. Cells	Mean GT* (min.)	Standard deviation (min.)
Exact	27	840	206.7	+ 12.2
Increase in Rel. Cell No.	5	600	200.4	+ 14.3

* Indicates the mean of the generation times as determined from the median generation time in individual experiments (exact method) and the mean of generation times as determined from the population doubling times in individual experiments employing the increase-in-relative-cell-number method.

It can be seen from Table 1 that the generation times determined by these two methods are in close agreement. The generation time derived from the population doubling times (200.4 min.) is expected to be shorter than the GT derived by the exact method (206.7 min.). This is due to the lognormal distribution of generation times in a population. A majority of the cells will have a short generation time with a narrow range of variation and "these cells will contribute more to the geometric increase in cell number than will the cells with longer and more spreadout generation times" (Nachtwey and Cameron, 1967, p. 234). For this reason the mean generation time determined by the exact method is more representative of the true generation time for these cells.

Division Time

The division time was defined as the time interval from the first appearance of a complete division furrow (furrowed on both sides) until cell separation. The division time (t_D) was determined by direct observation of individual cells undergoing division or by deriving it from the division index. The division index (DI) is the fraction of cells in a LGP population that are dividing (furrowed at any single moment). After correcting for the age gradient the DI may be used to determine the time fraction of the cell cycle taken by cells undergoing division. The basic formula for the age gradient correction is after Scherbaum

and Rasch (1957) as follows:

$$\frac{t_{\text{term}}}{\text{GT}} = \frac{\ln[(N_{\text{term}}/N) + 1]}{\ln 2}$$

which rearranged becomes

$$t_{\text{term}} = \frac{\text{GT} \cdot \ln[(N_{\text{term}}/N) + 1]}{0.693}$$

where t_{term} is the time spent in a terminal phase, GT = generation time, N_{term} = number of cells in some terminal phase of the cell cycle, N = total number of cells observed, ln = natural logarithm (\log_e), and 0.693 represents $\ln 2$. For example, if N = 300,

$$\text{DI} = \frac{N_{\text{D}}}{N} = \frac{14}{300}, \text{ and GT} = 207 \text{ minutes then}$$

$$\begin{aligned} t_{\text{D}} &= 207 / .693 \ln[(14/300) + 1] \\ &= (299) \ln(1.0467) \\ &= (299) (.0455) \\ &= 13.6 \text{ minutes} \end{aligned}$$

Post-DNA-Synthesis (G_2) Phase Time

Tritiated thymidine was introduced (at time = T_0) into a LGP culture at a final concentration of 5 $\mu\text{Ci/ml}$. Dividers were then picked from the LGP culture by micropipet at succeeding time intervals and autoradiograms prepared. The G_2 time was determined as the interval between the time of $^3\text{H-TdR}$ introduction (T^*) and the

time when 50% of the dividers were labeled minus one-half of the division time ($1/2t_D$). (See Figure 4).

Pre-DNA-Synthesis (G_1) Phase Time

Dividing cells were picked by micropipet from a LGP culture and introduced into medium containing $5\mu\text{Ci/ml } ^3\text{H-TdR}$. Autoradiograms of samples of post-dividers were prepared at 15 minute intervals thereafter. The G_1 time (t_{G_1}) was determined as the interval between the time of 50% cell separation and the time when 50% of the nuclei were labeled. (See Figure 5).

DNA Synthesis (S) Phase Time

The S-phase was determined by simply subtracting the times determined for division, G_1 , and G_2 from the generation time;

$$t_s = GT - (t_D + t_{G_1} + t_{G_2}).$$

The cell cycle of Tetrahymena pyriformis, GL-I, as dissected by the methods described above, is presented in Table 2.

Physiological Effects of HU and the Use of HU for Cell Cycle Analysis

The use of radioactivity labeled DNA precursors coupled with autoradiography forms the basis of several accurate methods of cell cycle analysis which yield highly reproducible results. (See previous

Figure 4. Determination of G_2 duration (t_{G_2}). The curve represents the accumulated results for the G_2 time controls of four experiments. Percent labelled dividers are plotted against the time after addition of the $^3\text{H-TdR}$. The data points have been weighted by the previously described interpolation method in order to account for differing sample sizes. Each data point represents a sample size of at least 30 cells. Tritiated thymidine was introduced at time zero (time = 0 min.). Assuming a division time of 13.6 min., the G_2 time equals 67 min. minus $1/2$ (13.6 min.) = 60.2 min.

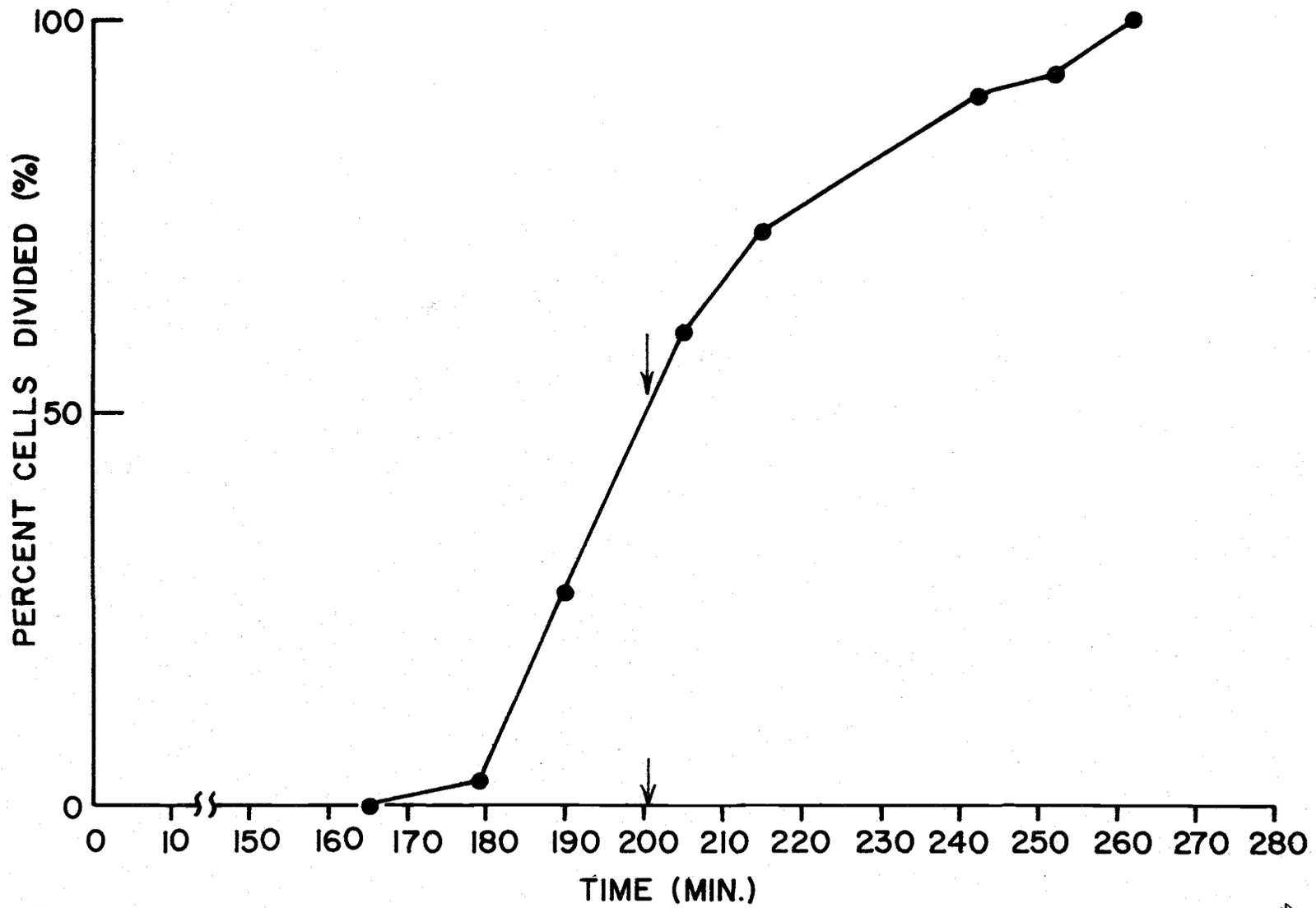


Figure 5. Determination of G_1 duration (t_{G_2}). The curve represents the results of a typical experiment determining G_1 time. The 50% labelling time is 46 minutes. The G_1 time is, therefore, 46 minutes. Each datum point represents a sample size of at least 15 cells. The time of 50% cell separation (T_d) and the time of addition of ^3H -TdR (T^*) was time zero (time = 0 min.).

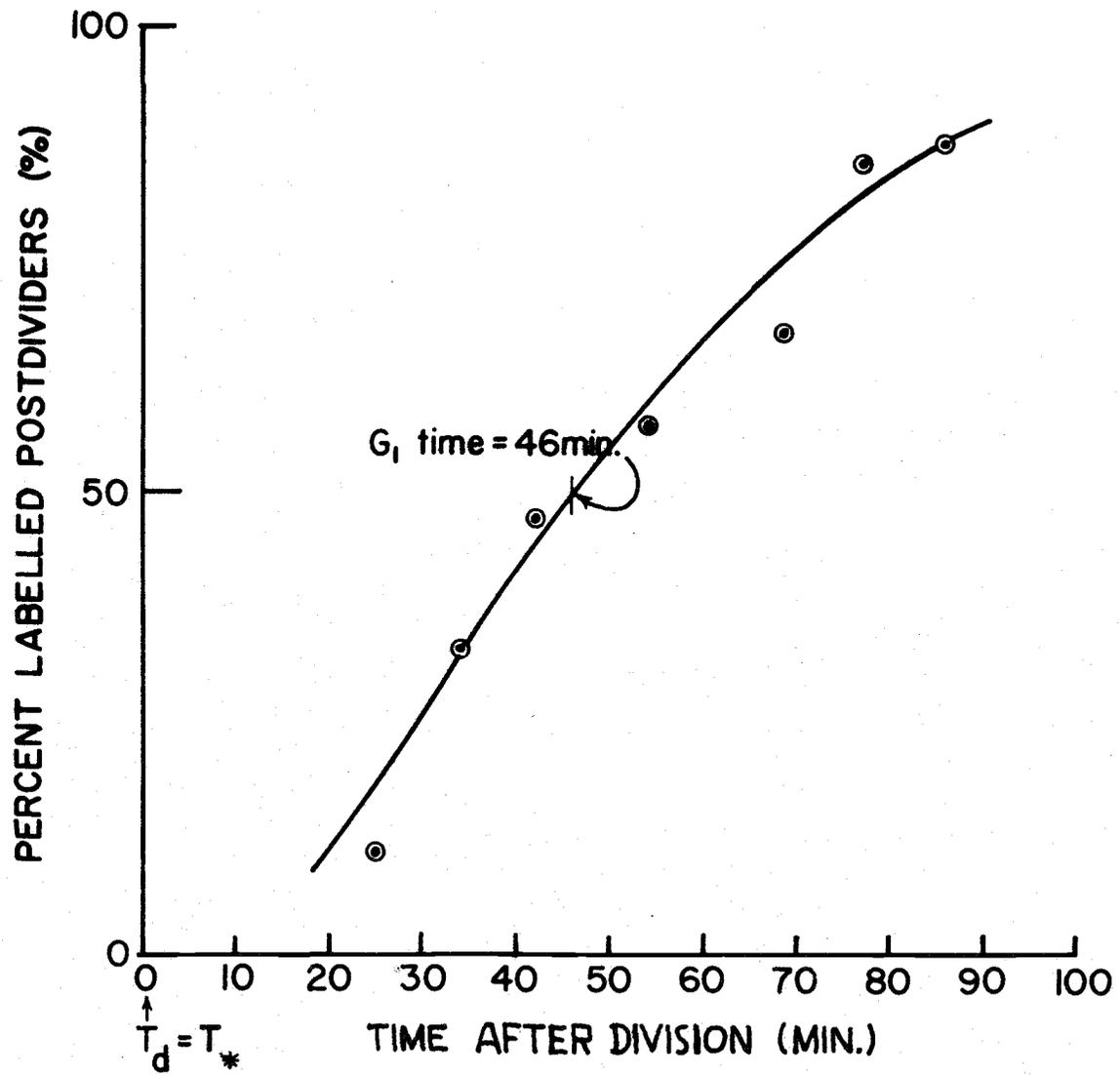


Table 2. Dissection of the cell cycle of Tetrahymena pyriformis (GL-I) under normal log phase growth conditions. ^a

Cell Cycle Phase	Methods	No. of Experiments	Average No. Cells Per Sample	Average Time \pm S. D. (min.)	% of Gen. Time
D	Direct observation	5	9	13.6 \pm 2.6	6.6
	D-Index	4	200	14.0 \pm 5.8	6.8
G ₁	Continuous Labeling	11	11	51.4 \pm 5.6	24.9
S	Subtraction ^b	--	--	86.3 -	41.8
G ₂	Continuous Labeling	21	17	55.4 \pm 5.7	26.8
GT	Exact Method	27	20	206.7 \pm 12.2	100.0

^aThe cells were in log phase growth at 24°C in Tyess medium.

^bThe division time used in the subtraction method was derived from direct observation (13.6 min.) because when derived by the D-Index method it had such a large standard deviation.

sections.) The primary disadvantages of these techniques are their relatively high cost and the great amount of time consumed in obtaining results. It is therefore beneficial to find a method of cell cycle analysis which retains the accuracy of radiotracer techniques but is less time consuming and less costly.

The established fact that, in many cell lines, HU blocks progression of only S-phase cells through the cell cycle has led to investigations with Tetrahymena pyriformis that are designed to reveal the exact position of the cell cycle that is affected by HU and the possibility of using HU in simple and inexpensive methods of cell cycle analysis. In addition, it was beneficial to investigate the metabolic effects of HU upon replicative DNA synthesis in order to better understand the S-phase arrest phenomenon, recovery from the S-phase arrest and the dynamics of DNA replication per se.

The Post-HU Block Period

Hydroxyurea at 5 to 25mM concentrations was found to arrest exponential growth, after some minutes delay, of Tetrahymena pyriformis populations. Concentrations less than 5mM were found to delay but not completely inhibit cell division and population growth. (See Figure 6.) A 100 mM HU concentration caused immediate death of the cells while a 50 mM HU concentration caused death after several hours delay. For subsequent studies a 10mM concentration

was used because it did not kill cells in normal log growth phase. (See Figure 8 and the discussion on page 59.)

It was initially observed that, if a synchronously dividing cell population was exposed to 10mM HU late in the cell cycle, a fraction of cells would proceed through the next cell division. (See Figure 6.) These cells were evidently past the HU-block in the cell cycle; it was hypothesized they were in G_2 and possibly in late S-phase when the HU was introduced.

A simple technique, which may be called the "time-percentage intersect method," was developed in order to define the temporal parameter of the post-HU-block period late in the cell cycle. Several samples of 25 to 30 dividers were picked from a LGP population and isolated into 0.1 ml drops of medium. Such selected dividers progress fairly synchronously through their cell cycle. These synchronous cell samples were then subjected to incubation in 10mM HU starting at various times during the latter half of their cell cycle. The percentage of cells in each sample that went on to divide, i. e., the percentage of cells beyond the HU-block, was then correlated with the time of HU introduction for each sample thus allowing the interpolation of a curve representing the HU-block/post-HU-block boundary (Figure 7). The curve was derived from the intersecting points of HU introduction time with the percentage of cells eventually

Figure 6. The effects of the addition of HU to the medium at various times of the cell cycle upon cellular reproduction in Tetrahymena pyriformis. The microdrop culture method was employed for convenient cell counting. Each curve represents cell population growth in microdrop cultures started at time zero (time = 0 min.) from 30 post-dividers. Thus the sample populations were synchronously dividing. The control generation time was 220 minutes in this experiment. Δ) control; \bullet) 2mM HU added 30 min. after the time of division (T_d); o) 10mM HU added 30 min. after T_d ; \blacksquare) 10mM HU added 115 min. after T_d ; \bullet) 10mM HU added 140 min. after T_d .

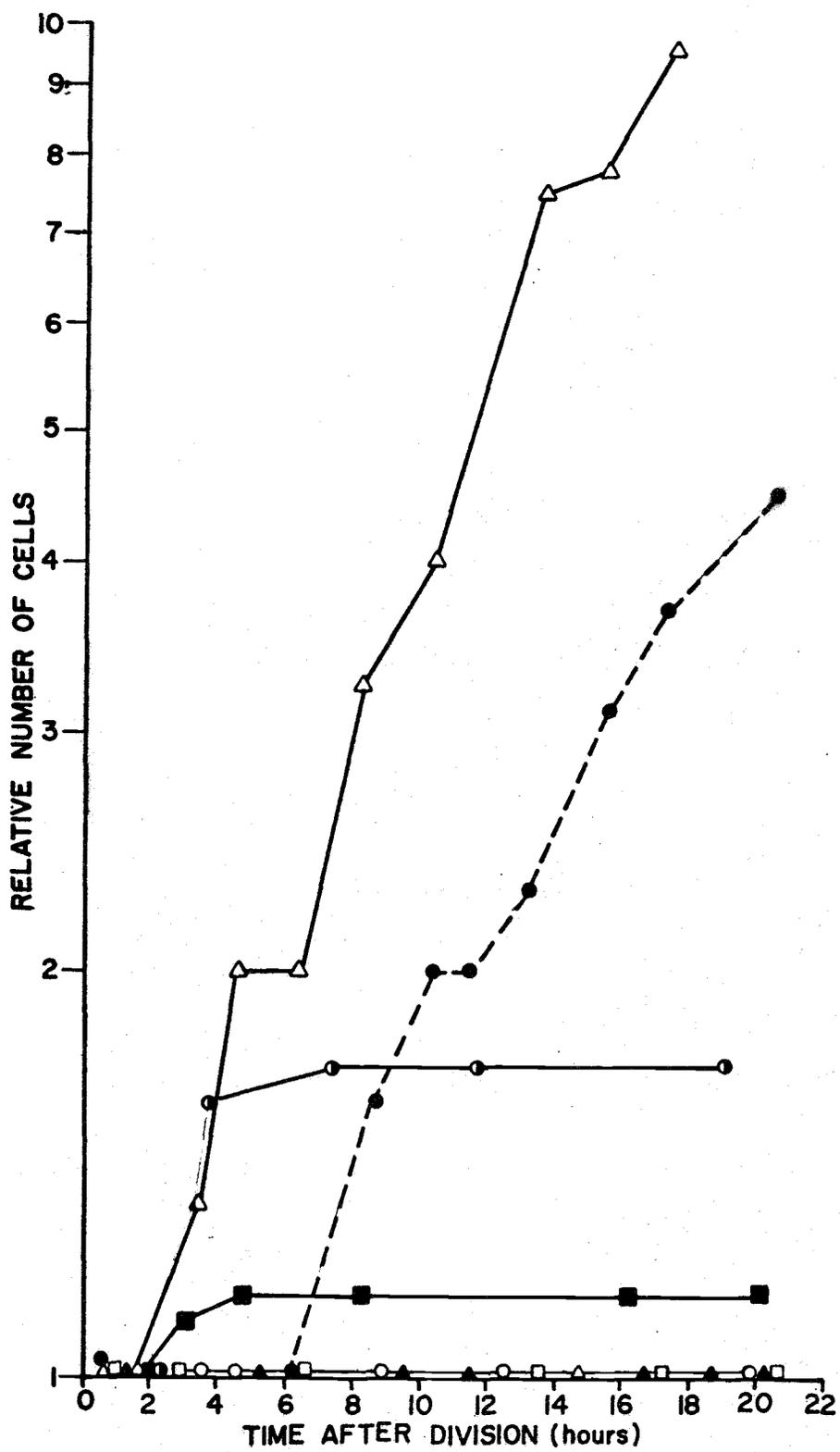
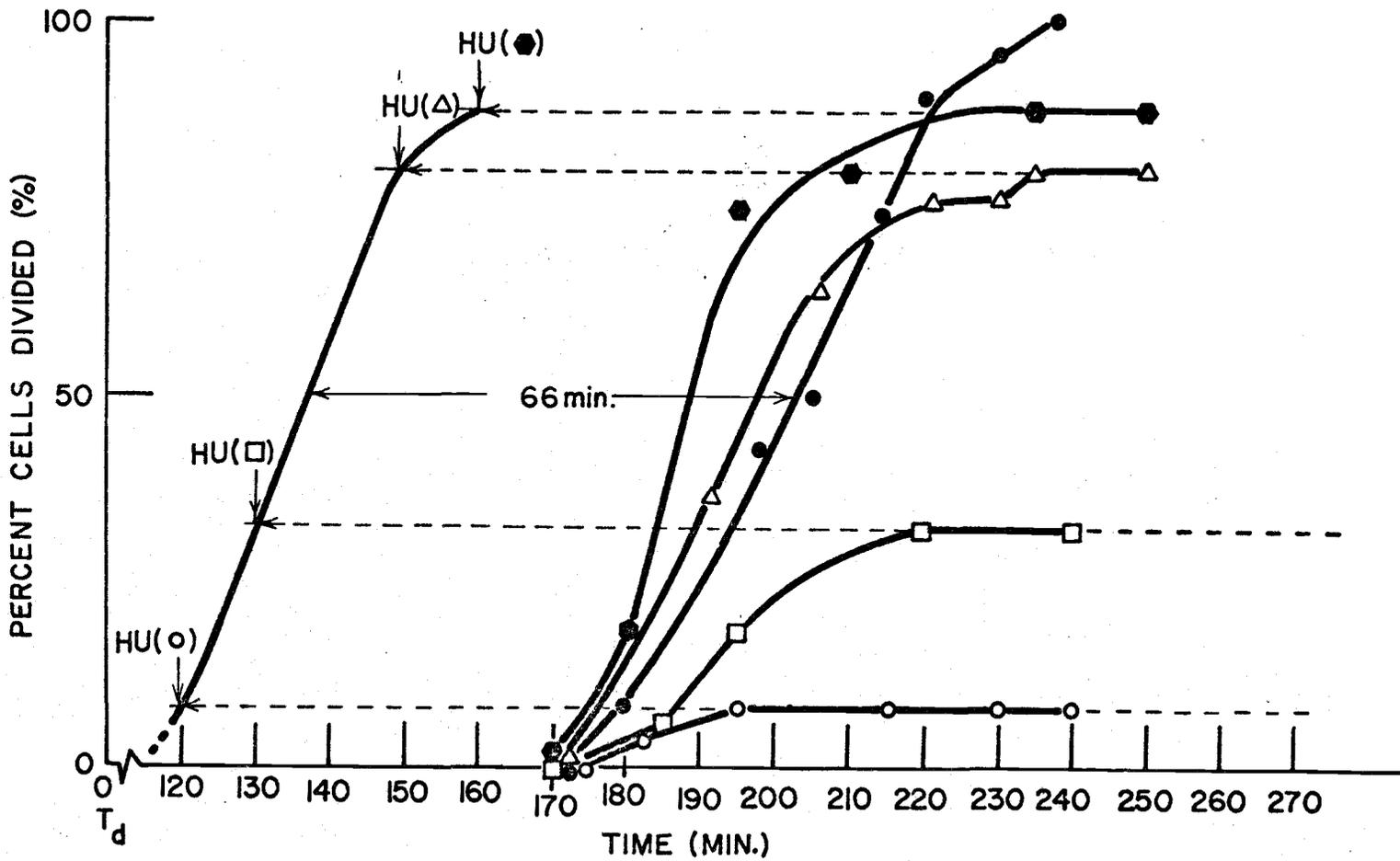


