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CELL CYCLE PHASE OF NONDIVIDING CELLS IN AGING HUMAN CELL CULTURES DETERMINED BY DNA CONTENT AND CHROMOSOMAL CONSTITUTION

Rosalind Marie Yanishevsky
(Ph. D. Thesis)

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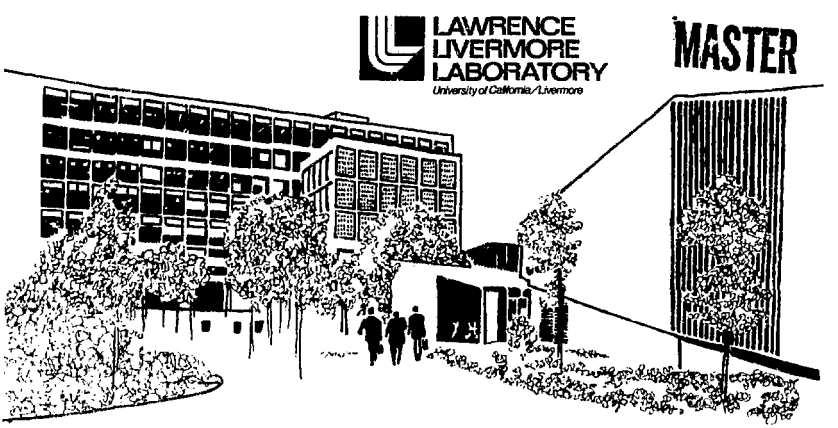

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ABSTRACT

CELL CYCLE PHASE OF NONDIVIDING CELLS IN AGING HUMAN CELL CULTURES DETERMINED BY DNA CONTENT AND CHROMOSOMAL CONSTITUTION

Rosalind Marie Yanishevsky
Vincent J. Cristofalo
Mortimer L. Mendelsohn

Human diploid cell cultures, strain WI-38, have a finite proliferative capacity and have been proposed as a model of biological aging. These cultures possess a subpopulation of nondividing (or out-of-cycle) cells that with culture age increases exponentially relative to the dividing cells. These studies involve the identification of the phase of the cell cycle wherein nondividing cells lose their ability to divide.

To identify the cell cycle phase of the nondividing cells, cultures of various ages were exposed to ^{3}HdT for 48 hours to label dividing cells, then the cycle phase was identified for individual cells by one of two methods, and finally, the proliferative status of the same cells was scored by autoradiographic evidence of ^{3}HdT uptake. The methods to identify the cycle phase were (1) determination of DNA stain content by Feulgen scanning cytophotometry, and (2) determination of chromosome constitution by the technique of premature chromosome condensation (PCC).

Preliminary experiments showed the effect of continuous exposure to various levels of ^{3}HdT on cell growth. High levels of ^{3}HdT

inhibited cell cycle traverse: the cell number and labeling index curves reached a plateau; the cell volume increased; the cells accumulated with 4C DNA contents and it appeared that they blocked in G_2 phase. This pattern is consistent with a radiation effect.

Cytophotometric-autoradiographic experiments showed that the majority of nondividing (unlabeled) cells had 2C DNA contents (where C is the haploid DNA content), and therefore were arrested in the diploid G_1 phase. Increasing culture age was associated with increasing numbers of nondividing 4C cells. These cells could be arrested as diploid in G_2. Alternatively, they could be arrested as tetraploid G_1, since increasing culture age was associated with the appearance of proliferating polyploid (8C) cells. Finally, the results suggested that the 2C and 4C cells for older cultures had an increased dispersion of DNA values than for younger cultures. This could be due to aneuploid DNA content, which may cause cell arrest.

Other cytophotometric-autoradiographic experiments utilized double labeling to identify S phase cells and thus permitted a quantitative evaluation of the dispersion [i.e., calculation of the coefficient of variation (CV)] of DNA values. If aneuploid DNA content caused cell arrest the CV would be greater for old cells than for young cells, and for nondividing cells than for dividing cells. The results showed that the CV of DNA values for 2C and 4C cells increased with age, and, in general, that the CV was similar for dividing and nondividing cells. Thus, aneuploid DNA content is

associated with aging. In addition, it appears that there is not a causal relationship for measurable aneuploid DNA content and cell arrest.