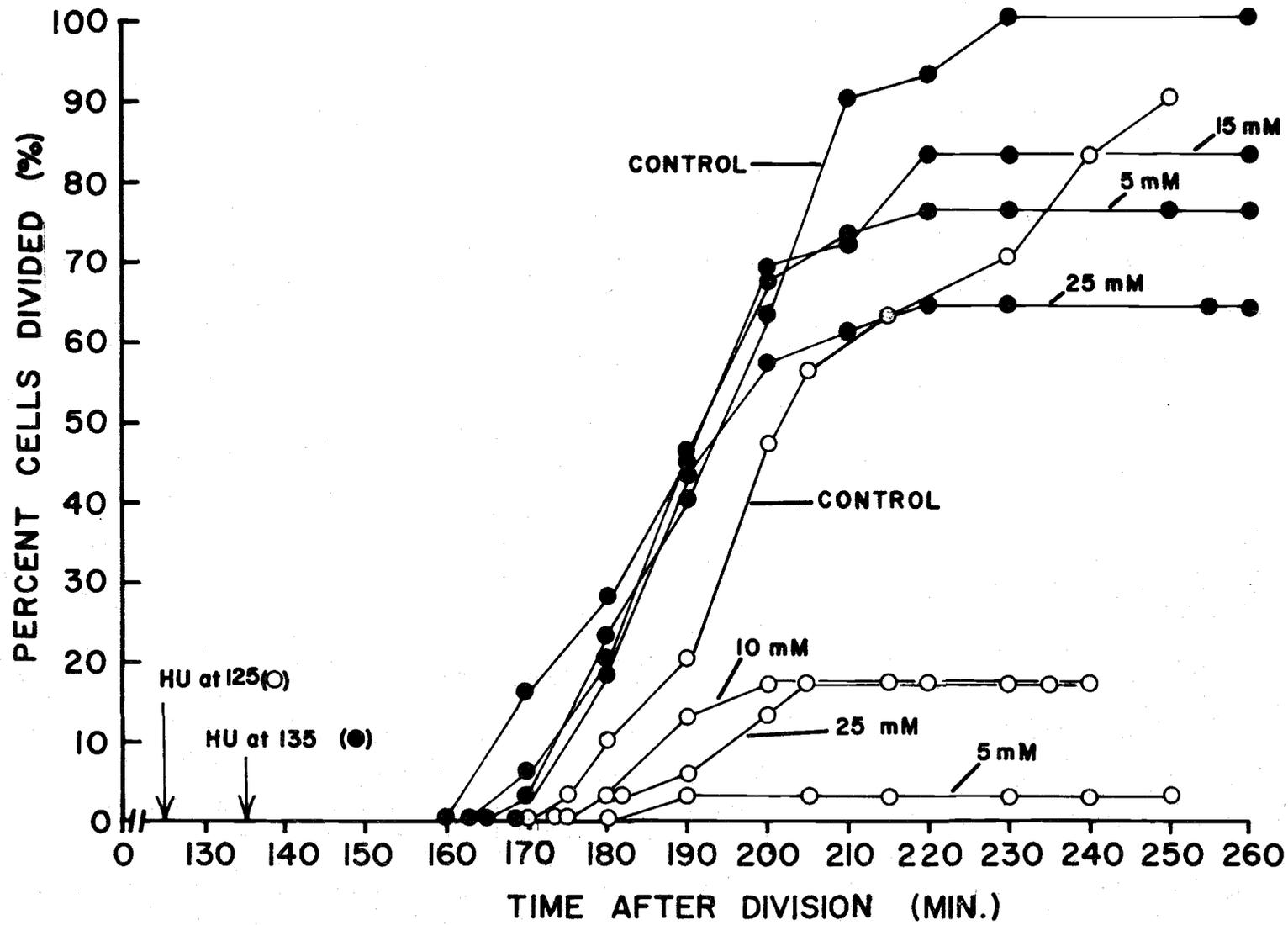
Figure 7. The time-percentage intersect method of determining the duration of the post-HU-block period. The curve on the left represents the HU-block boundary and was derived by connecting the points (\downarrow) representing the intersects of HU introduction times with the corresponding accumulated percentages of cell beyond the block (percent cells divided). The control (\bullet) curve indicates that the GT = 203 minutes. The HU-block boundary is estimated to be 66 minutes prior to the 50% cell division time. This correlates fairly well with the expected 69 minutes for the G_2 plus D time. (See Table 2.) o) HU introduced at time = 120 min.; \square) HU introduced at time = 130 min.; Δ) introduced at time = 149 min.; \bullet)HU introduced at time = 160 min.



dividing in each corresponding sample. It can be seen from Figure 8 that as time passes the percentage of cells beyond the HU-block increases until eventually the introduction of HU has no effect on any of the cells and 100% of them go on to divide. In every experiment this HU-block/post-HU-block boundary curve appeared to be the result of a lognormal distribution similar to that expected for the S/G_2 boundary (Nachtwey and Cameron, 1968). Therefore, the time when 50% of the synchronously dividing cells are beyond the HU-block can be subtracted from the total generation time to get the duration of the post-HU-block period. The post-HU-block period always corresponded well with the $G_2 + D$ time duration. Therefore, the HU-block/post-HU-block boundary corresponded equally well with the S/G_2 boundary as defined by classical means of cell cycle analysis. (See the legend under Figure 7 for further details.)

In order to better define the HU-block boundary it was necessary to determine whether or not the rate of entrance of HU into the cell was a significant factor in determining the fraction of cells allowed to progress beyond the HU-block. Thus it was necessary to see if the concentration of HU introduced had an effect on the time of blocking as reflected in the percentage of cells blocked. Experiments involving the introduction of different concentrations of HU, from 5mM to 25mM, at the same time in the cell cycle of synchronously dividing cells revealed no clearcut increase in the percentage of cells blocked with

Figure 8. The effect of HU concentration upon the time of HU-block as represented by the percentage of cells blocked. The solid circle curves (●) represent the results of an experiment where HU was introduced to a synchronously dividing population at 135 minutes after completion of division (late S and early G₂). The open circle curves (○) represent results from an experiment where HU was introduced at 125 minutes after the completion of cell division. The various HU concentrations for each sample are designated next to their respective curves. Each curve represents a starting sample of 30 cells.



increased HU concentration. These results are exemplified by the results of two such experiments presented in Figure 8. It is obvious from the graph that the percentage of cells blocked is random when related to the concentration of HU in the concentration range tested (5-25mM). It was therefore concluded that the use of 10mM HU was adequate for defining the boundaries of the HU-block.

A simpler method was devised for determining the duration of the post-HU-block period. This method merely takes into account how long exponential growth continues after HU is added to a LGP cell population. Since only post-HU-block cells will contribute to population growth through their ability to undergo division, the time elapsed from the addition of HU until the cessation of exponential growth defines the temporal parameter of the post-HU-block period. The HU was introduced into a LGP population at time zero ($t_0 = t_{HU}$) and, in order to facilitate ease in counting, the cells were then isolated into micro-drops of HU-containing medium as previously described in the Methods and Materials section. The cells were counted every 10 or 15 minutes and their exponential growth was compared to that of the control cells (which had similarly been prepared but without HU). After a period of time the HU-treated cells ceased to increase in number because all cells were effectively being blocked from further cell division. This growth cessation plateau was extrapolated back to the exponential growth curve of controls to determine the time point of

growth cessation. The post-HU-block duration was then defined as the interval from the HU introduction time to the intersect of the growth cessation plateau with the control growth curve (Figure 9). Although the exponential growth arrest method is not affected by the age gradient, it may be subject to some degree of error involved in locating the control curve. The probability for error here can be greatly alleviated by linear regression analysis.

Another method of determining the post-HU-block time period is through direct calculations that account for the cell cycle age gradient. The fraction of cells from a random sample that is in any one particular phase of the cell cycle is directly related to the time spent in that phase. So, if the GT is known, the duration of the specific phase under study can be roughly estimated from the product of the fraction of cells in the specific phase and the GT. However, it must be emphasized that this is only a rough estimation because it does not account for the age gradient that is characteristic of exponential cell proliferation. The age gradient is described by Nachtwey and Cameron (1968) as follows: "Because one cell at the end of its cell cycle divides into two cells, there are twice as many just-divided cells as there are cells just ready to divide. Between these two extremes, the frequency of cells of any particular age decreases exponentially." Figure 10 illustrates the relationship of the fractions of cells in the various phases relative to the total number of cells in the

Figure 9. The exponential growth arrest method of determining the duration of the post-HU-block period. The open circled curve (O) indicates growth in the presence of 10mM HU and the closed circle curve (●) represents growth in the absence of HU. The generation time for the control sample is 197 minutes. The HU growth cessation point intersects the control curve at a point equivalent to 67 minutes along the abscissa. The post-HU-block period is, therefore, approximately 67 minutes in duration.

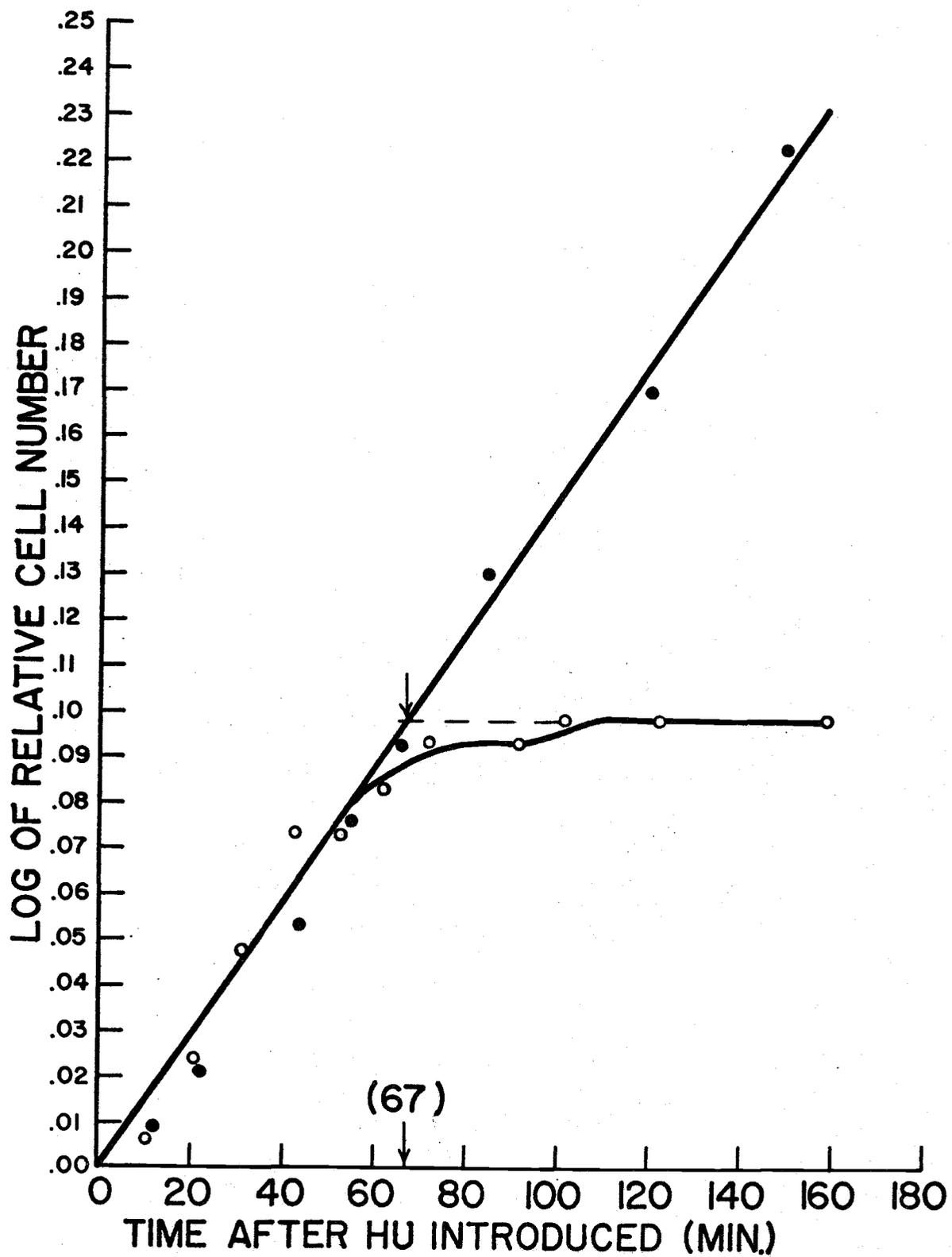
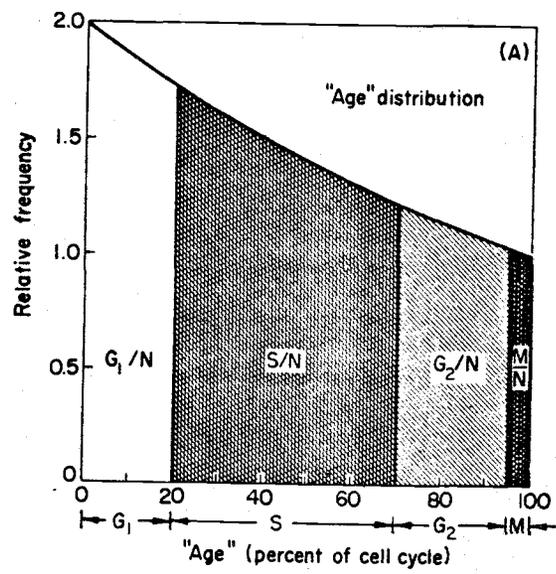


Figure 10. Age distribution in an exponentially proliferating cell population. G_1/N = fraction of cells in G_1 ; S/N = fraction of cells in S; G_2/N = fraction of cells in G_2 ; M/N fraction of cells in mitosis. (From Nachtwey and Cameron, 1968)



sample population. It becomes obvious from this relationship that the proportion of cells in any particular phase is not necessarily equal to the proportion of the GT spent in that phase. Therefore, the proportion of cells in a cell cycle phase can be used to determine the proportion of time spent in that phase only after a correction for the age gradient has been calculated. Using the basic formula previously described in the section titled Division Time and substituting the values gathered from the same experiment as that used to represent the exponential growth arrest method in Figure 9, the duration of the post-HU-block period may be calculated as follows:

$$t_{\text{post-HU}} = \frac{\text{GT} \ln[(N_{\text{post-HU}}/N) + 1]}{\ln 2}$$

$$t_{\text{post-HU}} = \frac{(198) \ln[(18/71) + 1]}{.693} = \frac{(198) \ln(1.254)}{.693}$$

$$t_{\text{post-HU}} = \frac{(198) \cdot 22670}{.693} = 64.8 \text{ minutes}$$

Eight experiments of the type described above, involving over eleven hundred cells, were conducted. The data from these experiments were combined after weighting to account for variation in sample size. The post-HU-block time from these combined data was calculated, using the age gradient formula above, to be 66 ± 12 minutes in cells with an average GT of 205 ± 12 min.

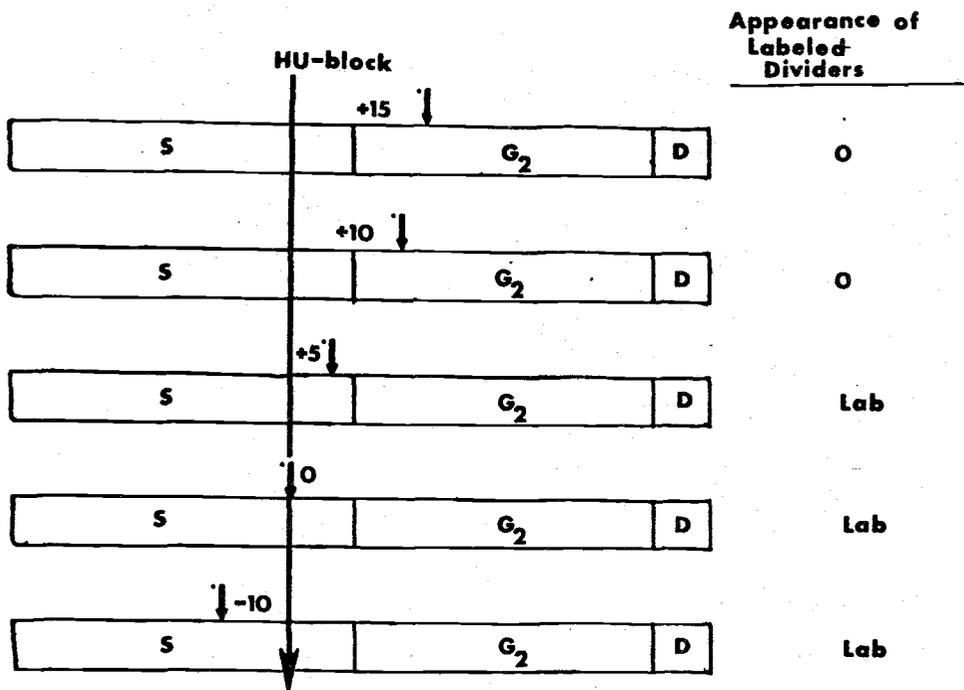
Extent of G₂ and Post-HU-Block Phase Homology

Experiments modified from the technique of Doida and Okada (1969) were designed in order to better resolve any temporal discrepancies that may exist between the S/G₂ and the HU-block/post-HU-block boundaries. These experiments involved comparing the fractions of cells beyond the HU-block that still incorporate ³H-TdR into their nuclear DNA of HU introduction as diagrammed in Figure 11.

Several preliminary experiments of the type diagrammed in Figure 11 showed that the HU-block occurred some time between five and ten minutes prior to the termination of S-phase: When HU was added to a LGP population at either the same time or at five minutes prior to adding the ³H-TdR, some labeled dividers were observed, but when HU was added ten or more minutes prior to the ³H-TdR no labeled dividers were seen.

In order to determine the fraction of cells in S-phase that are past the HU-block and the duration of the post-HU-block-S-phase, it was necessary to know the percentage of all cells in the post-HU-block period and the percentage of labeled cells in the post-HU-block period as well as the division time (t_D), G₂ time (t_{G_2}), and generation time (GT) of the cells under study. This was accomplished by adding the ³H-TdR and the HU to exponentially growing cells at the same point in

Figure 11. Determining the amount of coincidence between the S/G₂ border and the HU-block/post-HU-block border. The purpose of these experiments was to determine if any cells were synthesizing DNA after the HU was introduced and, if so, their frequency. The arrows mark the intervals (+15, +10, +5, 0, and -10 min.) between the addition of HU (long solid arrow) and the addition of H-TdR (*). These intervals were correlated with the appearance of labeled dividers during division. The above diagram indicates the HU-block to be a little over five minutes prior to the end of S-phase.



time and then isolating a random sample of at least one hundred cells into micro-drops under paraffin oil. Dividers were then counted at ten minute intervals and removed from the micro-drops for autoradiographic preparation. The percentage of dividers, both labeled and non-labeled, was then recorded (Figure 12). The post-HU-block time period was calculated from the total accumulated fraction of dividers, i. e. from the fraction of the treated population that was able to divide in HU, and compared to the G_2 plus D-phase time ($t_{D_2} + t_D$) which was determined from control samples of the population by the Continuous Labeling and the D-Index Methods previously described. The accumulated fraction of labeled dividers, after correcting for the age gradient, allowed the post-HU-block period to be temporally dissected into S-phase and post-S-phase portions. The S-phase portion, about 7 minutes, roughly accounted for the time discrepancy between the $G_2 + D$ period ($t_{G_2} + t_D = 60$ min.) and the post-HU-block period ($t_{\text{post-HU}} = 69$ min.). Table 3 summarizes the data from this and the preceding experiments and compares the time durations of the post-HU-block period and the G_2 plus D period. It can be seen from row 5 that the S-phase portion of the post-HU-block period accounts for about 7 minutes of the 206 minute generation time, or the terminal 8% of S-phase ($7 \text{ min}/86 \text{ min} \times 100$).

Figure 12. Resolving the exact position of the HU-block/post-HU-block border relative to the end of S-phase. The solid circles (●) represent the total fraction of dividers seen at various times after the addition of HU, the open circles solid line (-0-) represent fraction of labeled dividers, and the open circle broken line (--0--) curve represents the total minus the labeled dividers fraction for each time. The cross-hatched area represents the accumulated fraction of post-HU-block cells were still in S-phase at the time of HU addition. See text for further explanation.

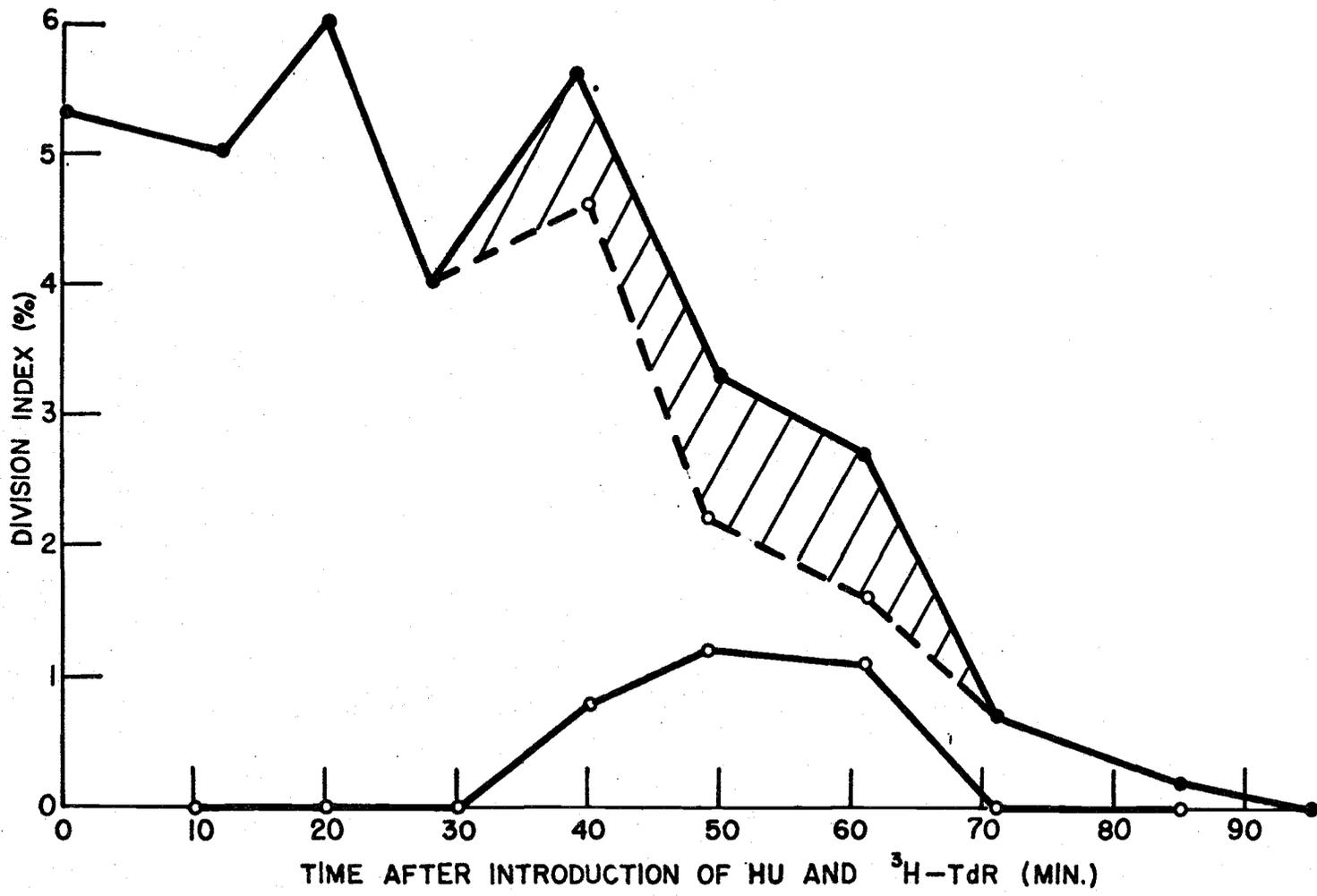


Table 3. A comparison of the durations of the post-HU-block period and the G₂ + D period.

Number of experiments	Total sample size	Accumulated dividers	Labeled dividers	GT ± (min.)	t _{G₂} ± (min.)	t _D ± (min.)	t _{G₂ + D} (min.)	t _{post-HU} (min.)	(t _{post-HU}) (min.)	t _{post-HU(S)} (min.)
1. 53	1000	-	-	204±13	55±6	14±3	69	-	-	-
2. 5	700	-	-	208±14	-	-	-	67±6	-	-
3. 1	71	18	-	198 -	-	-	-	67 -	-	-
4. 8	1174	294	-	205±12	-	-	-	66±12	-	-
5. 4	737	191	16	206±11	46	14±5	60	69±12	9 min.	7

Row 1 is control data derived by cell cycle analysis employing ³H-TdR uptake (Table 2). Row 2 indicates the duration of the post-HU-block (t_{post-HU}) derived by the time-percent intersect method (Figure 7). Row 3 shows the duration of the post-HU-block period derived by the exponential growth arrest (extrapolation) method (Figure 9). Row 4 presents a t_{post-HU} derived by the exponential growth arrest (calculation) method (page 68). Row 5 presents the results of four experiments designed to determine the degree of coincidence between the S/G₂ and HU-block/post-HU-block borders. These experiments combined ³H-TdR uptake with the HU-induced exponential growth arrest method to determine the total accumulated D-index (the sum of all the cells which undergo division divided by the total sample size) and the accumulated D-index with labeled macronuclei. Standard ³H-TdR cell cycle analysis controls reveal the duration of G₂ + D periods to be 60 minutes. The time fraction of post-HU-block period still undergoing DNA replication (t_{post-HU(S)} = 7 min.) compares well with the difference in time between the control G₂ + D time and the post-HU-block time (69-60 = 9 min.).

The results discussed above indicate that HU blocks cells in the first 92% of the S-phase. The results also indicate that HU has no significant effect on the progression through the G₂-phase.

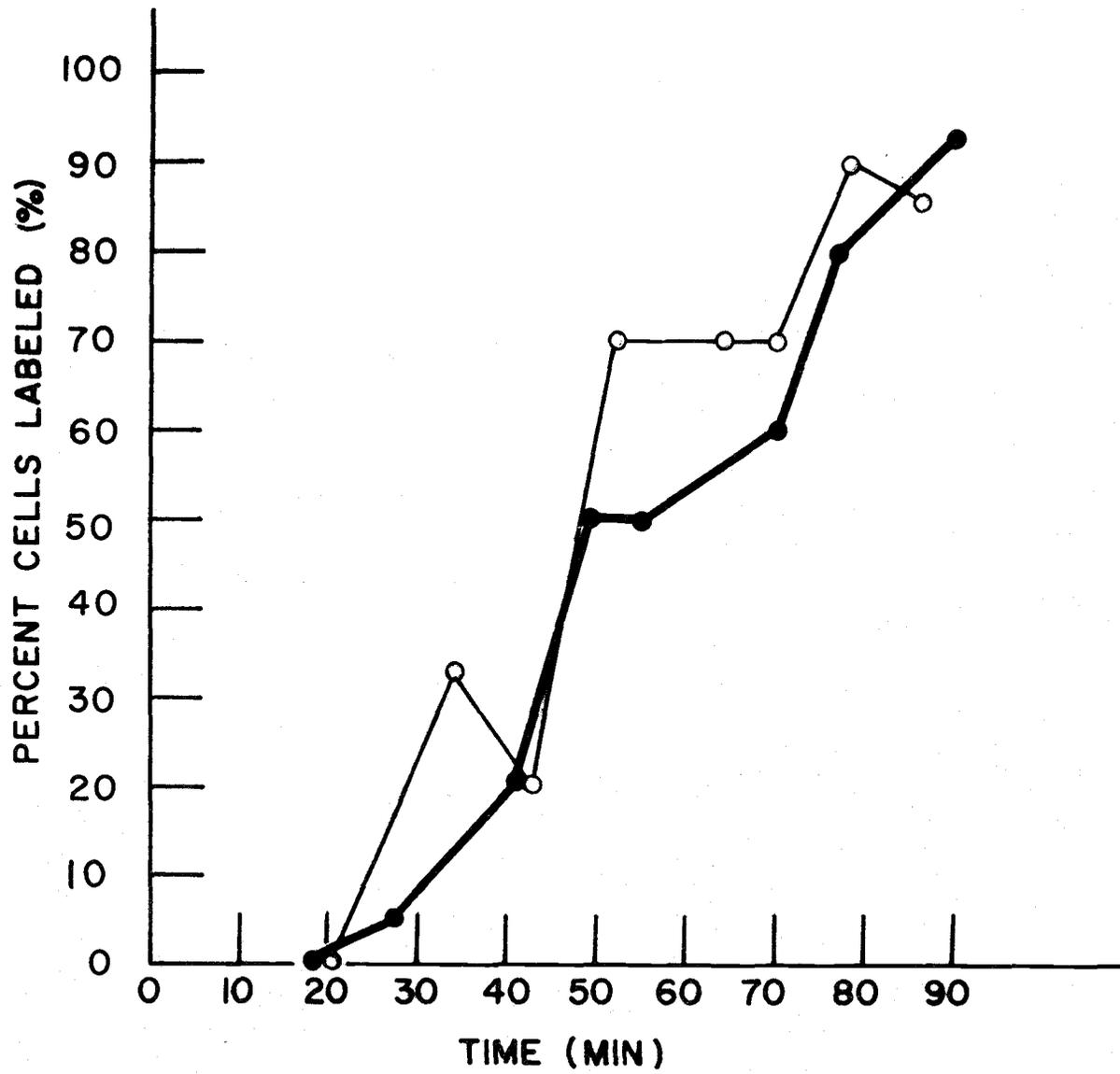
HU Effects on G₁ Cells

Experiments to ascertain the effects of HU on cells in the G₁-phase were conducted. Dividing cells were selected and pipetted into microdrops of medium containing HU and ³H-TdR. Control cells were pipetted into medium containing ³H-TdR alone. Samples were removed at 10 to 20 minute intervals following division and prepared for autoradiography to determine the presence of labeled nuclei, i. e. entry into S-phase. Figure 13 shows that cells in HU progress through G₁ and enter S-phase in the same time as cells not in HU, indicating HU has no effect on G₁ cells or on the passage of the G₁/S border.

HU Effects on DNA Synthesis

As pointed out above, cells can initiate DNA synthesis, i. e., incorporate ³H-TdR into DNA even in the presence of HU. Therefore, HU does not lead to the accumulation of Tetrahymena cells at the G₁/S border as it does with other cell types (Sinclair, 1967; Bacchetti and Whitmore, 1969). Moreover, some DNA synthesis can continue

Figure 13. The effect of HU upon the progression of cells through the G₁ period. The graph presents the results of standard G₁ time analysis for both control (●) and 10mM HU-treated cells (○). Postdividers were placed in 5uCi/ml ³H-TdR (and 10mM HU in the case of the HU-treated cells) and cell samples were removed at subsequent time intervals and prepared for autoradiography. The time of 50% cell separation for these synchronously dividing cell samples was time zero (time = 0 min.). Each point represents a sample size of 20 cells.



in HU-blocked cells. It has been determined that over 90% of the cells that accumulate at S-phase in HU-treated cultures are synthesizing DNA even after having been subjected to the HU treatment for several days. For example, one experiment involved the incubation of LGP cells in Tyess medium containing 10mM HU until most of the cells had collected in S-phase. The cell samples were then terminal-labeled for 15 minutes and prepared for autoradiography starting at 125 minutes, 170 minutes, 270 minutes, and 2 days after the HU had been introduced. The percent labeled cells for each HU exposure time was 84% (n = 86), 92% (n = 89), 97% (n = 74), and 99% (n = 80) respectively. Although the HU treated cells continued to synthesize DNA, they apparently did so at a lower rate. This was indicated by their decreased nuclear label intensity as compared to that of non-HU-treated controls.

Results of another experiment exemplify the typical difference in rates of ^3H -TdR uptake between HU-treated and untreated S-phase cells. In this experiment cells were incubated for 10 days in Tyess medium containing 10mM HU. They were then terminal-labeled, as were the non-HU treated LGP control cells, for various time durations. The data are presented in Table 4. In every experiment the HU reduced the DNA synthesis rate (as determined by grain counts) to about 20-30% of that of the non-HU treated S-phase cells.

Table 4. The effect of HU upon the rate of nuclear DNA synthesis in Tetrahymena pyriformis.

Treatment	³ H-TdR Incubation Time				
	7 min.	14 min.	30 min.	60 min.	3 hrs.
Non-HU Control	136	142	212	400	--
10mM HU	27	48	46	84	138
Rel. % HU-S-phase Lab.*	20%	34%	22%	21%	--

Average grain counts for normal LGP cells in S-phase are compared with those for HU treated S-phase cells which were incubated in HU starting 10 days prior to the introduction of ³H-TdR. Counts were made for 20 HU-treated and 20 control cells for each labeling period.

*The relative percentage of S-phase labeling in the presence of HU was determined by dividing the grain counts for control cells by the grain counts for HU-treated cells and multiplying by 100.

Experiments were designed to help elucidate the nature of the DNA synthesized during exposure to 10mM HU. Although it was not determined in the course of this study whether the label incorporation during HU-treatment was the result of replicative or repair DNA synthesis, the problem of how stable is the DNA which is synthesized during treatment with HU was challenged in terms of observing its relative turnover rate. An experiment was designed to answer the following question: In the presence of HU is the cell synthesizing some type of aberrant DNA that is repeatedly being detected and excised by a repair synthesis system or is this newly synthesized DNA fixed into the regular DNA complement of the cell? Labeling experiments, such as diagrammed in Table 5, showed that the intensity of

nuclear label was not apparently affected by pre- or post-treatment with HU. Only the amount of ^3H -TdR incorporation per se was affected by the presence of HU at the time of labeling. The results of treatment 1 indicate that HU-exposure does not result in the breakdown and complete loss of DNA synthesized previous to the time of HU addition. The results of treatment 2 indicate that cells incubated in the presence of HU still take up label albeit at a lower rate than when HU is not present at the time of labeling. The results of treatment 3 show that HU-treatment before, during, and after labeling does not affect the turnover rate of incorporated nucleotides in terms of label-index or intensity of label (when compared to the results of treatment 2). Therefore, DNA synthesized in the presence of HU does not apparently have a rapid turnover rate, although breakdown and resynthesis is not precluded.

Recovery from the HU Block

Hydroxyurea-blocked cells that were washed by a series of centrifugations and medium rinses recovered division activity after a certain lag or recovery period. The rate of recovery from HU exposure; i. e., the time from HU removal to division, was observed to be dependent upon the time in the cell cycle during which HU was first introduced. Synchronously dividing cells were exposed for a fixed

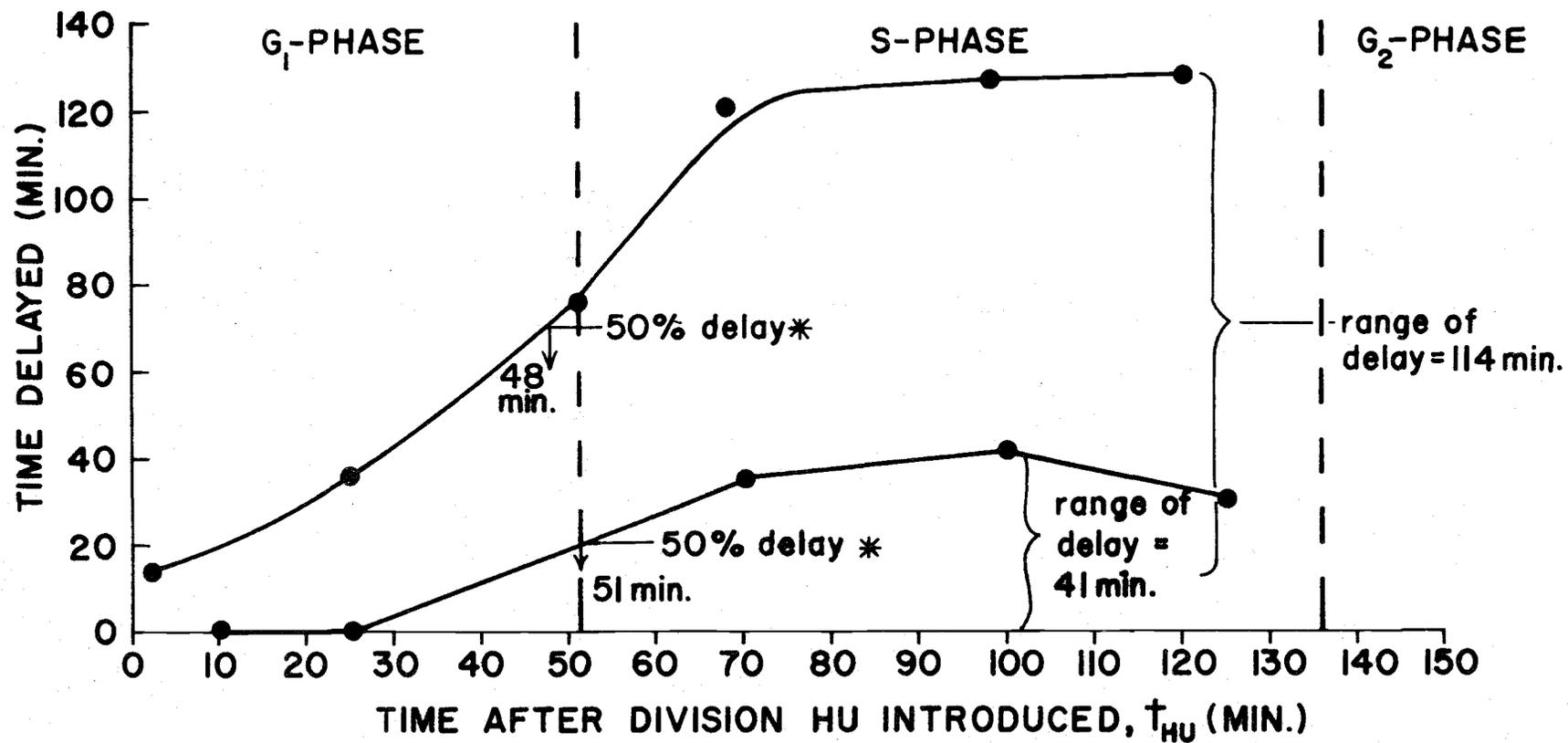
Table 5. Relative turnover rate of DNA synthesized in the presence of HU.

	Treatment	Fraction Cells Labeled	Percent Cells Labeled (%)
1*	$^3\text{H-TdR}$ (1 hr.) → wash → HU (4 hrs.) → Autoradiographs	53/53	100
2	HU (4 hrs.) → $^3\text{H-TdR}$ + HU (1 hr.) → Autoradiographs	69/71	97*
3	HU (4 hrs.) → $^3\text{H-TdR}$ + HU (1 hr.) → wash → HU (4 hrs.) → Autoradiographs	73/74	99*

* The cells of treatment 1 were "synchronized" by isolating dividers so that they were known to receive their pulse label during S-phase. Treatments 2 and 3 employed cells picked randomly from a LGP culture treated with HU 4 hours previously. The stars (*) indicate lightly labeled cells (about 20% as intensely labeled as treatment 1 cells) when $^3\text{H-TdR}$ incorporation is in the presence of HU.

period of time to 10mM HU introduced at various times during G_1 and S-phase. After a period of time, usually four and a half hours, the HU was removed from the medium by a series of centrifugation washes. The cells were then isolated in micro-drop cultures and periodically counted to determine the 50% cell separation time marking the first recovery division. The generation times of HU-treated cells, after subtracting out the time of exposure to HU, were compared to the control generation times in each experiment in order to detect differences in recovery times for cells exposed to HU at the different times in the cell cycle. The difference between the HU-treated and control cell generation times was designated the recovery delay time. Figure 14 presents the combined results of five experiments involving a 4 1/2 hour HU-exposure along with one experiment involving a 65 min. HU-exposure. The recovery delay times were minimal when HU was introduced during early G_1 and maximal when HU was introduced during S-phase. Introduction of HU during S-phase resulted in about the same amount of recovery delay no matter which portion of the S-phase the cells were in at the time the HU was first introduced. Notice that the half-maximal delay point, the midpoint in the increase of delay times from minimum to maximum, corresponds closely with the previously determined G_1 time of 51 ± 6 minutes. (See Table 2.) Whether this correspondence was merely coincidental or not was not determined during the course of this study.

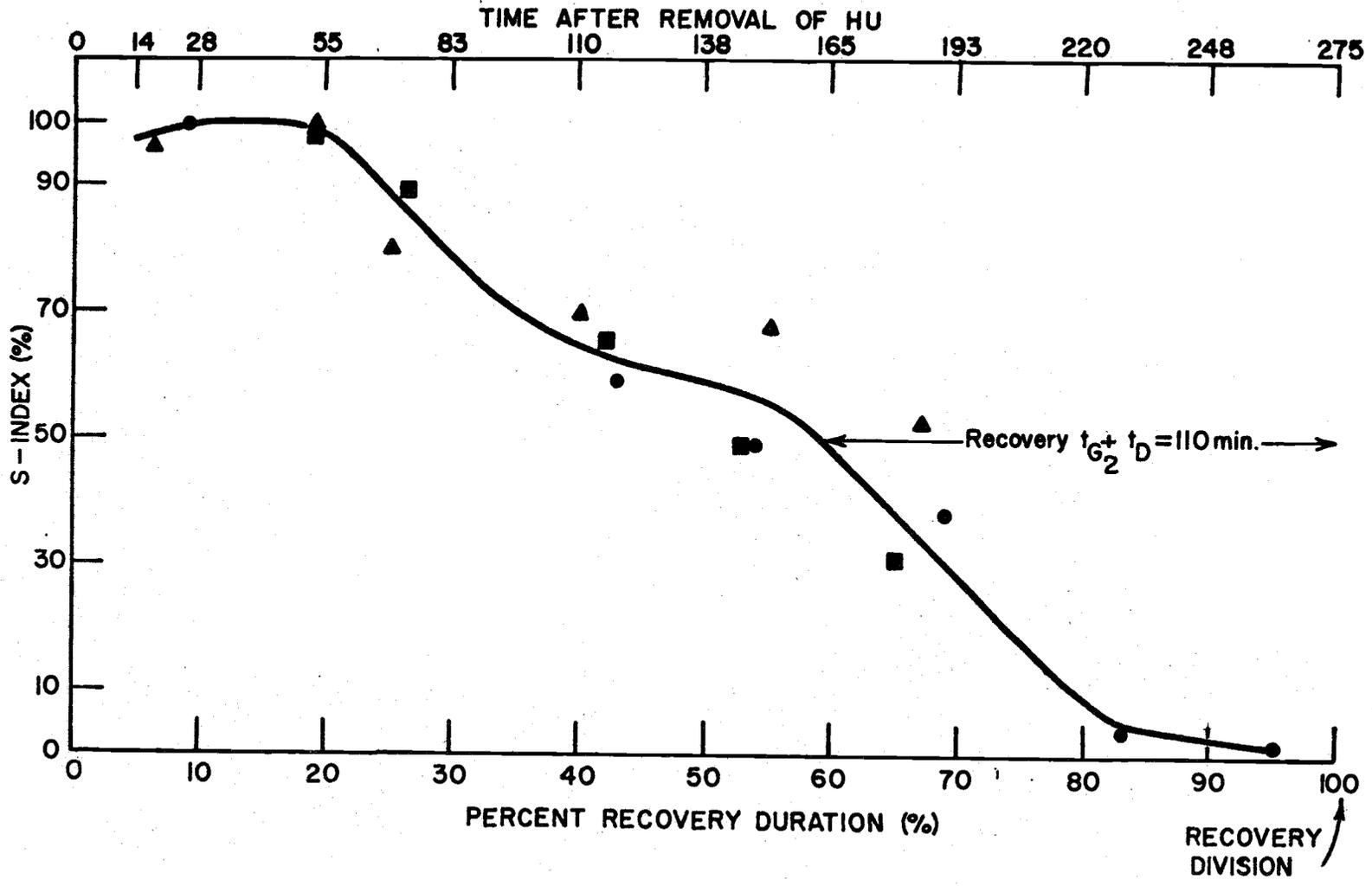
Figure 14. S-phase sensitivity to HU as marked by recovery delay. The upper curve represents the combined data of five experiments each involving a 4 1/2 hour exposure to HU. Each data point is derived from a combined sample size of no less than 90 cells. The lower curve represents the results of an experiment using a 65 minute HU incubation period. Each data point is from a sample size of 30 cells. The abscissa indicates the time during the cell cycle that the HU was introduced and the ordinate indicates the time difference between the combined control generation times and the generation times of the HU-treated cells minus the duration of the exposure. *The 50% delay point is the midpoint of the range of generation time delays from minimum to maximum for each curve.



The recovery period, after exposing a sample of LGP cells to HU for 10 1/2 hours, was analyzed to find when DNA synthesis occurred. This was accomplished by terminal-labeling samples of recovering cells at various times after the removal of hydroxyurea from the medium. (See Figure 15.) The results of these experiments indicate that recovery DNA synthesis begins almost immediately after the removal of HU and continues for most (165 minutes) of the recovery period. This DNA synthesis portion of the recovery period is followed by a long (110 minutes) $G_2 + D$ period. The rate of decrease in the S-index appears, for reasons unknown to the investigator, to be biphasic.

The question immediately comes to mind; Is the DNA which is synthesized in the presence of 10mM HU selectively excised during the recovery period after the HU has been removed? In other words, is this an aberrant species of DNA which must be removed before the cell can resume its normal cell cycle? One experiment that was conducted in order to help resolve this question involved labeling with $^3\text{H-TdR}$ a population of LGP cells which had been exposed to HU for four hours, removing the $^3\text{H-TdR}$ and the HU at the end of the fifth hour, and preparing one sample of these cells for autoradiography. The remaining cells were enriched with 0.8mM concentrations each of the four deoxyribonucleoside precursors of DNA in order to hopefully "chase" any reutilizable breakdown products (as well as residual or pooled radioactive precursors) from the cells. After

Figure 15. Cell cycle analysis of the recovery period. The recovery period is the interval from the time of HU removal to the time when 50% of the cells have divided. The S-indices were obtained by using 10 minute terminal pulses of $^3\text{H-TdR}$ at various times during the recovery period. This graph shows the results of recovery period analysis from three different experiments. ●, ($\bar{n} = 50$); ▲, ($\bar{n} = 30$); ■, ($\bar{n} = 30$). The average time for the recovery period was 275 minutes.

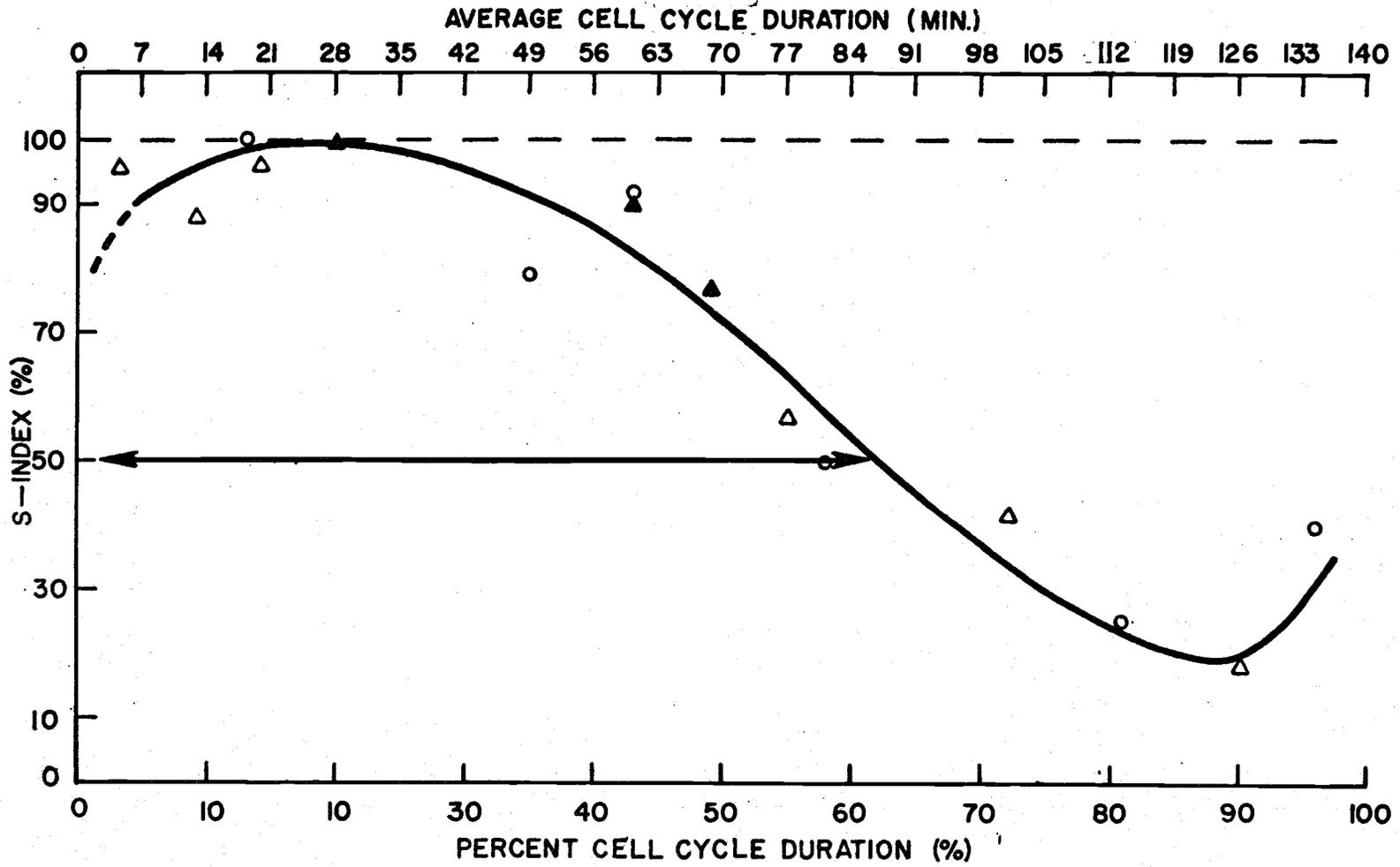


allowing these latter cells to progress 100 minutes (40%) into the recovery period, they were prepared for autoradiography. Grain counts for the two samples were then compared and it was found that there was no qualitative difference in label intensity for the two groups. Therefore, the DNA synthesized in the presence of HU evidently is not being selectively removed by recovering cells in the first 40% of their recovery period.

Early during the investigation of HU-block recovery it was observed that the first complete cell cycle following the recovery period was abbreviated by 50 or 60 minutes. This short cell cycle following recovery was analyzed for its G_1 , S and G_2 phase times by terminal-labeling cell samples at various times after the first recovery division. The specific experimental design is as follows: LGP cells were incubated in 10mM HU for 4 1/2 hours, washed and allowed to progress through the recovery period. Thirty cells were isolated into micro-drop cultures for monitoring the 50% recovery division time. From the remaining cells, samples of 30 to 50 cells were then isolated into drops of medium containing $5\mu\text{G/ml } ^3\text{H-TdR}$ for 10 minute terminal pulses at various times throughout the 1st cell cycle after the recovery division.

Figure 16 presents the results. It is readily noted that almost all cells are in S-phase 5 minutes after division; apparently the G_1 -phase is completely eliminated. The median S-phase time is 87

Figure 16. Analysis of the first complete recovery cell cycle following HU-treatment. The S-indices were obtained by using 10 minute terminal pulses of ^3H -TdR at various times during the cell cycle. The average generation time for these three experiments was 140 minutes. The median S-phase duration (arrow) is 87 minutes. Each data point represents a sample size of at least 20 cells ($\bar{n} = 20$).



minutes as determined from the interval between division and the time at which 50% of the cells no longer become labeled. The G_2 -phase plus division time is 53 minutes as determined from the interval between the end of S-phase and the time of cell separation. These two times compare fairly well with the times expected for these periods, 86 and 69 minutes respectively. (See Table 2 for these expected values.) The abbreviation of the first cell cycle after recovery division following a 4 1/2 hour exposure to HU is thus accounted for by its complete lack of a G_1 -phase.

It may be added that no significant difference in generation times for this first complete cell cycle were observed whether the cells had undergone an early recovery division (190 minutes after the wash) or they had undergone an average recovery period duration (275 minutes).

HU-Block Rescue by Deoxyribonucleosides

As pointed out in the Introduction, the primary mode of action of HU in blocking DNA replication in other cells is its inhibition of the reduction of ribonucleosides and ribonucleotides. Supporting evidence comes from the fact that in several cell lines studied the addition of deoxyribonucleosides reverses the HU-induced block of DNA replication (Adams and Lindsay, 1967; Weiss and Tolmach, 1967; Topping, 1968,

Jasty and Chang, 1970; Rosenkranz and Carr, 1970). It was of major interest to determine if in Tetrahymena the primary mode of action of HU was also the inhibition of reductase enzyme activity.

The addition of 0.5mM each of the four DNA-precursor deoxyribonucleosides (XdRs) to an HU-arrested cell population enabled these cells to resume normal growth and division. Results of experiments involving the XdR rescue of synchronously dividing cells in microdrops which were exposed to HU starting at different times in the cell cycle indicate that cells incubated in HU beginning in G_1 or early S-phase are rescued with little or no time delay while cells incubated in HU beginning in late S-phase are rescued with a significant delay. These rescue experiments also show that HU-treated G_1 cells do not readily progress into the portion of the cell cycle where rescue delay becomes significant. The details of these studies are shown in Figures 17 and 18 and discussed below.

Figure 17 shows the combined results of two experiments. The specific experimental procedures are explained in the legend. It can be observed that, when HU is added at division and is therefore present during G_1 (closed circles), not only is there no delay in the corrected generation times of these cells after XdR rescue, but there is a decrease in these corrected generation times. A simple and plausible explanation is that HU-treated cells are making progress through the cell cycle

Figure 17. S-phase sensitivity to HU as marked by recovery delay when the cells are rescued from the HU-block by 0.5mM XdRs. The graph indicates the results of two experiments normalized for times of XdR introduction by the point cluster method (see Methods and Materials). Each point represents a sample size of 60 cells. The curve marked by closed circles (●) shows the effect upon generation time (GT) when cells incubated in HU from the time of 50% cell separation (T_d) are rescued from the HU-block at various times by the addition of XdRs. The open circled curve (○) represents the effect of HU upon the GT when HU was introduced 90 minutes after T_d . The half-closed circled curve (◐) represents the effect of HU when it was introduced 135 minutes after T_d . The HU incubation times, indicated along the abscissa, were subtracted from the total generation times of rescued cells to get the corrected generation times. The generation time when HU and XdRs were simultaneously added is designated the control GT.

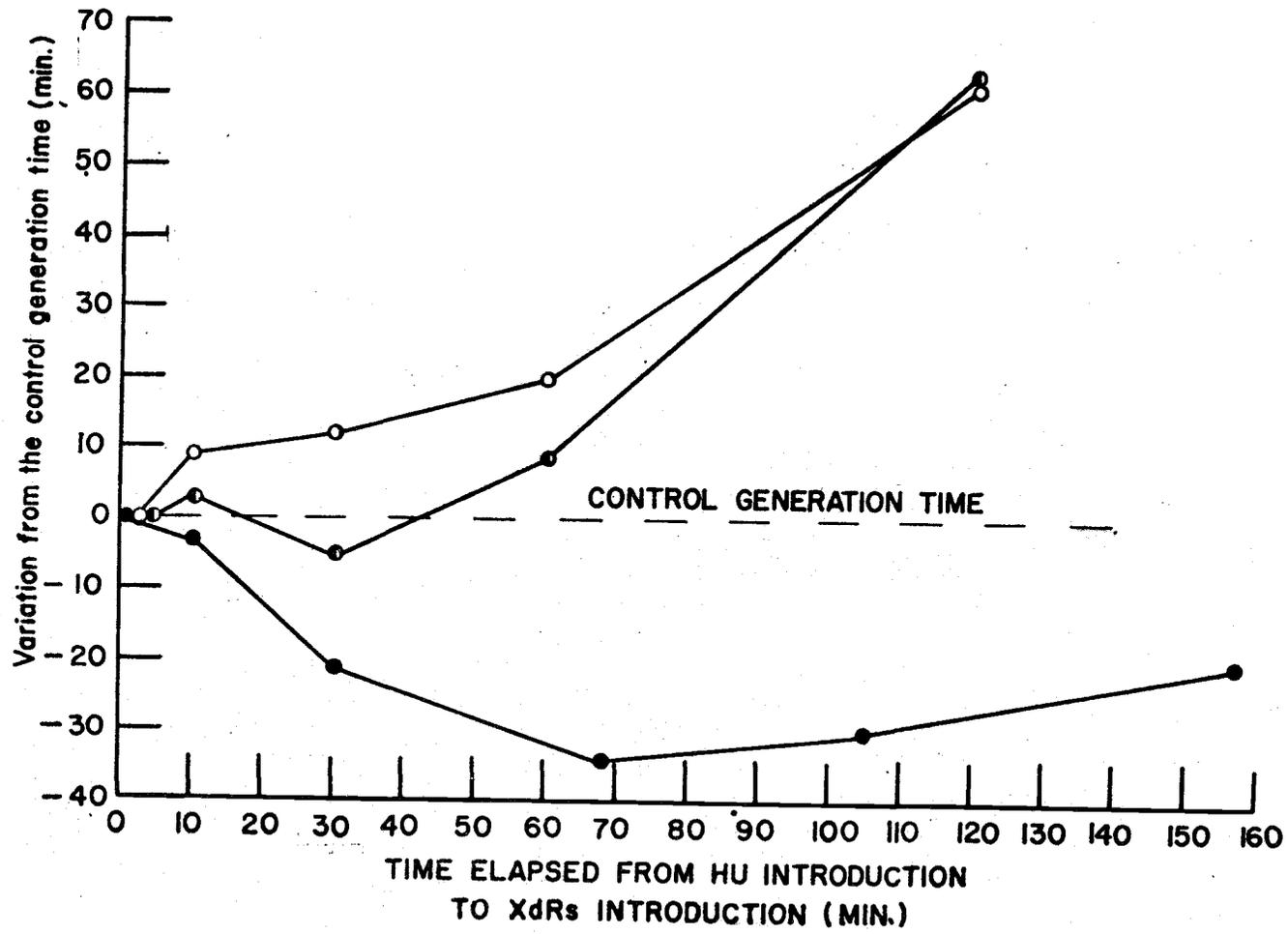
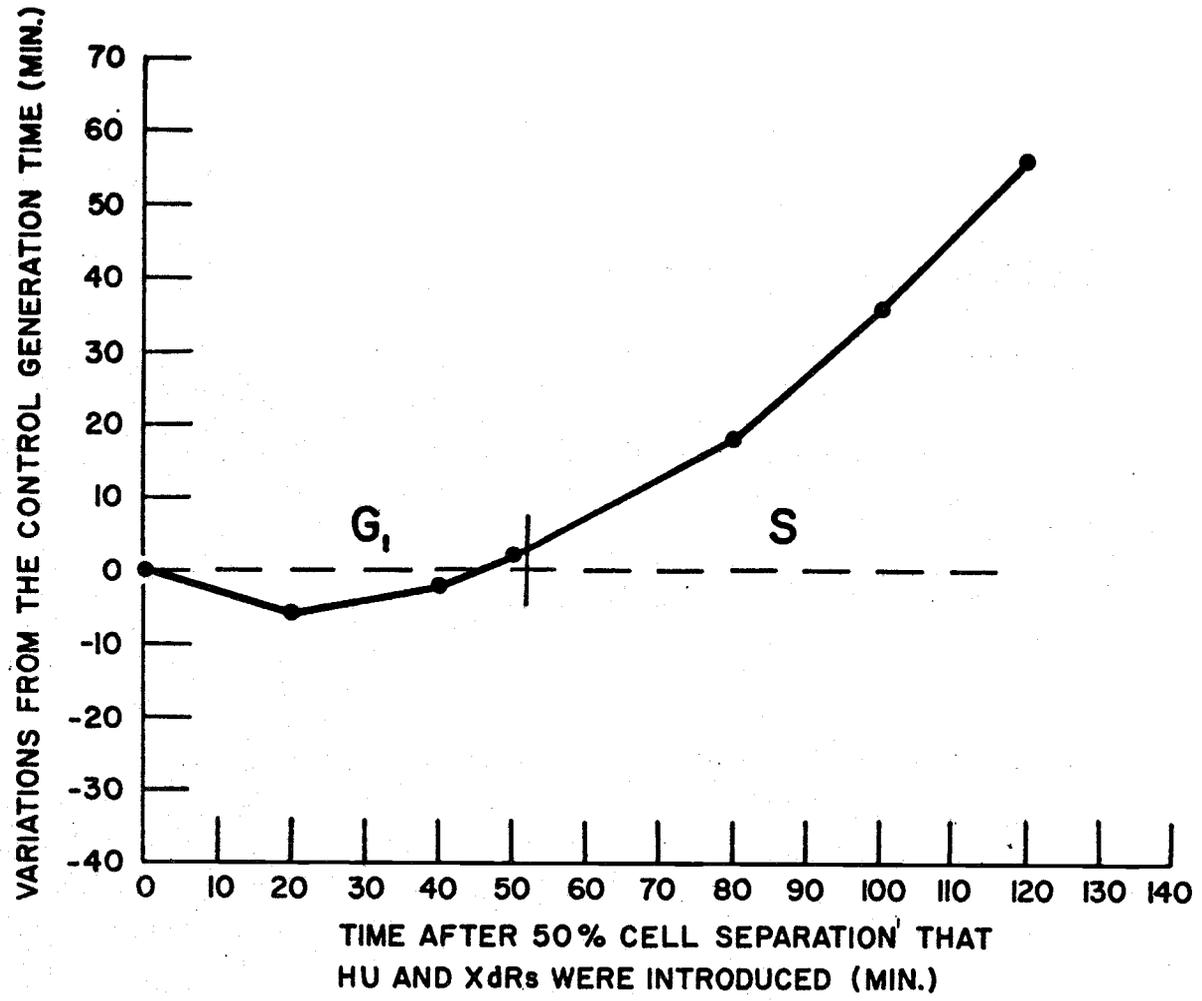


Figure 18. Recovery delay induced by the simultaneous addition of HU and XdRs to S-phase populations. HU at 10mM and all four DNA-precursor XdRs at 0.4mM were simultaneously introduced into the medium of synchronously dividing cells at various times during Div, G₁ and S. The generation time when HU and XdRs were added during division is designated the control generation time. Each data point represents a sample size of 60 cells from the combined results of two experiments.



during G_1 and then accumulate at the G_1/S border with little or no rescue delay being induced until finally some cells enter into S-phase and become sensitive to the presence of HU. It can be seen in Figure 17 (closed circle curve) that even after 157 minutes in HU the amount of recovery delay by those cells presumably entered into S-phase has not yet compensated for the decrease in corrected generation time stemming from the progression of HU treated cells through the G_1 -phase. It appears (curve labeled with open circles) that no delay is induced, even for those cells that were in early or mid S-phase when HU was added (at 90 minutes after division), until they have been incubated in HU for at least an hour. The most immediately sensitive portion of S-phase, as indicated by XdR rescue delay, seems to be late S-phase (half-closed circle curve).

Experiments were conducted in which HU and XdRs were added simultaneously to synchronously dividing G_1 and S-phase cells. The results showed that the later into S-phase the HU and XdRs were introduced, the greater was the excess delay in generation time (Figure 18). The simultaneous introduction of HU and XdRs also causes a small (10-15 min.) initial increase in generation times but, in order to more clearly identify the cell phase sensitivity response to HU and XdR addition, this difference was eliminated from the graph by designating the generation time resulting when HU and XdRs were added at division as the control generation time.

In one experiment, control generation times were determined for cells in XdR-enriched complete medium as well as the regular complete medium. When XdRs were introduced 90 minutes into the cell cycle (mid-S-phase) the generation time was unaffected (200 min.) but when the XdRs were introduced at 5 and 35 minutes into the cell cycle (G_1 -phase) the generation time was reduced to 160 minutes in both instances. This surprising discovery led to additional investigations described in the section: Effects of XdR-Enrichment Upon Exponentially Growing Cells, page 104.

Morphological Changes Induced by HU

Halo Effect

When log growth phase cells which had been incubated in HU for at least 100 minutes were vitally stained with euchrysrine, it was observed by means of fluoromicroscopy that the nuclei of some of these cells had undergone a morphological transition. (See Frontispiece.) The photomicrographs in Figure 19 compare a normal LGP S-phase cell (A) with an S-phase cell that had been exposed to 10mM HU for four hours. (B) Photomicrograph B shows that HU-treatment can induce a halo of bright fluorescing spherules surrounding a central body of chromatin.

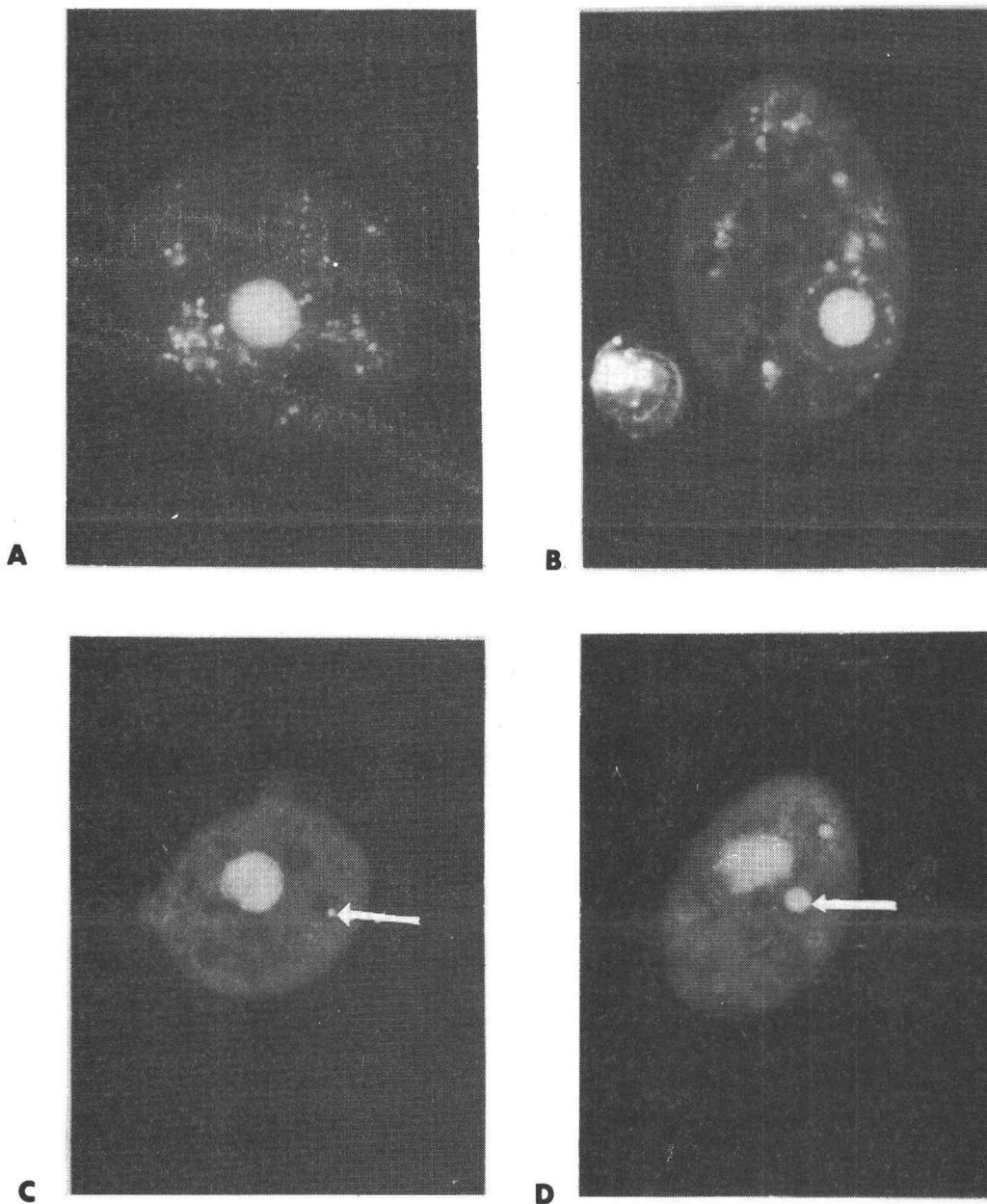


Figure 19. Fluorescence photomicrographs (B+W) of euchrysrine stained cells. A) a control S-phase cell. B) An S-phase cell which has been treated with HU for 2 hours. Notice the halo of spherules surrounding the central body of nuclear material. C) A post-divider (G₁) cell showing a bright extrusion body (arrow). D) A post-divider from the first HU recovery division with its large extrusion body (arrow).

Measurements of the nucleus, halo, and central body of normal and HU-treated cells stained with euchryesine were made using an ocular micrometer. The averages are listed in Table 6.

Table 6. The Morphological Effects of HU on *Tetrahymena pyriformis*.

Treatment	N	Cell Length ± s (microns)	Nuclear Dia. ± (microns)	Halo Dia. ± s (microns)	Central Body Dia. ± s (μ)
None	75	61 ± 11	17 ± 1	--	--
HU (3 hrs.)	45	68 ± 8	--	18 ± 2	13 ± 2

Table R-9. The nuclear diameter, halo diameter, and central body diameter measurements were always the maximum diameter reading of these generally spherical structures.

Cell Phase Sensitivity to Halo Induction

An experiment was designed to compare the frequency of HU-halo induction of S-phase cells with that of non-S-phase cells treated with 10mM HU for the same period of time. A group of LGP cells was incubated for 110 minutes in HU and then stained with Euchryesine and observed by fluoromicroscopy. The duration of exposure was sufficient such that most of the cells had collected in S-phase by the time of observation. It was found that about 50% of the cells revealed the HU-halo effect. Another sample of LGP cells from the same population was treated with HU and after 65 minutes all the dividing cells (post-HU-block cells) were isolated and continued to be incubated in HU for 45 more minutes before being stained and observed as described above. This procedure insured that the cells observed in this

latter experimental group were in the non-S-phase portion (G_2 , D, and G_1) of the cell cycle during virtually the entire 110 minute HU-exposure period. There were no HU-haloes observed in this group. Therefore, only S-phase appears to be sensitive to HU exposure as indicated by halo induction. Thus the morphological criterion for S-phase sensitivity to HU support the previous observation of increased sensitivity to HU during S-phase as measured by recovery delay (page 81).

Although S-phase appears to be the sensitive phase for halo induction, the fraction of cells with haloes cannot be easily used for the determination of the S-phase duration. The percentage of LGP cells with haloes after 110 minutes of exposure ($\sim 50\%$) is greater than the percentage of cells in S-phase at the start of the HU-treatment ($\sim 43\%$). Therefore, haloes might be induced in cells that progress from G_1 into S-phase after exposure starts. It is also apparent from numerous observations that S-phase cells require a certain amount of time, at least an hour, in HU before the halo effect becomes evident. These characteristics of halo induction do not preclude the possible use of the halo effect in determining the fraction of cells in S-phase from which the S-phase duration may be calculated. Future studies will focus upon the use of the halo effect in S-phase analysis.

Morphological Characteristics of HU-Recovery

Cells were exposed to HU for several hours, washed to remove the HU, and allowed to recover their ability for normal growth and division. These recovering cells were studied fluoromicroscopically in order to characterize morphologically the recovery period. Several experiments of this type, usually involving a 10 1/2 hour exposure to HU, were conducted. Three basic observations resulted from these experiments: 1) In all cases the HU-induced halo persisted, even after the HU was removed, until the first recovery division, at which time it permanently disappeared; 2) There was a significant increase in the frequency of cells which carried extranuclear chromatin bodies (extrusion bodies) over that of regular LGP post-dividers (70-90% vs. 20-40%); 3) The size of these post-recovery-division extrusion bodies (2 to 4 μ) was much larger than those normally seen in LGP cultures (0.5 μ). Figure 19 (D) is a photomicrograph of a G₁ cell following the first recovery division which shows an enlarged extrusion body; Figure 19 (C) shows an extrusion body in an untreated cell from an LGP culture.

The Effects of XdR-Enrichment Upon
Exponentially Growing Cells

Acceleration of Exponential Growth

When 0.8mM of all four DNA-precursor deoxyribonucleosides were added to exponentially growing cells in complete medium the generation time decreased by about 20 percent. This decreased GT was demonstrated in experiments where a number of cells were randomly picked from an XdR-enriched exponentially growing population and placed into XdR-enriched microdrops to facilitate cell counting. The cells were periodically counted until they had passed through three generations (8x the original sample size). The exponential increase in cell number was then plotted against time on semi-log axes and the generation time directly derived from the doubling time of the linear portion of the growth curve. (Non-linearity of the initial portion of the growth curve commonly results from synchrony artifactually induced in picking the starting random cell sample as discussed in Methods and Materials, page 19). Figure 20 presents the data from a typical experiment of this type and Table 7 summarizes the data from three such experiments. It can be seen that autoclaving the XdR's evidently lessened their capability of inducing growth acceleration while introducing imbalanced XdR-enrichment (omission of thymidine) significantly inhibited growth rate.

Figure 20. Acceleration of exponential growth by deoxyribonucleoside enrichment. Exponential growth curves for a non-XdR enriched sample, ●; an XdR enriched (0.8mM) sample, ○; and a sample enriched with 0.8mM autoclaved (20 minutes) XdRs, ⊙. The samples were from the same original log growth phase culture. The generation times listed in Table 7 for this experiment (042271) were derived from the doubling times of the linear portion (latter portion) of the curves. Thus the time elapsed for a relative cell number of 4 (time = 430 min.) to double to a relative number of 8 (time = 655 min.) for the control curve is equal to $655 - 430 = 225$ minutes. The initial sample size for each growth curve was 35 cells.

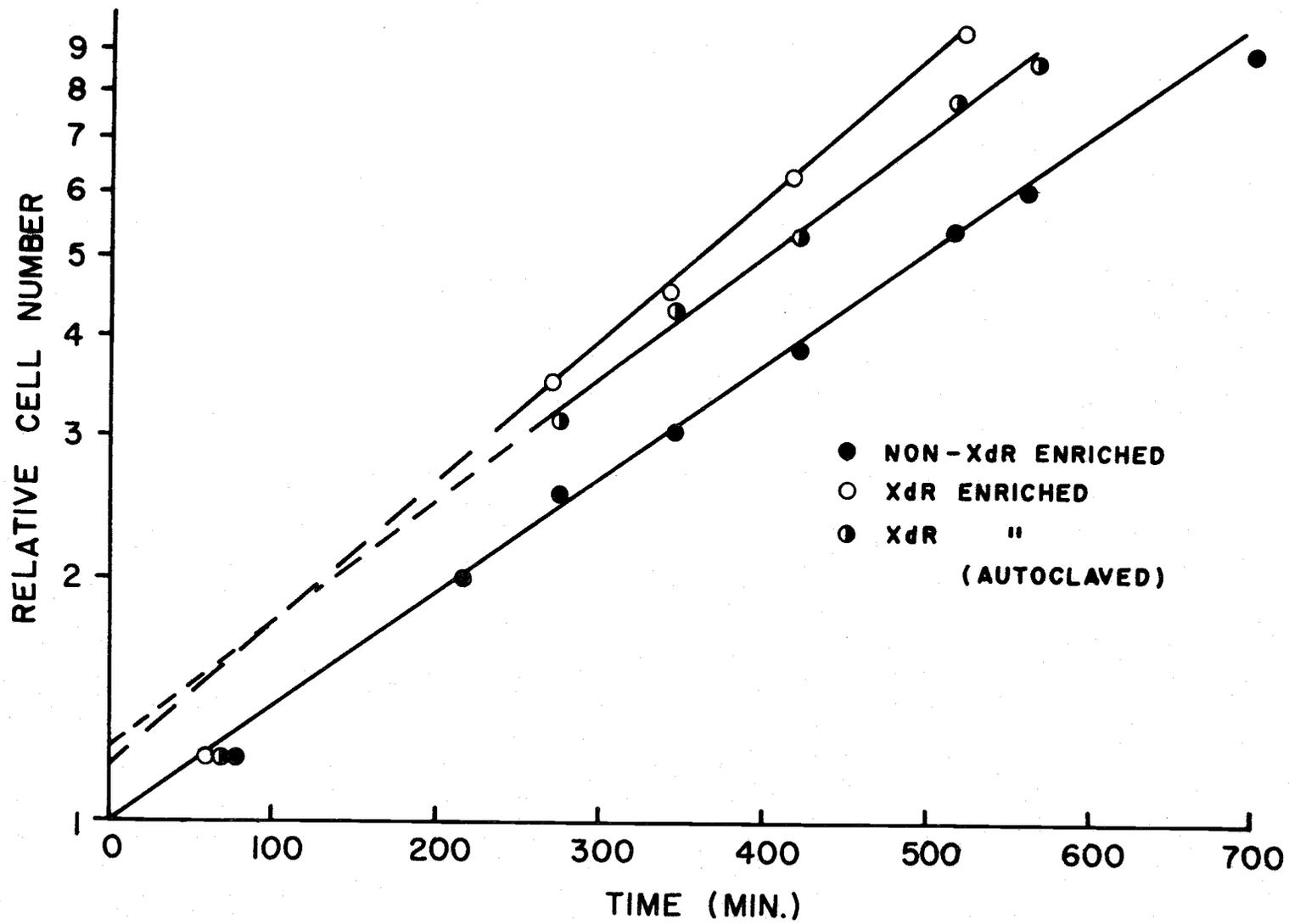


Table 7. The effect of deoxyribonucleoside enrichment (0.8mM) upon the generation time of Tetrahymena pyriformis.

Experiment	Treatment	Generation Time (T)	Percent of Control T (%)
041971	Control	261 minutes	100
041971	0.8mM XdRs	223 minutes	85
041971	*0.8mM XdRs	241 minutes	92
042271	Control	225 minutes	100
042271	0.8mM XdRs	170 minutes	75
042271	*0.8mM XdRs	195 minutes	87
042771	Control	200 minutes	100
042771	0.8mM XdRs	160 minutes	80
042771	#0.8mM XdRs - TdR	290 minutes	145

The abbreviation XdRs stands for deoxycytidine (CdR), deoxyadenosine (AdR), deoxyguanosine (GdR) and thymidine (TdR). These deoxyribosides were sterilized by filtration through a 0.45 μ Millipore membrane filter (Sybron Corp., Rochester, N. Y.). *Indicates non-filtered autoclaved deoxyribosides. #Indicates 0.8mM addition of AdR, CdR and GdR but no additional TdR added.

Similar experiments with various concentrations of the four deoxyribosides ranging from 0.004 μ M to 400 μ M revealed a threshold growth response with the transition from normal to the abbreviated generation time occurring at approximately the 2.0 μ M concentration. (See Table 8.)

Table 8. Determining the concentrations of excess XdRs which affect the generation time of Tetrahymena pyriformis.

Concentration of Additional XdRs (μ M)	Generation Time (min.)	Percent of Control Generation Time (%)
None (control)	205	100
0.004	199	97
0.4	220	107
2.0	183	89
4.0	175	85
40.0	179	87
200	172	84
400	176	86

Combined results of two experiments concerning the effect of XdR concentration on generation time. All data were normalized to a control generation time of 205 minutes.

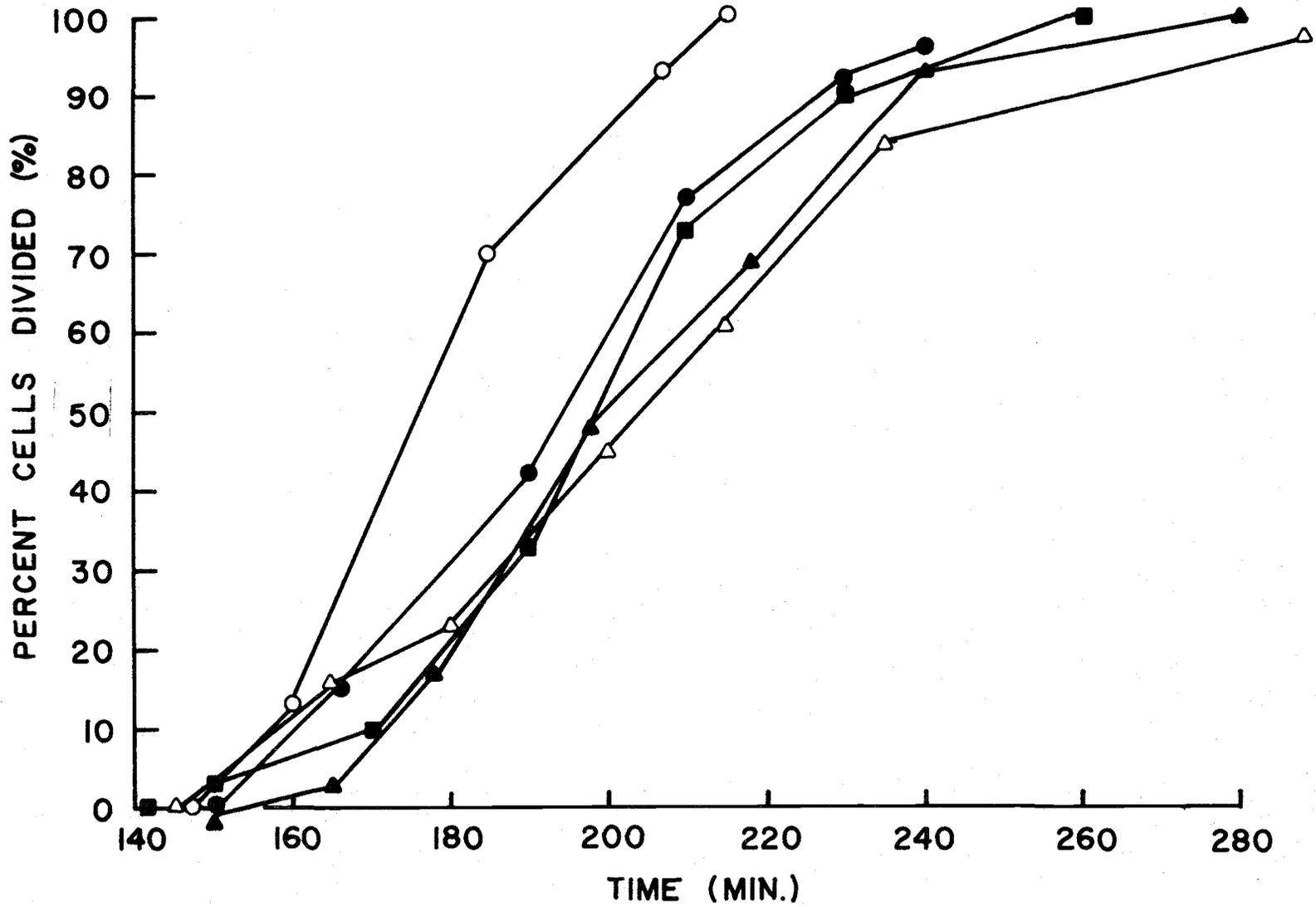
Cell Phase Response to Excess XdRs

Experiments were conducted to determine: 1) if the growth acceleration response to excess XdRs is immediate due to their direct involvement in DNA synthesis metabolism or delayed through the formation and maintenance of endogenous pools prerequisite to

the rapid synthesis of DNA and; 2) which portion of the cell cycle, if not every portion, is responding to the presence of excess XdRs. The first problem was attacked by determining whether or not the presence of excess XdRs during one cell cycle affects the generation time of the following cycle. That is to say, do the excess exogenous XdRs affect an increase in the endogenous deoxyribonucleotide pool formation and is this pool maintained through cell division to be used in affecting the rapid growth of the following cell cycle?

Random exponential growth Tetrahymena cultures were exposed to 0.5mM XdRs for 40 minutes and then washed to remove the excess XdRs. Dividers were then picked and isolated from the main culture at various times after the removal of the excess XdRs and subcultured in microdrops to determine the subsequent generation times. The furrowed state served as a morphological marker, thus enabling the selection of cells pulsed with excess XdRs at a specific known time of their cell cycle relative to division. The generation times of the cell cycle following the XdR pulse were compared to the control generation times. Figure 21 shows the results of one such experiment and Table 9 presents the combined data from four such experiments. These data indicate possibly some rapid growth from excess XdRs taken up and pooled during the previous G_2 , however, 6% reduction in GT is not nearly as great as the 15-20 percent reduction in

Figure 21. The effect of excess XdRs in portions of one cell cycle upon the generation time of the next cell cycle. Generation time plots for cell samples (N = 30) that were pulsed for 40 minutes with 0.5mM XdRs at different times during the previous cell cycle. ● = control (no XdRs added), ○ = XdRs pulsed in G₂, ▲ = XdRs pulsed in late-S, △ = XdRs pulsed in mid-S, ■ = XdRs pulsed in early -S.



generation time observed under conditions of continuous excess XdR's exposure. It is concluded that the effect of exogenous XdR's is expressed primarily during the cell cycle which the XdR's are present.

Table 9. The effect of excess XdRs in portions of one cell cycle upon the generation time of the next cell cycle.

Treatment	Time of XdRs Pulse in the Previous Cell Cycle (min.)	Generation Time (min.) of Subsequent Cycle	Percent of Control Gen. Time (%)
Control	--	206	100
G ₂ pulse	151-191	196	94
Late-S to Early G ₂ pulse	113-153	206	100
Mid-S to Late-S pulse	98-138	208	101
Early-S pulse	60-100	208	101

The generation times of cell samples exposed to a 40 minute pulse of 0.5mM XdRs at various times during the preceding cell cycle. The time of the XdRs pulse was determined as described in the text. These data are the combined averages from four experiments. The average sample size for each experimental condition is one hundred cells (N = 100).

Several types of experiments were conducted in order to determine whether or not the growth acceleration response to excess XdR's was a general effect on all portions of the cell cycle and, if not, which specific portions were being affected. One type of experiment involved continuous exposure of synchronously growing cell populations to XdRs starting at different times in the cell cycle. Dividers were picked from logarithmic growth phase cultures in order to obtain synchrony and, after various known time intervals, the cells were pipetted into microdrops of medium supplemented with 0.2 to 0.4mM

XdRs. The cells were then periodically counted to obtain generation time data and thus to determine if any specific portion of the cell cycle responded to the presence of excess XdRs and, if so, to identify that portion. The results of a typical experiment of this type are graphed in Figure 22. It can be noted that the presence of excess XdRs in G_1 and early S-phase resulted in reduced generation times while an excess of XdRs in late-S does not affect the generation time.

The combined results of six such experiments are graphed in a manner which enables easy identification of the cell cycle portion (late-S and G_2) which does not respond to excess XdRs (Figure 23). The graph reveals that, no matter if the XdRs are added just after division or in late G_1 , the generation time is decreased by about the same amount of time. After entering S-phase, the response to excess XdRs diminishes up to mid-S-phase when no distinguishable difference in the generation times of XdR-treated and non-treated cell populations is detected. It appears from these data that both G_1 and early S-phase are sensitive to the presence of excess XdRs with early S-phase showing the greatest sensitivity.

In order to demonstrate specifically which portion of the cell cycle, G_1 or early S-phase, contributed the major growth response to XdR enriched medium, an experiment involving continuous and pulse exposures to excess XdRs was conducted.

Figure 22. Continuous exposure to excess XdRs (0.4mM) starting at 5 min. (○), 20 min. (▲), 35 min. (△), 52 min. (⊠), 65 min. (□), 80 min. (■), 93 min. (⊙), and 110 min. (⊕). The control generation time (●) is 210 minutes. The initial sample size of each curve was thirty cells (n = 30).

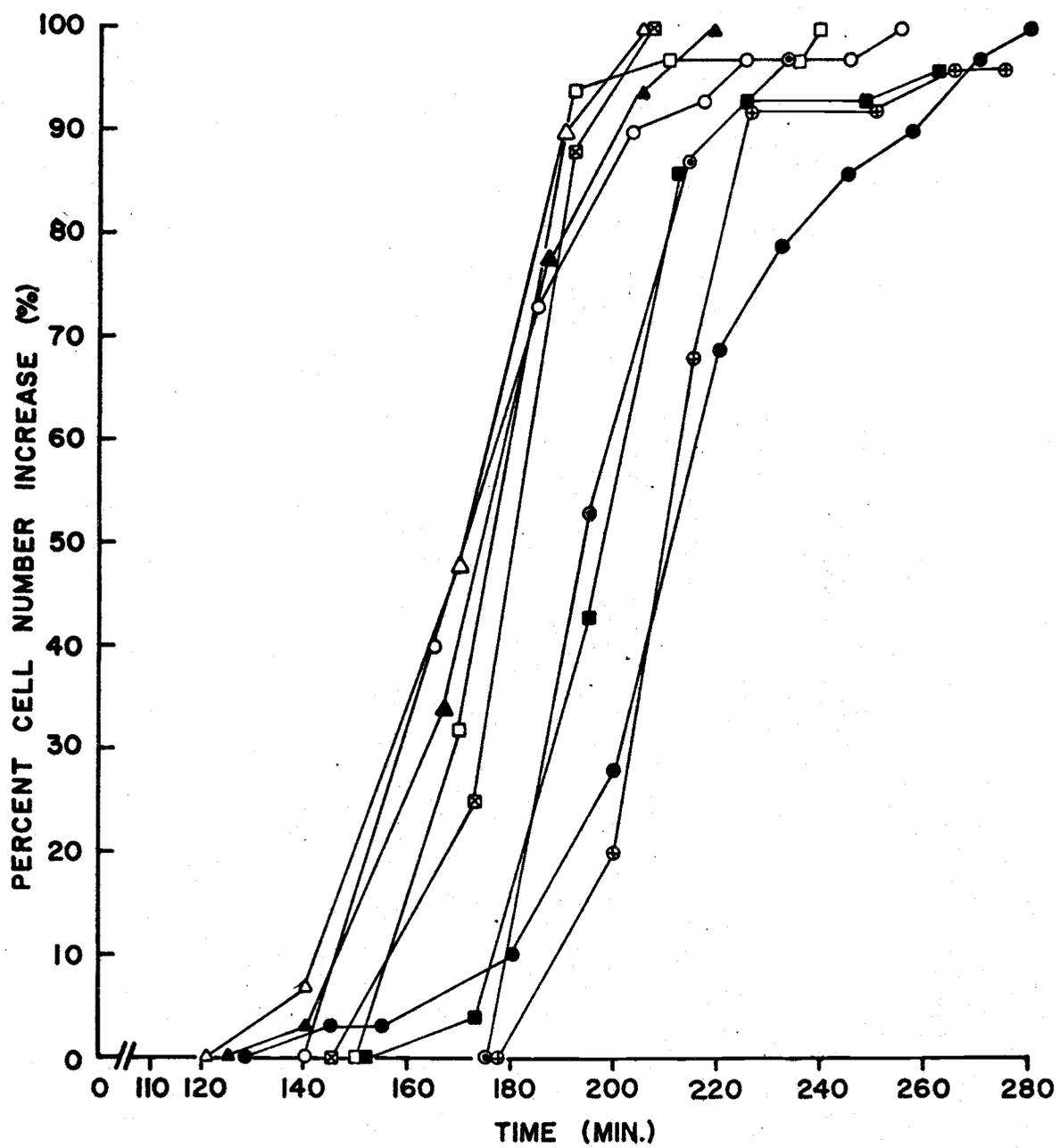
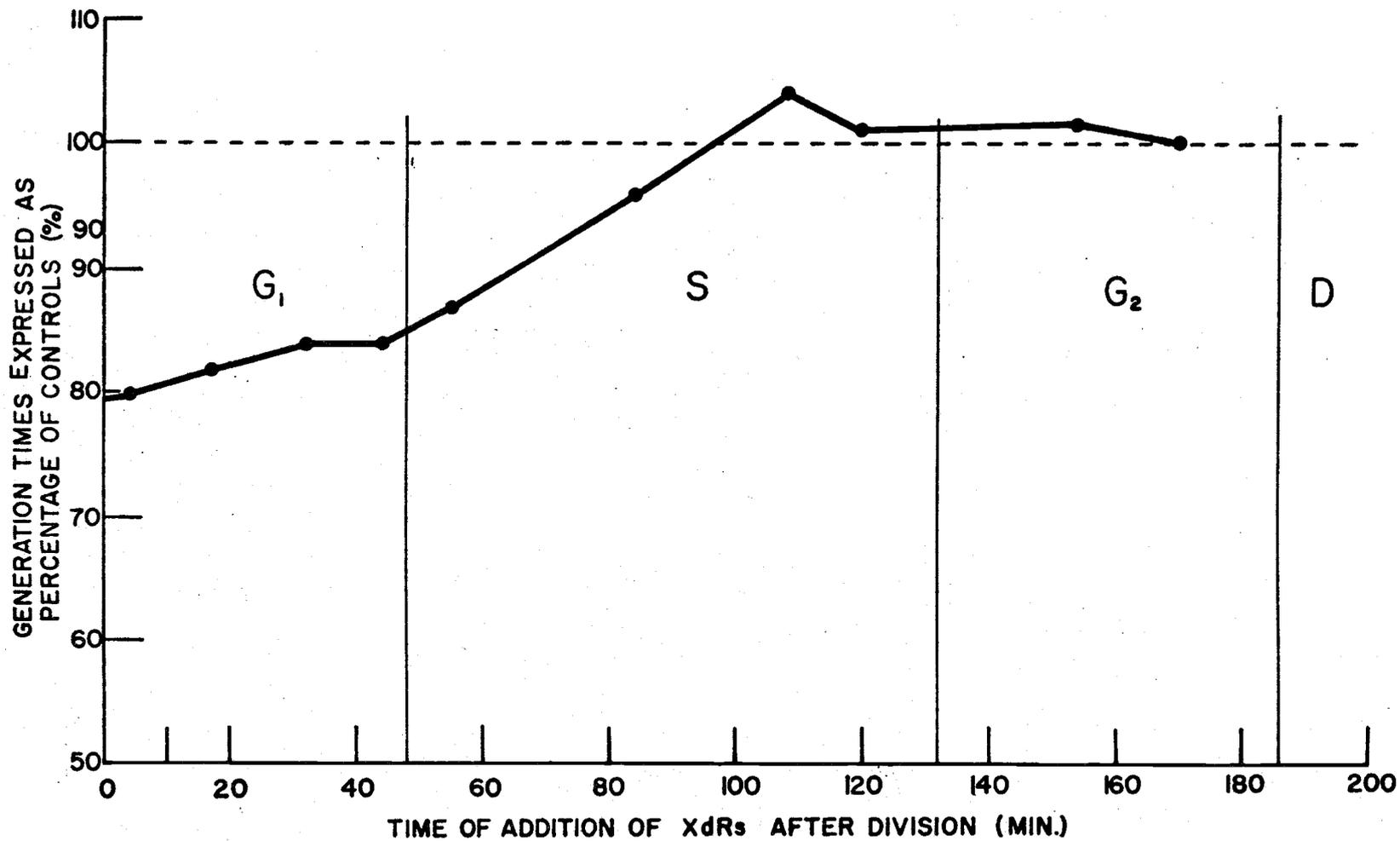


Figure 23. Combined results of experiments involving continuous exposure to XdRs. The results of six experiments combined by the point cluster normalization method. Each data point is derived from a combined sample of over 150 cells. The 200 minute cell cycle is derived from the average of the six experiments. The percentages expressed along the ordinant are the combined percentages of the controls of each individual experiment.



The durations of the first few generations of cells transferred to fresh medium after being in maximum stationary phase are reduced at the expense of G_1 (as determined by cell cycle analysis with $^3\text{H-TdR}$). This peculiarity allowed these cells to be utilized in experiments that could determine if the generation time of G_1 -less cells could still be reduced by the presence of excess XdRs. Dividing cells from a culture that was in maximum stationary growth phase were transferred to fresh medium and divided into control subcultures for standard $^3\text{H-TdR}$ labeling cell cycle analysis and into experimental subcultures for examining the effects of excess XdRs upon the cell cycle of these rapidly growing cells. It was found that the GT of the control cells not given XdRs was 164 minutes (G_1 was about 6 minutes, S-phase about 104 minutes, and $G_2 + D$ about 54 minutes). The GT of the cells continuously exposed to excess XdRs for one generation was reduced even further to 115 minutes! Dividing cells picked from these early post-stationary phase cultures and then pulsed with excess XdRs for 30 minutes at various times during S-phase responded by a reduction of GT only when pulsed during the first half of S-phase. These results indicate that the early S-phase is the only period that is affected by the presence of the XdRs. The results are illustrated graphically and schematically in Figures 24 and 25 respectively.

Figure 24. Effect of excess XdRs upon the generation time of cells devoid of a G_1 -phase. The generation times for cells exposed to excess XdRs continuously and for 30 minute pulses at various intervals during S-phase are derived from the 50% cell division points on the curves above. Each curve was from an initial sample of thirty cells ($N = 30$). Non-XdRs control (0); XdRs continuous (●); 5 to 35 min. XdRs-pulse (Δ); 20 to 50 min. XdRs pulse (\blacktriangle); 40 to 70 min. XdRs pulse (\square); 60 to 90 min. XdRs pulse (\blacksquare); 80 to 110 min. XdRs pulse (\blacklozenge).

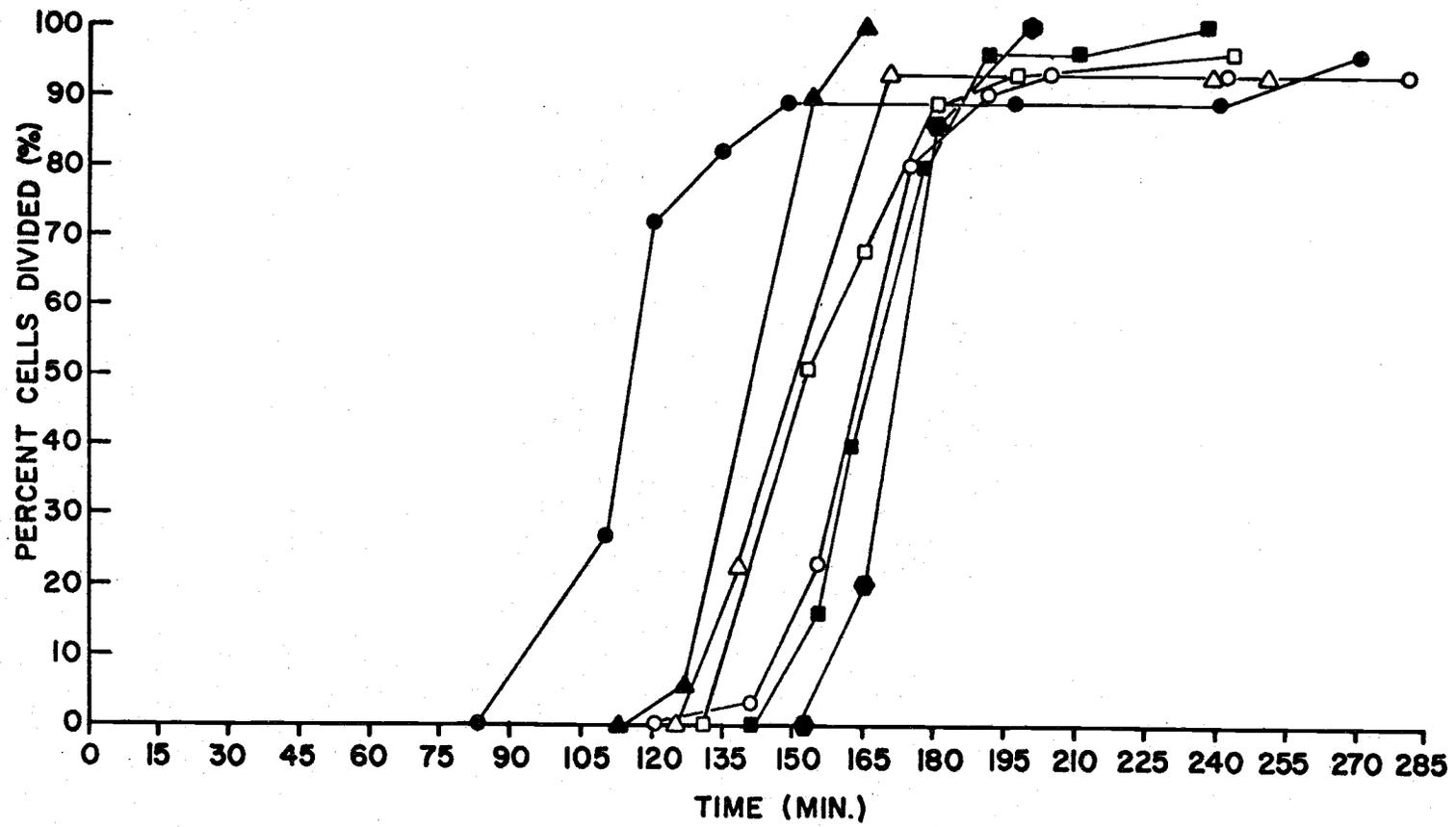
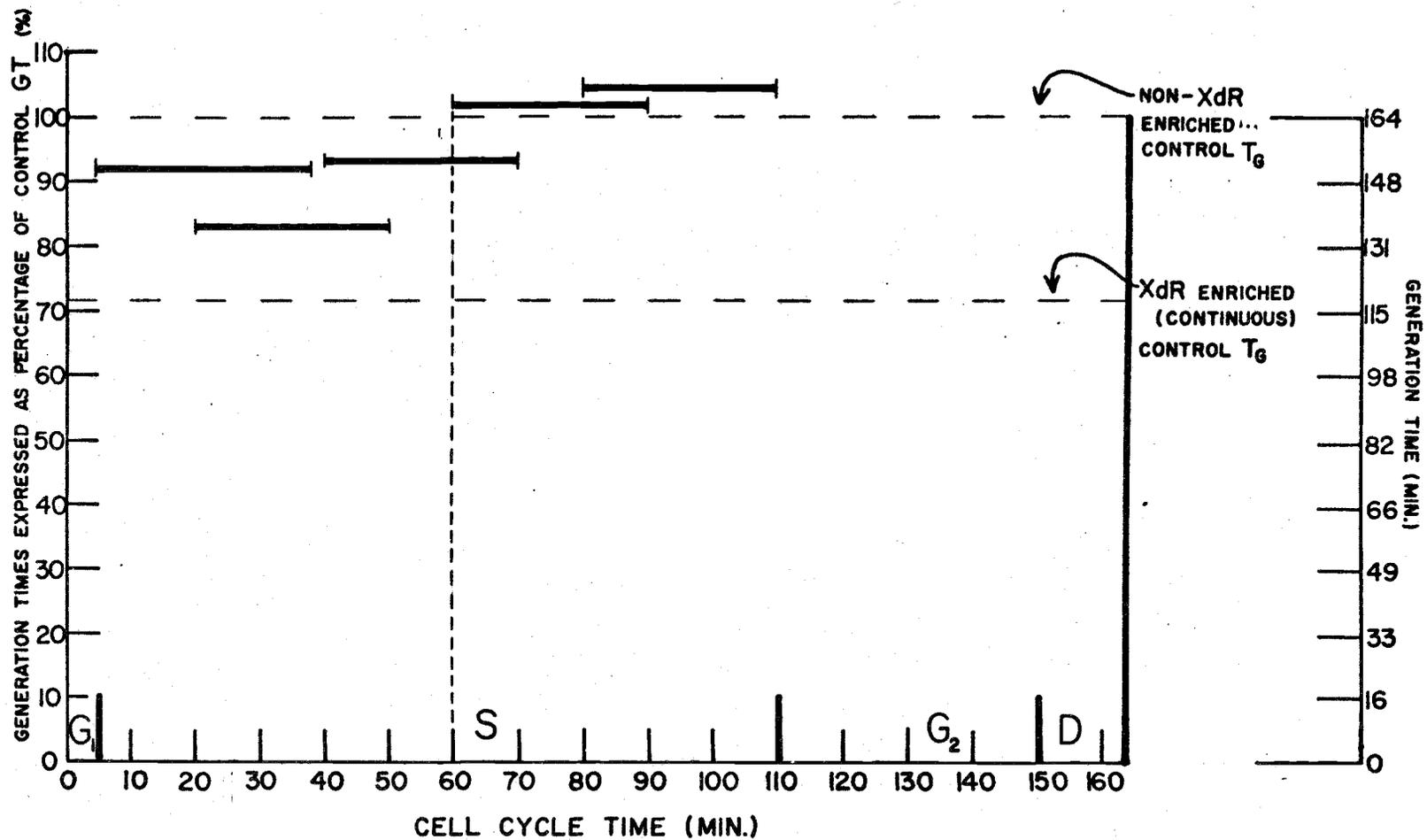


Figure 25. Effect of excess XdRs upon the generation time of cells devoid of a G₁-phase. The data from Figure 24 are schematically presented. The dark horizontal bars indicate the time of the cell cycle during which samples of synchronously dividing cells were given 30 minute pulses of XdRs. The position of each bar along the ordinate indicates the resulting generation time of each experimental group. The dashed lines indicate the control generation times for cells in non-enriched Tyess and for cells enriched with 0.4mM XdRs. The vertical dotted line partitions S-phase into a portion sensitive to the presence of XdRs and a portion not sensitive to the presence of excess XdRs.



Phosphorus-33 Cell Cycle Analysis

The results of experiments involving continuous exposure and pulse exposures of exogenous XdRs described above indicate that early S-phase is the portion of the cell cycle that primarily responds to the excess XdRs by a reduction of the generation time. These experiments, however, do not actually determine which portion of the cell cycle is being circumvented when excess XdRs are present because they do not preclude the possibility that the cells may take up the XdRs at one time and utilize them at another time. Thus, in the presence of excess XdRs the circumvented portion of the cell cycle may not be identical to the cell cycle portion that directly responds to their presence.

In order to determine which specific portion of the cell cycle is being circumvented one may employ radiotracer methodology; however, classical techniques involving ^3H -TdR are of no avail due to the diluting effect of the high concentration of unlabeled thymidine already present in the XdRs-enriched medium. In order to alleviate this problem it was necessary to use a radionuclide labeled DNA-precursor other than a deoxyribonucleoside or -nucleotide. Phosphorylation of nucleosides is a prerequisite step to the eventual formation of the phosphodiester linkage in the sugar-phosphate backbone of the polynucleotide DNA molecule. Recent commercial

availability of phosphorus-33 (^{33}P), a low energy beta emitter ($E_{\text{max}} = 0.248\text{mev}$), made it the radiotracer of logical choice for use in autoradiographic investigation.

Cell cycle analysis, involving the use of ^{33}P and classical autoradiographic methodology, was conducted on cells exponentially growing in medium enriched with XdRs. Figures 26 and 27 graphically illustrate the results of experiments defining the duration of G_1 in the former and G_2 in the latter. These experiments reveal that both G_1 and G_2 are unaffected by exposure to XdRs in excess of the normal concentration in Tyess medium and that, therefore, it is S-phase that is reduced in duration.

Figure 26. G_1 analysis with phosphorus-33. Continuous labeling with ^{33}P (30 Ci/ml) yielded the curves shown. The ^{33}P was introduced to synchronously dividing LGP cells at the 50% cell separation ($T_D = 0$) and cell samples were removed and prepared for autoradiography at subsequent time intervals. Each data point represents a sample of twenty cells. The G_1 time for the control cells (\bullet) is 56 min. and for the XdRs enriched cells (\blacktriangle) is 55 min. The generation times were $T_{\text{control}} = 200$ min. and $T_{\text{XdR}} = 144$ min.

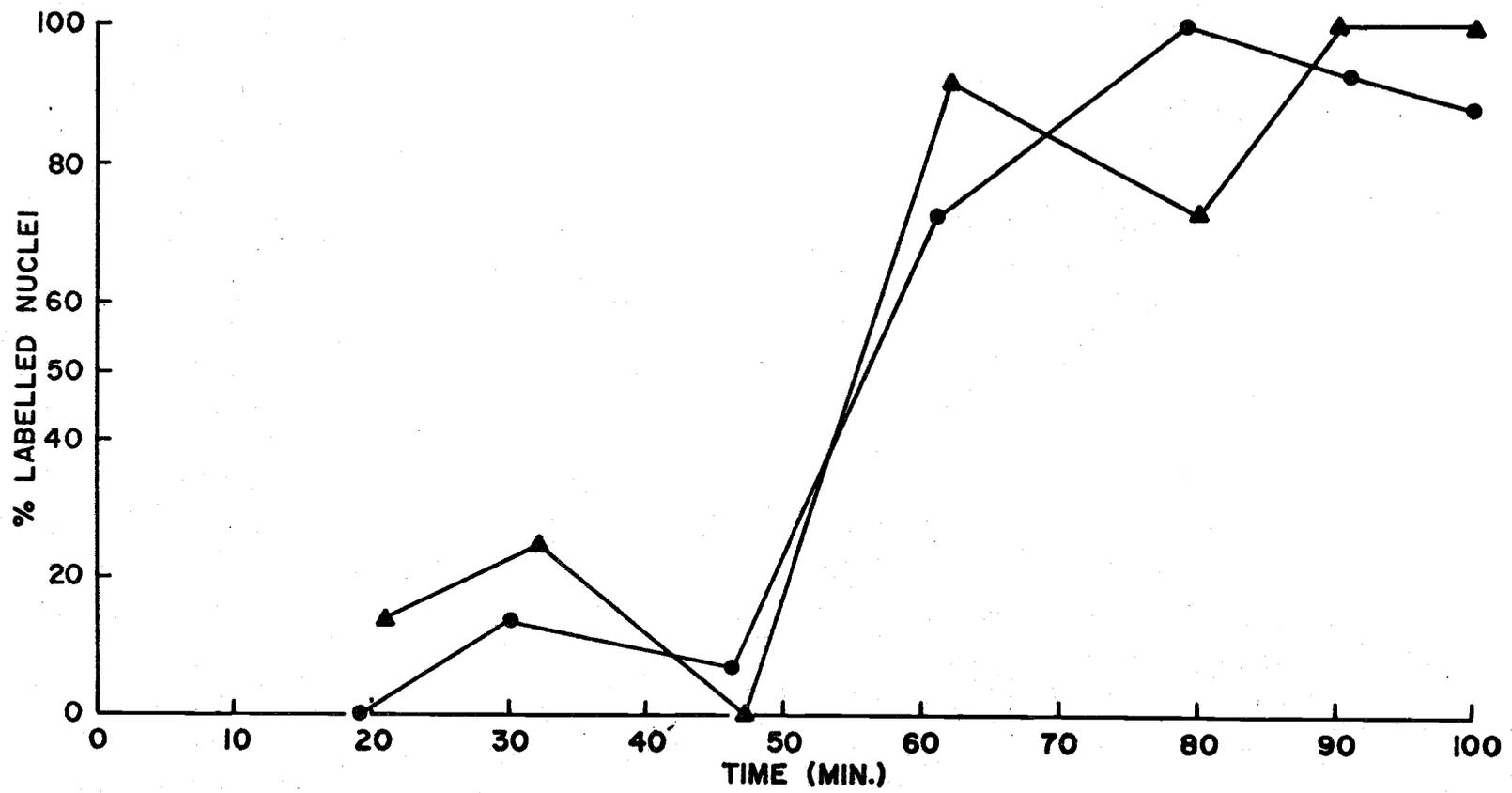
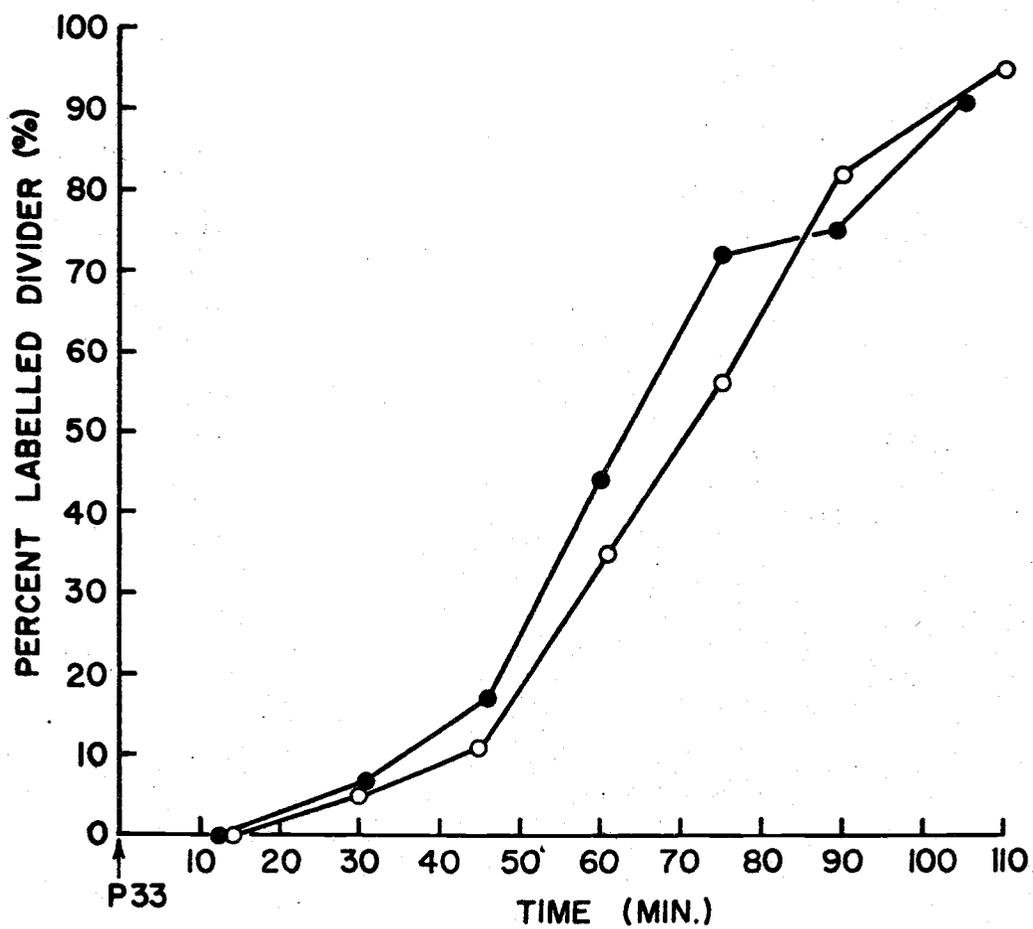


Figure 27. Continuous labeling G_2 analysis involving autoradiographic monitoring of ^{33}P into the nuclear DNA produced the labeling curves shown. The $t_{G_2} + 1/2 t_D$ for the control (○) is 71 min. and for the cells exposed to 0.2mM XdRs (●) is 63 minutes. This difference does not appear to be significant. The generation times were $T_{\text{control}} = 200$ min. and $T_{\text{XdRs}} = 165$ min.



DISCUSSION

The results of this study reveal that HU arrests all cells from further progress through the cell cycle once they have entered S-phase. Although the cells remain arrested in S-phase they continue to incorporate a reduced but significant amount of tritiated thymidine into their DNA. The HU-block may be reversed by either removing the HU from the culture medium or adding a 0.5mM concentration of each of the four DNA-precursor deoxyribonucleosides (XdRs). Upon removal of HU or addition of XdRs, recovery from the HU-block is not immediate but is characterized by a recovery delay period which is S-phase specific. That is; when the HU-block is reversed, a delay in excess to the expected time remaining in the cell cycle is induced specifically in cells which were in S-phase at the time HU was first added. S-phase specificity is also observed for the induction of morphological changes in the nuclei of HU-treated cells. The effects of HU are discussed in the following section entitled Effects of Hydroxyurea.

The fact that HU-treatment results in physiological and morphological responses that are S-phase specific makes plausible the use of HU for cell cycle analysis. The use of HU for cell cycle analysis is discussed in the section entitled Cell Cycle Analysis with Hydroxyurea.

Exponentially growing cells treated with HU for a significant time are, upon removal of the HU, able to recover and re-enter exponential growth after a significant delay. Cells recovering from the HU-block immediately begin synthesizing DNA in great quantity before they undergo their first recovery division. After dividing, these cells enter a short cell cycle abbreviated at the expense of G_1 -phase. The cell cycle kinetics of the recovery period and the abbreviated cell cycle following recovery division are analyzed and discussed in relation to a nuclear-DNA content to cytoplasmic mass ratio mechanism involved in the initiation and control of cell growth, DNA replication, and cell division in Tetrahymena pyriformis. This discussion is located in the section entitled Cell Cycle Analysis of Cells After HU-Treatment.

It was found that, when complete medium (Tyess) was enriched with 0.5mM of each of all four DNA-precursor XdRs, the generation time of exponentially growing cells was reduced by 20%. It was determined that early S-phase was being accelerated and it was in this portion of S-phase that the uptake, incorporation, and possibly pool formation of XdRs occurred. These results are discussed in the section entitled Effects of Excess XdRs.

Effects of Hydroxyurea

DNA Synthesis

Hydroxyurea blocks Tetrahymena cells from further progression through the cell cycle if they are in almost any portion of S-phase. This S-phase block is accompanied by a partial suppression of DNA synthesis (Table 4). The HU-block can be quickly reversed by the addition of excess XdRs to the medium, thus indicating that the primary site of HU's blocking action in Tetrahymena, as in many organisms, is the inhibition of the reduction of ribonucleotides to deoxyribonucleotides. (See HU-Block Rescue by Deoxyribonucleotides, page 92). These results have recently been confirmed in Tetrahymena by Cameron and Jeter (1972).

It is important to note that HU does not completely suppress DNA synthesis during S-phase (Table 4). Indeed, Cameron and Jeter (1972) reports that even at a 50mM concentration, HU does not completely inhibit DNA synthesis in the two strains of Tetrahymena pyriformis (GL-C and HSM) with which they worked. McDonald (1972) also reports the uptake of ^{14}C -TdR in significant amounts into the nuclear DNA of S-phase Tetrahymena pyriformis (GL) cells treated with HU. In mammalian cell systems, in vivo experiments indicate a reduction of DNA synthesis to 1 to 29% of the controls (Painter and

Cleaver, 1967; Bacchetti and Whitmore, 1969; Cleaver, 1969; Prempre, 1969; Coyle and Strauss, 1970; Connell, et al., 1971; Lieberman, et al., 1971). The nature of this DNA synthesis was explored in some of these studies.

Cleaver (1969) employed radiotracer labeling, BUdR density labeling, and DNA characterization on CsCl gradients to reveal that the DNA synthesis that does occur in the presence of HU during S-phase is non-semiconservative. The fact that a significant amount of ^3H -TdR incorporation into DNA occurs in S-phase cells treated with HU coupled with the observation that this DNA synthesis is of the non-semiconservative type (confirmed by Regen, et al., 1971) supports previous studies which show that HU induces direct damage to DNA (See the Introduction). This leads to the interpretation that the DNA synthesis during HU-arrest is repair synthesis; not replicative DNA synthesis. That excision repair synthesis is not inhibited by HU has already been discussed in the Introduction.

An alternative explanation is that HU is unable to completely suppress semiconservative DNA synthesis. An incomplete type of replicative (semiconservative) DNA synthesis may occur that is limited in rate and progress by the direct inhibition by HU of DNA ligase enzyme activity. Coyle and Strauss (1970) have demonstrated, using ^3H -TdR labeling and alkaline sucrose gradient techniques, that only small

fragments of DNA are synthesized in the presence of HU and upon removal of the HU these fragments are converted into the rapidly sedimenting native DNA. No evidence was given whether this fragment DNA was the result of semiconservative replication or not. These fragments may be the "Okazaki fragments" normally involved in replicative DNA synthesis (Okazaki, et al., 1970). Hydroxyurea may be inhibiting normal replicative DNA synthesis by blocking the step involving the DNA ligase enzyme which results in the inhibition of the joining of newly synthesized single strand fragments (Okazaki fragments) to the native DNA.

A third hypothesis, also largely derived from the data of Coyle and Strauss(1970), is that HU allows some limited semiconservative DNA synthesis to go on as long as any deoxyribonucleosides and deoxyribonucleotides are available. The inhibition effect of HU upon the reduction of ribonucleosides and ribonucleotides may result in the synthesis of partial or incomplete Okazaki fragments at the sites of DNA replication. Thus the newly synthesized DNA strands may be a series of polynucleotide fragments interspersed with gaps resulting from the partial availability of deoxyribonucleosides and -tides for DNA synthesis. These gaps could also be the basis of the S-phase specific induction of single strand breaks reported by other investigators (See the Introduction).

The hypothesis that HU inhibits the action of DNA ligase is largely refuted at the outset by the numerous observations of XdR-induced reversal of the HU-arrest. If HU inhibits the ligase step, then how can the addition of excess XdRs overcome this effect? It seems, therefore, that the newly synthesized DNA fragments which occur in S-phase cells treated with HU are associated with either/both non-semiconservative (repair) or/and disrupted semiconservative DNA synthesis.

The question of how much DNA is actually synthesized in the presence of HU is fundamental to understanding the actual nature of this S-phase DNA synthesis. Preliminary investigation along these lines conducted by Michael Salamone (1972) of this laboratory in cooperation with the laboratory of David C. Shepard at California State University at San Diego has revealed interesting results. Using the two wavelength microspectrophotometric technique, his experiments revealed that after maintaining Tetrahymena cells in HU for 10 1/2 hours they still possess about the same relative DNA content as control G₁ cells. The relative DNA content for cells in G₁ is 1, for cells in G₂ approximately 2, and for cells maintained in HU for 10 1/2 hours slightly greater than 1. Thus, it appears that the actual amount of DNA synthesized in the presence of HU is very slight and, therefore, is probably the result of repair DNA synthesis.

Recovery Delay

The results presented in this paper show that cells treated with HU for several hours do not immediately recover the capacity to progress at a normal rate through the cell cycle to division. Upon removal of HU, there is a time lag before they undergo the first recovery division. This time lag is termed the recovery delay. The results show that recovery delay is greatest for cells that are in S-phase at the time of HU introduction (Figures 14 and 17). The recovery delay is about the same after HU treatment initiated at any time in the S-phase (Figure 14).

Similarly, the recovery delay for cells rescued from the HU-block by the addition of XdRs is greatest when the HU treatment is initiated during any portion of S-phase. Figure 17 graphically illustrates that when HU incubation is of significant duration, i. e., 120 minutes, then recovery delay is maximum for cells whether they were in early or late S-phase when the HU was introduced.

When the XdRs are added to the medium simultaneously with the addition of HU a delay in cell cycle progress is induced which becomes most apparent when the HU and XdRs are added during the second half of S-phase (Figure 18). S-phase is, therefore, partitioned into a nonsensitive and a sensitive portion in terms of induction of cell cycle delay by simultaneous exposure to HU and XdRs. This partitioning of

S-phase is similar to the partitioning of S-phase according to the time when XdRs are taken up and incorporated into the DNA, which is discussed in a later section. (See Effects of Excess XdRs.) Cell cycle delay may be the result of two different phenomena: 1) the repair of simple single-strand breaks in the DNA induced by HU and 2) a paucity of kinase enzymes that are involved in phosphorylating the exogenously supplied deoxyribonucleosides to deoxyribonucleotides. As indicated in the section, Effects of Excess XdRs, certain kinase enzymes may not be readily available during late S-phase. Thus, the increasing delay in the second half of S-phase, observed when HU and XdRs are added simultaneously, may reflect the cyclic availability of kinases involved in the conversion of XdRs into DNA.

Morphological Changes Induced by HU

1. Halo Effect

The results of fluoromicroscopic investigation using euchryesine dye reveal a striking morphological response in Tetrahymena cells to the presence of 10mM HU. The major portion of chromatin condenses forming a central chromatin body which is enclosed by a network of chromatin aggregates. This outer complex of chromatin aggregates, which are apparently associated with the nuclear membrane, is called the "halo." The bright green color of these nuclear membrane associated aggregates is identical to the color of the central chromatin

body in HU-treated control cells. According to Allison and Young (1969), double stranded nucleic acids show bright green fluorescence while single stranded nucleic acids show yellow to orange fluorescence with euchry sine. This is the criterion for identifying these bodies as aggregates of chromatin. It is noteworthy that electron microscopic investigation employed by Gale, et al. (1964) revealed that nuclear material of HU-treated Pseudomonas aeruginosa cells was either virtually absent or coalesced into electron-dense spherical bodies.

The "chromatin aggregates" that form the halo in HU-treated cells may actually be the "nucleolar fusion bodies" described by previous investigators. Under exponential growth conditions hundreds of nucleoli are evenly distributed over the inner surface of the nuclear membrane of Tetrahymena (Elliot, 1963; Cameron and Guile, 1965). These nucleoli then fuse into large bodies called nucleolar fusion bodies during late exponential growth or heat-shock synchronization (Cameron and Guile, 1965; Cameron, Padilla and Miller, 1966). Electromicrographs, phase photomicrographs, and photomicrographs of Feulgen stained cells show nucleolar fusion bodies apparently complexed with the inner surface of the nuclear membrane in stationary phase and heat-shock recovering cells (Cameron, Padilla and Miller, 1966; Nilsson and Leick, 1970). Similar to chromatin material, these

nucleolar fusion bodies contain proteins, RNA, and DNA (Charret, 1969). Nilsson and Leick (1970) postulate that the complex of fusion bodies becomes more distinct and detached from the chromatin during the stationary phase of growth because the DNA is non-replicating and therefore condensed.

The halo formed during the HU-induced inhibition of DNA replication and cell division in Tetrahymena may be merely a more extreme or amplified form of the formation of nucleolar fusion bodies that occurs when cell division is inhibited during stationary phase or heat-shock treatment. The halo effect has also been observed, using euchryesine dye, in Tetrahymena which were X-irradiated during heat-shock recovery with an acute dose of 7.5 kilorads (Hodge, 1971a, b).

The fact that the halo effect was neither observed by Hodge (1971a) in non-irradiated, heat-shock synchronized cells nor myself in non-HU-treated stationary phase cells suggests a qualitative difference between the formation of nucleolar fusion bodies as reported by others (Cameron and Guile, 1965; Cameron, Padilla, and Miller, 1966; Nilsson, 1970; Nilsson and Leick, 1970) and the HU or X-irradiation induced formation of the "halo" observed with euchryesine stain. Both of the agents (HU and X-rays) that have been observed to initiate the halo effect in Tetrahymena are also known to induce damage to DNA thus providing the basis for a real qualitative difference between the

halo effect described in this study and the peripheral formation of nucleolar fusion bodies described by others.

2. Physiological Significance of the Halo Effect

Some important questions may be posed. What is the physiological significance of the halo effect? How does this morphological response to HU relate to what is already known about the effects of HU upon the cell? Does the apparent separation of the major portion of chromatin from the nuclear membrane reflect an actual uncoupling of membrane associated DNA synthesis? Let me attempt to answer this last question first. The previous observations that HU inhibits semiconservative but not non-semiconservative DNA synthesis along with the observations that newly synthesized DNA is fragmented in the presence of HU during S-phase (Rosenkranz, 1966; Coyle and Strauss, 1970) point to the consideration that HU may be inhibiting one of the enzymes specifically involved in replicative DNA synthesis. The DNA polymerase (and possibly the DNA ligase) involved in replicative (semiconservative) DNA synthesis is now believed to be membrane associated (Smith et al., 1970; Knippers and Stratling, 1970) so perhaps the halo effect is visible evidence of a physical uncoupling of replicative DNA synthesis from the nuclear membrane in S-phase cells. However, this tempting hypothesis seems quite unlikely in light of the

fact that the halo effect remains after the HU is washed from the cells until the first recovery division. As I've already indicated (see Effects of Hydroxyurea), autoradiographic and cytospectrophotometric investigations reveal a large amount of DNA synthesis, the major portion of which is probably semiconservative, occurring during the recovery period. Thus, the fact that the halo effect is still present in cells which are apparently replicating their DNA contradicts the idea that the halo effect is the result of a physical uncoupling of replicative DNA synthesis.

Now we can return to the initial questions concerning the physiological significance of the halo effect in conjunction with what is already known about the effects of HU. First of all, the halo effect is a cellular response to known DNA damaging agents (HU and X-irradiation). The halo effect may therefore be some kind of cellular response to situations of acute damage induction to DNA. Perhaps it is a morphological reflection of a physiological response in the form of massive mobilization of excision repair activity. HU was found to induce the halo effect only in cells undergoing S-phase. The X-ray induced halo effect does not appear to be S-phase specific (Hodge, 1971a, b), while the HU induced halo effect is S-phase specific. This may merely be a reflection of the fact that X-irradiation is not phase specific in its direct induction of damage to DNA, while HU is

phase specific. The DNA may become more vulnerable to HU during S-phase because of "unmasking" of DNA-associated proteins, such as histones, during DNA replication. Thus, the DNA becomes more accessible to the damaging effects of HU in the regions where DNA replication is being initiated, resulting in increased damage and, therefore, increased repair activity in these regions. The maintenance of the halo effect throughout the recovery period may be due to a continuation of increased excision repair activity by the cell in order to correct damage which was previously introduced in the presence of HU.

Cell Cycle Analysis with Hydroxyurea

It is clear from the results of this study that HU does not affect the durations of G_1 , G_2 , or D phases in Tetrahymena pyriformis. The fact that HU specifically blocks progression of S-phase cells through the cell cycle coupled with the fact that dividing cells are morphologically distinguishable from nondividing cells allows the use of HU in simple and time-conserving procedures for determining G_2 and possibly G_1 times in Tetrahymena pyriformis as steps in cell cycle analysis. In addition, the S-phase specificity of the HU-induced halo effect allows S-phase cells to be morphologically distinguishable from non-S-phase cells, thus creating a potential for using HU in a simple method of determining the duration of S-phase.

G₂ Analysis

The results of this study indicate that HU blocks all cells in S-phase except for a small fraction which are evidently near termination of DNA replication. This S-phase specificity allows the use of hydroxyurea as a simple and rapid method for analyzing G₂ times. The most economical method, in terms of time, work, and money, for determining a close estimate of the G₂ time of Tetrahymena is the exponential growth arrest method using HU. (See Figures 9 and Table 3). The exponential growth arrest method simply consists of adding 10mM HU to a sample of exponential growth cells and then determining the accumulated fraction of cells that continue to divide. This fraction, along with the generation time, which is determined from a control sample of the same population of exponential growth cells, are used to calculate the time duration of the post-HU-block period, which is in close agreement with the G₂-phase duration derived by standard ³H-TdR labeling procedures.

The results of this study showed that only about 8% of S-phase is beyond the HU-block (page 72). This small fraction of labeled cells which are able to divide is probably not a result of any lag period involved in HU reaching its target sites (permeability factor) because there is no relationship between the concentration of HU used and the fraction of cells beyond the block (Figure 8). The fraction of S-phase

cells beyond the HU-block is probably a reflection of the deoxyribonucleotide pool size in these late S-phase cells. The initial action of HU is to block the reduction of ribonucleotides; however, some DNA synthesis may continue as a result of the availability of deoxyribonucleotides which were formed and pooled prior to the introduction of HU into the system. This could account for the observation that cells in the terminal 8% of S-phase are seen to slip past the HU-block in this study.

The exponential growth arrest method of determining G_2 -time may also be applicable to other cell systems. Several factors must be considered, however, before application of the method can be considered valid. The ability of HU to block essentially all cells undergoing DNA replication may be quite variable among various cell lines and growth media. For example, the use of HU may not be feasible for G_2 analysis in mouse L-cells where the terminal 30% of S-phase is evidently beyond the HU block (Bacchetti and Whitmore, 1969). The extent to which a particular cell line can form a deoxyribonucleotide pool and rely on this source for DNA synthesis may greatly affect the amount of DNA replication that can continue after the introduction of HU into the medium. Endogenous pool formation is also largely dependent upon the exogenous environment of the cell. Thus, the DNA-precursor content of the growth medium may become an important factor in influencing the size of cellular deoxyribonucleotide

pools. In addition the extent to which the cell must complete the replication of its genome before it can continue on and divide may be an important factor in determining the feasibility of cell cycle analysis with HU. Indeed, it has been reported that early G_2 cells (chinese hamster) had difficulty in traversing to mitosis in the presence of HU (Sinclair, 1967). Thus, post S-phase factors involved in maintaining progress to division are evidently affected by HU in this case. Still another factor to be taken into consideration is cell permeability to HU and the rate at which HU moves from outside the cell to its target sites within the cell. In connection with this permeability factor, the concentration of HU employed may be important in minimizing any lag period between the time HU is introduced and the time it induces its inhibitory action.

A recent paper by Degnen and Newton (1972) describes the use of HU-induced arrest of exponentially growing bacteria (Caulobacter) as a means of determining the duration of the G_2 -period. The drug evidently arrests all cells from further progress that have not completed synthesis of the DNA chromosome, thus allowing its use to analyze the G_2 -phase time. The use of HU for G_2 analysis in Tetrahymena and Caulobacter should encourage further investigation concerning the application of this time saving and simple method of G_2 analysis to other cell lines.

G₁ Analysis

In Tetrahymena the S-phase is definitely the sensitive phase of the cell cycle in terms of recovery from HU-exposure as indicated by the amount of time elapsed between the removal of HU and the first recovery division. Figure 14 shows that in synchronously growing populations, recovery delay time does not significantly increase until the HU addition time is in late G₁ and/or early S-phase. Recovery delay then reaches a maximum and remains there for populations into which HU was added during S-phase. These results suggest the possibility of using HU to analyze the duration of G₁.

The G₁ time can be defined as the duration from division until the time of 50% increase in recovery delay times (Figure 14). Thus, if HU is introduced into a cell population in late G₁ and early S-phase, there will be an increase in the recovery delay directly proportional to the fraction of the population that was in early S-phase at the time of HU introduction. When the cell cycle progresses to a state where 50% of the population has entered S-phase while 50% still remains in G₁-phase when HU is introduced, then the recovery delay time will be 50% of the maximum recovery delay time seen when 100% of the cells have entered into the S-phase. This 50% HU-recovery delay time then marks the end of G₁ and the start of the S-phase.

The validity of this method for determining G_1 time rests upon the pivotal assumptions that 1) the S-phase sensitivity to HU as measured by recovery delay coincides closely with S-phase as defined by the traditional radiotracer methods and 2) the cells that are not in S-phase at the time of HU introduction are blocked from entry into S-phase, at least for the duration of the HU-treatment.

In relation to the second assumption above (2), labeling experiments indicate that HU-treatment has no effect on the duration of the G_1 -phase (Figure 13), while XdR rescue experiments indicate that HU-treated G_1 cells do accumulate, possibly at the G_1/S border, for a significant period of time before moving into S-phase (Figure 17). This apparent paradox may be explained by postulating that the HU-nonsensitive/-sensitive border does not coincide with, but comes slightly after the G_1/S border. Whatever the case may be, it is clear that before it can be considered valid, the recovery delay method of G_1 analysis must be put to test under different experimental situations and repeated enough times to determine whether there is a statistically real relationship between the G_1 time measured by radiotracer techniques and the G_1 time as measured by this method.

As to the mechanism of the HU-induced excess delay during S-phase, it has already been pointed out in the Introduction that numerous studies indicate that S-phase is the portion of the cell cycle that is sensitive to HU in terms of the induction of lethality, chromosome

aberrations, and degradation of DNA. These effects are likely the result of a direct action of HU on DNA. Similarly, it is probable that a direct effect of HU upon DNA is involved in recovery delay in Tetrahymena. The HU may be inflicting damage to the DNA of cells in S-phase while those cells that are in G_1 at the time of addition of HU tend to collect at the G_1/S border and transgress into S-phase at a very slow rate (Bacchetti and Whitmore, 1969). The cells with the greatest amount of HU-inflicted damage, that is, the cells that were in S-phase during the time of HU introduction, have to undergo the greatest amount of DNA repair before they can go on to divide. This additional DNA repair may result in the excess recovery delay observed in these cells.

S-phase Analysis

The S-phase specificity of HU-induced halo formation allows the possibility that the halo effect may be used as a means of cell cycle analysis. If the S-index can be obtained directly from the "halo-index," that is, the percentage of cells expressing the halo effect, then the duration of S-phase can also be determined (assuming the generation time is known). One problem foreseen in using this method of S-phase analysis stems from the fact that halo induction does not become visible until after about 2 hours treatment with HU. This problem may, however, be eliminated if non-S-phase cells collect at the G_1/S border and are delayed in entering S-phase (the HU sensitive

phase) as appears to be the case in the recovery delay response to HU treatment (See Results and Discussion sections on recovery delay, XdRs rescue and G_1 analysis). If non-S-phase cells were delayed from entering S-phase, then the halo-index could still be used, even after a 2 hour delay in its actual expression, to determine the original S-index. Of course, in determining the actual S-index from the percentage of cells expressing the halo effect after 2 or 3 hours of HU treatment one would be required to correct for dilution of the S-index caused by the continuing division of cells that were in G_2 at the time of HU introduction. This is simply accomplished by observing the increase in relative cell number after HU introduction and mathematically taking this into consideration by subtracting a proportional number of cells from the non-haloed fraction before determining the fraction of haloed cells. At present the validity of S-phase analysis with HU has not actually been put to test and it remains to be seen whether or not HU can be used for such analysis.

Cell Cycle Analysis of Cells After HU-Treatment

When Tetrahymena cells are treated with HU for a period of time and then the HU is removed, the cells are able to recover from the HU-block and subsequently divide. After dividing they enter a cell cycle which is significantly shorter in duration than control generation

times. Cell cycle analyses with ^3H -TdR of the recovery period and the cell cycle following recovery division were conducted. The results of these analyses aided in characterizing certain events involved in recovery from the HU-block. During the course of these investigations it was discovered that the cell cycle kinetics of cells recovering from the HU-block follow a pattern that supports a nucleo-cytoplasmic mass ratio model for the control of cell growth, DNA replication and division in Tetrahymena.

When Tetrahymena cells are maintained in HU for a period of time, e.g., 10 1/2 hours, and then the HU removed from the medium, they are able to recover their capacity to proliferate after a period of time. The duration from the time of HU removal until the time of 50%-cell separation has been designated the recovery period. The recovery period has been dissected into a DNA-synthesis period and a post-DNA-synthesis period, both of which are of greater duration than the normal S-phase and G_2 -phase. The DNA-synthesis period begins almost immediately upon removal of the HU (Figure 15) and continues through the first 60% (165 min.) of the recovery period.

In terms of recovery delay we have seen that S-phase is the most sensitive portion of the cell cycle to HU exposure. (See Figures 14 and 17). The S-phase specificity for recovery delay is perhaps due to the post-HU-treatment repair of damage which was induced in the

DNA by HU during S-phase. Thus, a fraction of the DNA synthesis observed during the recovery period could be due to excision-repair and/or post-replication repair of DNA (Lehmann, 1971). However, the major amount of DNA synthesis that occurs during the recovery period is probably of the replicative (semiconservative) type.

Nuclear DNA/Cytoplasmic Mass Ratio Control of DNA Replication and Cytokinesis

The DNA synthesis portion of the recovery period lasts 165 minutes (compared to 85 minutes for the average S-phase of exponentially growing cells). Recent microspectrophotometric studies of the DNA content of cells recovering from HU-treatment conducted here and in the laboratory of David C. Shepard have yielded interesting results. These investigations indicate that the DNA content, which is at a relative value of 1 after 10 hours treatment in HU, increases during the recovery period to a relative value of about 4 prior to the first recovery division. The observation that the nuclear DNA content of cells recovering from a 10 hour HU-treatment triples or quadruples rather than just doubles was rather unexpected initially, but when placed in conjunction with other observations becomes quite logical. McDonell (1972) reported that in the typical cell cycle of Tetrahymena pyriformis the ratio of cytoplasmic dry mass to nuclear-DNA is always the same just prior to division (late G₂) and just after division (early G₁).

When *Tetrahymena* cells were treated with HU for a significant period of time they were always observed to enlarge significantly. A quantitative example of this observation is presented in Table 6. After only 3 hours treatment with HU the average cell has increased in length from 61 to 68 microns. After 10 1/2 hours treatment the cells appear much larger. An increase in cytoplasmic volume in the presence of HU has been previously observed and measured in mouse L cells (Rosenberg and Gregg, 1969).

The observation that a specific nuclear DNA content to cytoplasmic mass ratio is attained in pre- and post-dividers coupled with the observations that the DNA content quadruples in the cytoplasmically enlarged cells recovering from the HU-block together support the hypothesis that the nuclear-cytoplasmic ratio is a major factor in the control of DNA synthesis and cell division in Tetrahymena. The following paragraphs explain the nuclear-cytoplasmic ratio hypothesis and discuss, in the context of that hypothesis, the characteristics of recovery from the HU-block.

The principle of "Kernplasmarelation" (nuclear to cytoplasmic mass ratio) as a limiting factor to the growth of individual cells was first postulated in 1908 by R. Hertwig (as cited by Mazia, 1961). Hertwig postulated that, given a specific size nucleus, a mass of cytoplasm can grow only to a limited extent and that the capacity for

growth is refreshed once the nucleus has divided. When the nuclear to cytoplasmic ratio falls below a certain value, physiological instability sets in which precipitates cell division. In 1928 Max Hartmann (as cited by Mazia, 1961) generated data from experiments with Ameba proteus that supported the nucleo-cytoplasmic mass ratio as a factor limiting cytoplasmic growth. He induced "experimental immortality" by amputating cytoplasm at intervals, permitting the cell to regenerate cytoplasm before the next amputation. As long as he kept this up (several months) the cells survived without division. He concluded that cell division would not be necessary for biological immortality if cells could naturally slough off cytoplasm and regenerate new cytoplasm before reaching the nuclear to cytoplasmic mass ratio that is critical to the induction of cell division. These results were extended by David Prescott (1956a, 1956b, 1959) who also worked with Ameba proteus. Using a Cartesian diver balance, Prescott measured cell mass between divisions. Normally cells reached twice their post-division (daughter cell) mass before they divided. If, however, sister cells were unequal in cytoplasmic mass, they did not each double in mass but both the small and the large daughter grew to the mass of their parent before dividing again. The generation time was inversely proportional to the cytoplasmic mass of the cell at the start of its cell cycle (post-division daughter cell). These data strongly support the

hypothesis that a critical nuclear/cytoplasmic mass ratio must be achieved before these cells undergo division.

The proponents of the nucleo-cytoplasmic mass ratio as a controlling factor of cell growth and reproduction do not attempt to argue that it is the ratio per se that is programmed to stimulate or inhibit growth and reproduction. Instead it is postulated that cytoplasmic growth can normally continue until it begins to increase beyond the sphere of nuclear influence (Hertwig, 1908). Thus, cell growth is limited to the amount of cytoplasm that can be "administered" by the nucleus. As growth increases beyond a certain point the increased requirement for protein synthesis may go beyond the capacity of the nuclear membrane-enclosed genome. In support of this view are the numerous observations of giant cells resulting when the genome is amplified, for example, by polyploidy, polyteny, or by selective gene amplification in vertebrate oocytes. The increase in cytoplasmic mass may be limited by the number of genes that can be transcribed for the synthesis of enzymes necessary in catalyzing the metabolic processes that are required to maintain the dynamic equilibrium of the living cell. An increase in the total number of transcription sites for the synthesis of proteins necessary to support the metabolic and structural requirements of the cytoplasm allows a concurrent increase in the cytoplasmic mass of the cell. Therefore, the "giant effect" of polyploid, polytene,

and oocyte cells is an expected phenomenon when considering the nucleo-cytoplasmic mass ratio as a controlling factor of cell growth and reproduction.

The data gathered from monitoring the cytoplasmic dry mass, nuclear DNA content, and analyzing the cell cycle of Tetrahymena cells recovering from exposure to hydroxyurea strongly support the nuclear/cytoplasmic mass ratio model of control of cell growth and reproduction in this organism. During the HU-treatment, replicative DNA synthesis is inhibited while RNA and protein synthesis are not inhibited (see Introduction), thus resulting in the arrest in growth of the nuclear DNA mass while the cytoplasmic mass continues to grow. This unbalanced growth or uncoupling between nuclear and cytoplasmic growth results in a low nuclear to cytoplasmic ratio. When the HU is removed, the cells can recover the capacity to divide only after achieving the correct nucleo-cytoplasmic ratio by increasing their DNA content.

The first cell cycle following the recovery period after 10 1/2 hours HU-treatment is abbreviated at the expense of the G₁-period (Figure 16). This is similar to the first cell cycle following heat-shock synchrony in Tetrahymena pyriformis (GL) (Sherbaum and Zeuthen, 1954).¹ Also, the first few cell cycles of Tetrahymenae

¹The heat-shock synchronization method for Tetrahymena has been exploited by many investigators and has generated a large literature, of which key reviews are by Zuethen (1964), Scherbaum (1964),

which have just left the maximum stationary growth phase and are entering the logarithmic growth phase are abbreviated at the expense of G_1 . According to A. Zetterberg (as cited by Zuethen, 1970), a short cell cycle at the expense of G_1 is also a typical feature in oversized mammalian cells. In addition, shortened cell cycles have been noted in yeast after removing a DNA replication block (Mitchison and Creanor, 1971), in Physarum after U. V. induced division block (Sachsenmaier, et al., 1970), and in Tetrahymena after U. V. induced division block (Shepard, 1965).

Enlarged cells re-entering exponential growth phase after treatment with HU (or heat shocks) have evidently achieved the necessary conditions to allow them to immediately begin DNA replication upon termination of the treatment and again immediately enter S-phase upon completion of the first recovery division. In recovering from HU-arrest, the cells must synthesize and accumulate the enzymes involved in replicative DNA synthesis during the HU-treatment and/or during the recovery period. (In this connection it is pertinent that HU has

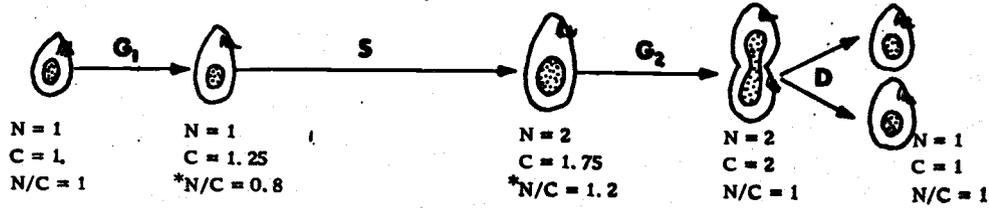
Zuethen and Rasmussen (1971), and Mitchison (1971). The heat-shock treatment uncouples the synchrony between DNA replication and division. During the series of heat-shocks the cells continue to enlarge both in nuclear and cytoplasmic mass. After the final heat-shock the cells undergo a recovery period during which 60 to 70% of them are synthesizing DNA. It is postulated that once DNA replication has been initiated it must continue to completion before division can occur (Anderson, Brunk, and Zuethen, 1970; Jeffery, 1972). The first recovery division is followed by a short cell cycle characterized by the partial or complete absence of a G_1 period (Hjelm and Zuethen, 1967).

been observed to induce an increase in the synthesis of thymidine kinase in cultured human Chang liver cells (Eker, 1968) and an increase in the synthesis of ribonucleotide reductase in Chinese hamster cells (Murphree, et al., 1969). In the case of Tetrahymena the oversized cells appear to return to the expected size of logarithmic growth phase cells after the first one to four recovery cell cycles. In light of this observation, a short cell cycle following the first recovery division is not unexpected. But it may be asked: Why does DNA replication occur at all in these cells? Why is the G_1 -less cell cycle not abbreviated even more by lacking an S-phase too? A possible explanation for the necessity of DNA replication in the first recovery cell cycle takes into account the nuclear to cytoplasmic ratio theory for the control of DNA replication and cell division.

In light of my own observations and those of MacDonell (1972) and Salamone (1972), I have constructed a diagram (Figure 28) which accounts for the occurrence of DNA replication, without any G_1 period, both during the recovery period and the first cell cycle following the recovery period, and which meets the requirements of a nuclear/cytoplasmic-ratio model of control for DNA synthesis and cell division. The diagram in Figure 28 includes nuclear DNA content values (N) and dry mass values (C) which are relative and wherever possible were derived from experiments involving microspectrophotometric measurements conducted by MacDonell (1972) and Salamone (1972),

Figure 28. A model for the control of the initiation of DNA replication and cytokinesis based upon nuclear DNA content/cytoplasmic mass ratios. The diagram indicates relative cytoplasmic mass (C) values, relative nuclear DNA content (N) values, and nuclear DNA content to cytoplasmic mass ratios (N/C) for the cell at various stages of the cell cycle both before (A) and after treatment with 10mM HU (B). The N/C values are derived from the data presented in Table 10. Where the data was incomplete or unavailable hypothetical N/C values are listed and marked by an asterisk.

A. Exponential Growth Cell Cycle.



B. HU-Treatment and Recovery.

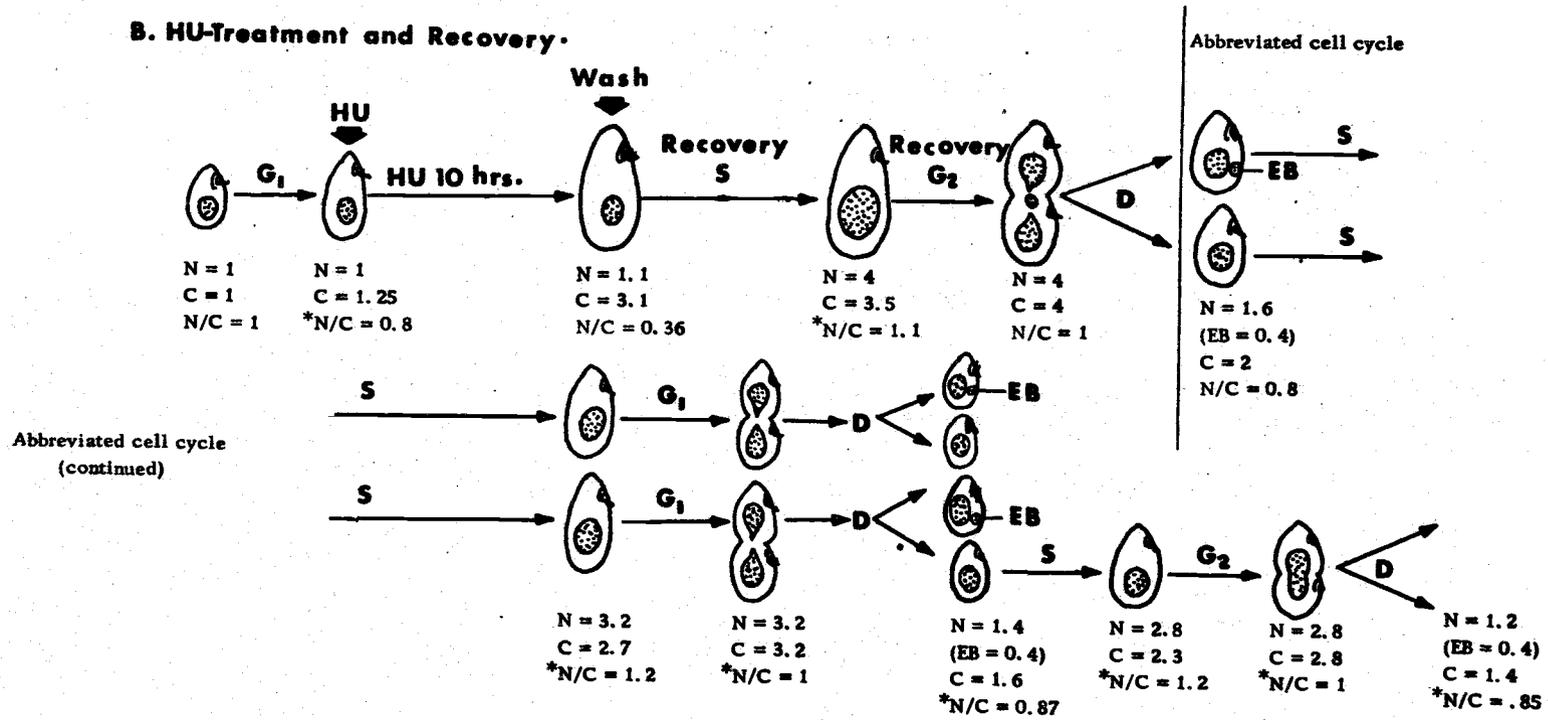


Table 10. The effect of HU exposure (10 hours) on the relative DNA content (N), dry mass (C), and DNA/dry mass ratio (N/C) during and after the release of the HU block in *Tetrahymena pyriformis*.

Experimental Situation	Dry Mass x 10^{-9} g ^a	Relative Dry Mass (C) ^b	Microspectro Readings for Nuclear DNA ^a	Relative Nuclear DNA Values (N) ^b	Relative DNA/Dry Mass Ratios (N/C)
Control post-division cells					
McDonell	1.61	1.0	70.4	1.0	1.0
Salamone	-	-	63	1.0	1.0 ^d
Control pre-division cells					
McDonell	3.25	2.0	134.2	2.0	1.0
Salamone	-	-	-	-	-
HU blocked cells (10 hours)					
McDonell	4.02 ^c	3.1	-	-	-
Salamone	-	-	67	1.1	0.36 ^d
Pre-recovery div. cells					
McDonell	6.42	3.9	273	3.9	1.0
Salamone	-	-	267	4.2	0.93 ^d
Post-recovery div. cells					
McDonell	-	-	-	-	-
Salamone	-	-	101	1.6	0.8 ^e
Post-recovery div. ECBs					
McDonell	-	-	-	-	-
Salamone	-	-	26	0.4	0.2 ^e

^aThe raw data in the first and third columns are mean values from samples, $n \geq 24$ for McDonell (1972) and $n \geq 12$ for Salamone (1972).

^bAll relative dry mass and nuclear DNA values were weighed from a relative value of 1 for early post-divider control cells.

^cThis value was derived from a different experiment than the rest of McDonell's data in that column.

^dThese DNA/dry mass ratios were derived by using Salamone's nuclear DNA content values and McDonell's dry mass values.

^eThese DNA/dry mass ratios are approximations based upon Salamone's data and the relative dry mass value of ~2 expected for these post-recovery division cells.

whose data are presented on Table 10. The nucleo-cytoplasmic ratios (N/C) are also relative values taken directly from Table 10. However, the N/C values for various times in the cell cycle where no experimental data are available (for example, the G_1/S and S/G_2 transitions) are hypothetical. These N/C ratios are designated with an asterisk in the diagram.

The diagram in Figure 28A is of a typical cell cycle in exponentially growing Tetrahymena. The cell cycle starts with $N/C=1$. After the cell increases its cytoplasmic mass to a certain point (during G_1) DNA replication is initiated because the low N/C ratio ($N/C=0.8$) sets the intracellular condition that allows the "triggering" of DNA replication. The DNA synthesized after one round of DNA synthesis overcompensates for the cytoplasmic growth that has concurrently taken place resulting in a high N/C ratio ($N/C=1.2$). The high N/C ratio triggers the intracellular events that prepare the cell for division (G_2 period) while cytoplasmic growth still continues until just prior to division. The fact that cytoplasmic growth is of a constitutive nature, continuing until division or just prior to division in Tetrahymena, is well documented (Cameron and Prescott, 1961; Prescott, 1961; Lovlie, 1963; Summers, 1963; Schmid, 1966). Near the end of the division preparation period (G_2) the cytoplasm has grown to a point ($C=2$) that yields a nucleocytoplasmic ratio of one ($N/C=1$), thus allowing the cell to enter division. The cell then divides, not

necessarily equally (McDonald, 1958; Cleffman, 1968), to yield daughter cells whose nuclear to cytoplasmic ratios are, on the average, the same ($N/C=1$) as that for their parent divider cell. Unequal distribution of macronuclear DNA to the daughter cells without a compensating unequal distribution of cytoplasm may result in some variation in individual cells from the average nuclear to cytoplasmic ratio of 1. This variation results in a variation in the amount of growth required before DNA replication is allowed to begin, which is, in turn, expressed as a variation in G_1 times. The fact that G_1 is the most variable portion of the cell cycle is well documented for a variety of cells (Mitchison, 1971) and is consistent with the nucleo-cytoplasmic model of control over the initiation of DNA replication and cell division.

In summary, for exponentially growing cells when cytoplasmic growth results in the nuclear to cytoplasm ratio decreasing to a certain point, then DNA replication is initiated, resulting ultimately in a high nuclear to cytoplasmic ratio. This high N/C ratio, e. g., $N/C=1.2$, stimulates the cell to prepare for division. Cytoplasmic growth continues until a nucleo-cytoplasmic equilibrium expressed in the model as a nuclear to cytoplasmic ratio of 1, is reached, at which time the cell enters division yielding daughter cells whose average N/C is also 1. So, we can conceptualize a linear series of relationships between the cytoplasmic mass, nuclear DNA content

and cell division in the sequence; cytoplasmic growth→nuclear DNA replication→division.

The above model is consistent with a number of observations. A block in cytoplasmic growth can result in a block of both nuclear DNA replication and cell division. A block in DNA replication will not immediately block cytoplasmic growth but will block division. A block in division will not necessarily block cytoplasmic or nuclear DNA growth; e.g., colchicine-induced endomitosis results in giant polyploid cells, and heat-shock synchronization of Tetrahymena blocks division but allows cytoplasmic growth and DNA replication to continue (Anderson, Brunk, and Zeuthen, 1970; Zeuthen and Rasmussen, 1971).

Mitchison (1971) summarizes several experiments that confirm the above relationships. He divides the cell cycle into two basic sub-cycles, the "growth cycle" and the "DNA-division cycle", citing several examples supporting the existence of a close interdependence between DNA replication and cell division while at the same time showing the great degree of dissociation that exists between cytoplasmic growth and the DNA-division cycle. Several nuclear transplant experiments are in agreement with the mechanism by which the nuclear/cytoplasmic ratio controls the initiation of DNA replication and cell division. It appears in both Physarum (Guttes and Guttes, 1968) and Amoeba (Ord, 1969) that G_2 nuclei do not synthesize DNA when transferred to

S-phase cytoplasm, while S-phase nuclei continue to synthesize DNA when transplanted to G_2 cytoplasm. Interpreted in the context of a N/C ratio control mechanism, the G_2 -nucleus/S-cytoplasm cell has a high N/C ratio that is expected to inhibit initiation of DNA replication while the S-nucleus/ G_2 -cytoplasm cell has a low N/C ratio which is expected to stimulate DNA replication. [Ord's (1969) results are, however, in complete disagreement with the earlier investigations of Prescott and Goldstein (1967) who also worked with Amoeba. They found that when S-phase nuclei were transferred to G_2 cytoplasm usually no DNA replication occurred, while in the reciprocal transplant experiment (G_2 nuclei in S cytoplasm) DNA synthesis occurred in most of the cells. Prescott and Goldstein's (1967) sample sizes were, however, less than half that of similar experiments by Ord (1969).] In Stentor G_1 nuclei transplanted to G_2 cytoplasm are induced to enter an accelerated DNA replication phase while S and G_2 nuclei do not synthesize DNA when transferred to G_1 cytoplasm. Interpreted in the context of a N/C ratio control mechanism, the G_1 -nucleus/ G_2 -cytoplasm cell has a low N/C ratio which is expected to stimulate DNA replication and the G_2 -nucleus/ G_1 -cytoplasm cell has a high N/C ratio which is expected to inhibit DNA replication.

Killander and Zetterberg (1965) have postulated that DNA replication is initiated only after cells have grown to a certain "critical" mass. Using interference microscopy to determine total dry mass

of individual cells (mouse-L fibroblasts) and the Feulgen microspectrophotometric technique of determining DNA content, they found that, although the variation in cellular dry mass was great among postmitotic cells, the variation in cellular dry mass among cells just starting the S-period was significantly low. These data along with the finding that the variation in G_1 times is proportionate to the variation in the mass among postmitotic cells led Killander and Zetterberg (1965) to postulate their "critical mass" model of initiation of DNA replication. The variation in G_1 times is due to the variation in the amount of growth in cells that must take place before the critical mass, which triggers DNA replication, is reached. They found that the average cell increased its mass by a factor of 1.25 before the onset of DNA replication and increased its mass by a factor of 2 before cell division took place. However, they point out that if all individual cells, independent of their initial mass, would always increase their mass by a factor of 2, then size heterogeneity would become amplified in the population after a few generations. This was not found to be the case in their population of cells over a 3 year period of monitoring cellular distribution of mass, thus supporting the critical mass model of S-phase initiation.

It should be made clear that Killander and Zetterberg (1965) were using a stable diploid cell line that revealed a low coefficient of

of variation in the DNA content of G_1 cells. Thus, their data indicating that the cells must grow to a critical mass before the initiation of DNA replication may also be interpreted in terms of nuclear DNA to cytoplasmic mass ratios. The nuclear DNA content is stable and constant and, therefore, the variation in cell mass is essentially variation in cytoplasmic mass. The fact that a critical cell mass must be attained before the initiation of DNA synthesis may be only incidental to the more important requirement that a specific nuclear DNA to cytoplasmic mass ratio (N/C ratio) be attained. Thus, Killander and Zetterberg's (1965) work may be interpreted as supporting a "critical N/C ratio" control model of DNA replication just as logically as it may be interpreted as supporting a critical mass model of the control of DNA replication.

The effects of HU on cell division can also be explicated in terms of N/C ratios. A hydroxyurea-induced block of DNA replication subsequently results in a block in cell division, but cytoplasmic growth continues in the presence of this 'DNA-division' block. Figure 28B illustrates the nucleo-cytoplasmic relationships that might fulfill the requirements of a control mechanism involving the nuclear DNA to cytoplasmic mass ratio for initiating DNA replication and cell division. The diagram indicates N/C ratios both during and after the HU-treatment through two recovery cell cycles. Again, most of the N values and ECB values (explained below) are relative values derived from the

actual investigations of McDonnell (1972) and Salamone (1972).

The model diagrammed in Figure 28B thus represents a synthesis of observations and hypotheses that relate the effects of HU on cell division to the nucleocytoplasmic mass ratio control mechanism.

Figure 28B illustrates the fact that during the HU-treatment, DNA replication is blocked but cytoplasmic growth continues resulting in unbalanced growth such that the N/C ratio is significantly decreased to a value of 0.36. (See Table 10.) After the HU is removed DNA replication immediately takes place resulting in a counterbalance phenomenon that increases the N/C ratio to a hypothetical value of $N/C=1.2$ which is high enough to allow the triggering of preparation for division. The continuation of cytoplasmic growth brings the N/C ratio to the observed value ($N/C=1$) that initiates nonreversible entry into division. At this point the cells are oversized both in cytoplasmic mass ($C=4$) and DNA content ($N=4$) and the subsequent recovery cell cycles are involved in rapidly returning the cells to the lower cytoplasmic and DNA contents normally found in an exponentially growing population. Two features accommodate the attainment of this objective; these being, the shortening of the cell cycle and the increased formation of large extra-nuclear chromatin bodies (ECB's). The shortening of the cell cycle serves to cut down the amount of cytoplasmic growth that can take place between divisions, thus returning the cells to their original level of cytoplasmic mass. Extra-nuclear

chromatin bodies, sometimes called 'extrusion bodies' are extranuclear packets of DNA that originate by 'pinching off' from the macronucleus during division. The fate of the ECB DNA is unknown but it is probably denatured by nucleases and either expelled from the cell or recycled in the form of deoxyribonucleotides within the cell. After 10 1/2 hours HU treatment I have noted, in post-recovery division cells, an ECB frequency of about 60% whereas the frequency of post-divider cells containing at least one ECB in normal exponential growth populations is only 20 to 30%. In addition the diameter of these spherical bodies increases from about 0.8 microns in exponential growth post-dividers to 3 to 5 microns in post-recovery dividers after a 10 1/2 hour HU treatment. Salamone (1972) reports an average DNA content of these enlarged HU-recovery ECB's to be about two fifths that of the average G_1 nucleus. The formation of ECB's then serves as a mechanism to reduce the nuclear DNA content of recovering cells back to the original value typical of exponentially growing cells. Indeed, Walker and Mitchison (1957) have previously suggested "nuclear budding" as a mechanism to adjust the amount of nuclear DNA in Tetrahymena.

Labeling experiments which I conducted also support this view of the function of ECB formation. Relative grain counts between ECB's and nuclei were compared in post-recovery-division cells which were labeled during the HU treatment in one group and labeled during the

recovery period in another group. It was found that, although in both groups the ECB's seemed to be more intensely labeled, there was no relative difference between the two groups in the relative ECB to nuclear grain counts. This is interpreted as being evidence that the DNA found in the ECB's is not a specific type of DNA, such as HU-damaged DNA that is being selectively excluded from the genome, but is a nonspecific sample of the nuclear DNA. Thus, the importance of ECB formation is related to quantitative, not qualitative, parameters.

After the first recovery division, the nuclear DNA content of the daughter cells is reduced by the formation of ECB's from the expected relative value of 2 to a value somewhere between 1.0 and 2.0. I have assigned values of 1.6 to the nuclei and 0.4 to the ECB's of these post recovery division cells. These values were derived from actual data (listed in Table 10). Actually, if the parent dividers ($4.0N$) of these cells form an average of one ECB ($0.4N$) each, as observations indicate, then $4.0 - 0.4 = 3.6N$. This $3.6N$ divided in half should yield daughter nuclei with average N values of 1.8. The discrepancy between the actual ($1.6N$) and the expected ($1.8N$) data is not great enough to warrant concern, but what is noteworthy is that the loss in nuclear DNA content by ECB formation is not accompanied by an equivalent reduction in cytoplasmic mass during division. The N/C ratio, therefore, is low (0.8). The low N/C ratio triggers almost

immediate initiation of DNA replication, thus bypassing the G_1 growth period. DNA replication is still required in order to achieve a high enough nuclear to cytoplasmic ratio to allow cell division to take place, but the reduction in cytoplasmic mass results from the restriction of cytoplasmic growth to a shorter time duration, i. e., the G_1 -period has been eliminated from the cell cycle. After one round of DNA replication the N/C ratio is high enough to trigger preparation for division, etc.

The diagram in Figure 28B indicates that the original (pre-HU-treatment) cytoplasmic and nuclear DNA content will be reached after about three complete cell cycles following the first recovery division. Due to the asymmetrical division of the macronucleus, the actual number of abbreviated recovery cell cycles probably varies from cell to cell resulting in a wide range of G_1 times and a breakdown in division synchrony after the first one or two cycles. The assumption in the diagram that the original cytoplasmic and nuclear DNA content will be attained after three recovery cell cycles is for the sake of simplicity. Variations in the actual number of recovery cell cycles should have no effect on the basic N/C ratio model proposed for the control of the initiation of DNA replication and cell division in cells recovering from HU arrest. I should again caution the reader that the data supporting the schema diagrammed in Figures 28A and 28B are not yet complete.

Effects of Excess XdRs

The combination of experiments involving continuous and pulse exposure to XdRs along with the ^{33}P autoradiographic experiments revealed that excess XdRs shorten the generation time by shortening the S-phase. Early S-phase showed the major response to the presence of excess XdRs while late S-phase showed no shortening response. S-phase is, therefore, dissected into a portion that responds to the presence of excess XdRs and a portion that does not respond. The most feasible explanation for this differential response is that during early S-phase, XdRs are both incorporated directly and pooled for later incorporation into DNA. This may be a reflection of the cyclic availability of kinase enzymes involved in phosphorylating the XdRs. This hypothesis has been previously discussed in conjunction with the differential pattern of division delay response to the simultaneous addition of HU and XdRs during early and late S-phase (p. 135). A more thorough understanding of the heterogeneity in early and late S-phase of Tetrahymena pyriformis awaits studies involving monitoring the activity of various DNA metabolic enzymes such as thymidine kinase during S-phase, along with monitoring the nucleotide pool sizes during S-phase.

SUMMARY

1. The present study involved the use of radionuclides, hydroxyurea (HU), and DNA-precursor deoxyribonucleosides (XdRs) in determining their various effects upon the cell cycle and in analyzing the cell cycle per se of the ciliated protozoan Tetrahymena pyriformis (GL-I).

2. The basic approach was to define the physiological and morphological effects of the challenging agent (HU and XdRs), to determine which portion(s) of the cell cycle was sensitive to the challenging agent and to then test the feasibility of employing the agent for use in analysing the cell cycle.

3. One objective of this study was to determine the primary effect of HU upon the physiology of the organism. Hydroxyurea (10mM) was found to arrest all cells from further progress into the cell cycle once they had entered S-phase. Autoradiographic analysis indicates that HU-blocked cells continue to synthesize DNA but the grain count is about 1/5 that of untreated S-phase cells. The HU-block can be reversed by either removing the drug from the culture medium or by the addition of 0.5mM each of the four DNA-precursor XdRs to the medium. Reversal of the HU-block by addition of XdRs supports the results of previous work which indicated the primary action of HU is the inhibition of the reduction of ribonucleosides (-tides) to deoxyribonucleosides (-tides).

4. Another objective of this study was to determine the existence of any additional effects HU may have upon the physiology of the organism. Synchronously dividing populations were blocked by the addition of HU at various times during the cell cycle. The block was then removed by either washing out the HU or adding XdRs to the medium. It was found that, for cells which were in S-phase at the time of HU addition, an excessive delay in recovering the ability to divide was induced. This S-phase sensitivity to HU may be due to the repair of single-strand breaks in the DNA which were induced solely during S-phase.

5. It was also found during the course of this study that HU induced changes in the morphology of the cell. Fluoromicroscopic examination of euchryesine-stained cells exposed to HU for a minimum time of 2 hours revealed the appearance of a morphologically altered macronucleus. This phenomenon, called the "halo effect," is characterized by the formation of apparently membrane-associated chromatin aggregates surrounding a constricted chromatin mass. The induction of the halo is S-phase specific. When the HU-block is removed by centrifugation washing the halo remains until after the cells have undergone the first recovery division. The halo effect may be a morphological indication of the cells repair response to the damaging effects of HU upon the DNA of the macronucleus.

6. Another objective of this study was to attempt to make use of the S-phase specificity to the effects of HU for simple means of cell cycle analysis. It was found that HU blocks normal progression of cells through the initial 92% of S-phase but does not affect cells in the remaining portion (8%) of S-phase or in G_2 . Thus, the HU-block/post-HU-block boundary coincides closely (within about 6 minutes) with the S/G_2 boundary thereby establishing the potential use of HU in analyzing the duration of G_2 . A simple means of G_2 analysis with HU is described. The possibility of using HU in the analysis of G_1 and S-phase is also discussed.

7. It was found that the addition of all four DNA-precursor XdRs (0.5 mM) to exponentially growing populations increased their rate of growth. Subsequent studies involving cell cycle analysis and autoradiography with phosphorus-33 and cell phase sensitivity to the presence of excess XdRs revealed that the XdRs were taken up and incorporated at an increased rate during the initial 50% of S-phase. It is postulated that this was the result of cyclic availability of deoxyribonucleoside kinase enzymes during early S-phase in Tetrahymena.

8. Another objective of this study was to analyze the cell cycle of cells recovering from a period of incubation in the presence of HU. When HU was removed after 19 hours of treatment the cells underwent a period of recovery lasting 275 minutes before undergoing their first

recovery division. Tritiated thymidine uptake showed that the first 60% of the recovery period is taken up by cells undergoing DNA synthesis. After the recovery division the cells enter a cell cycle which is abbreviated at the expense of G_1 . These results coupled with the results of other investigations lend plausibility to a model for the control of the initiation of DNA replication and cytokinesis based on the ratio of nuclear DNA content to cytoplasmic mass in Tetrahymena. The model is presented and discussed.

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