The chromosomes of nondividing WI-38 cells were examined by fusing senescent cultures with mitotic HeLa cells to induce PCC. Nondividing cells condensed their chromatin to form PCC as readily as dividing cells. The efficiency of PCC was independent of cell cycle phase, as shown by comparison of observed frequencies with expected frequencies. Ninety-six percent of the nondividing cells had single chromatids and were therefore in G_1. The remaining 4% had anomalous chromatid pairing. Typical G_2 configurations (double chromatids) were observed only among dividing cells. These data indicate that nondividing cells with 4C DNA contents shown in the cytophotometric-autoradiographic experiments are arrested in tetraploid G_1 phase.

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DEDICATION

TO MY TEACHERS

who took what was given
who gave what could not be taker.

Idries Shah
"Tales of the Dervishes"

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There are many teachers who have helped me in a myriad of ways. My parents continually inspire me by the examples of their lives. Doctors Vincent Cristofalo and Mortimer Mendelsohn have taught me the scientific process from their individual vantage points. Their insight, critical advice and encouragement helped me crystallize the ideas presented in this dissertation. Among my other teachers I thank Dr. Anthony Carrano for his enthusiastic support and active interest; Dr. Brian Mayall for many stimulating and enjoyable discussions; Lee Thorn who has helped me to synthesize and organize my thoughts; members of the Wistar Institute, especially Doctors David Kritchevsky and George Rothblat for their personal understanding and guidance; members of the Biomedical Division of the Lawrence Livermore Laboratory, especially our resourceful library staff, Dr. Dan Moore, II for his help with statistical analyses and, in particular, Sylvia Crooker for her skill and perseverence in typing this manuscript. I thank all these people for their friendship.

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PREFACE

The contents of this dissertation represent work done by the authoress at the Wistar Institute of Anatomy and Biology (Philadelphia, Pennsylvania), and the Lawrence Livermore Laboratory (Livermore, California). Portions of this work were published as follows:

(1) Graul, R.M,[a] Mendelsohn, M.L., Mayall, B.H. and Cristofalo, V.J.: Cellular DNA: A cytophotometric and autoradiographic analysis of aging in WI-38 cells. Gerontologist, 12: 35, 1972 (abstract).

(2) Yanishevsky, R. and Carrano, A.V.: Prematurely condensed chromosomes of aging human cell cultures. J. Cell Biol., 63: 383a, 1974 (abstract).

(3) Yanishevsky, R. and Carrano, A.V.: Prematurely condensed chromosomes of dividing and nondividing cells in aging human cell cultures. Exptl. Cell Res., 90: 169-174, 1975.

(4) Yanishevsky, R., Mendelsohn, M.L., Mayall, B.H. and Cristofalo, V.J.: Cytophotometric and autoradiographic studies of dividing and nondividing human diploid cells in culture. In Vitro, 8: 428, 1973 (abstract).

(5) Yanishevsky, R., Mendelsohn, M.L., Mayall, B.H. and Cristofalo, V.J.: Proliferative capacity and DNA content of aging human diploid cells in culture: A cytophotometric and

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INTRODUCTION

The conversion of cells from the dividing to the nondividing state is a common occurrence in nature. For example, cells may cease dividing in response to environmental stress, as they mature and differentiate, or as they senesce. Human diploid cultures, strain WI-38, present an intriguing model for the study of the loss of proliferative capacity. Soon after being established in culture (phase I), these populations proliferate vigorously and can be serially subcultivated (phase II); but with successive subcultivations, the growth rate progressively declines (phase III) and ultimately the culture cannot be propagated (Hayflick and Moorhead, 1961; Hayflick, 1965). It has been suggested that the number of doublings the culture attains is intrinsically controlled and that the limited doubling potential is a cellular expression of aging. The work presented here involves the identification of the phase of the life cycle wherein WI-38 cells lose their ability to divide.

The framework for present-day concepts of the life cycle of eukaryotic cells is based on the classical work of Howard and Pelc (1951, 1952). They discovered that deoxyribonucleic acid, or DNA, was synthesized in interphase (as distinct from mitosis) and only during a limited, discrete part of the interphase. Thus, they extended the cell cycle from a two-phase system (mitosis and interphase) to a

four-phase system: mitosis (M), pre-synthetic phase (G_1), DNA synthesis phase (S), and post-synthetic phase (G_2).

Out-of-cycle (nondividing) cells predominate in many normal tissues of the adult organism (e.g., Epifanova and Terskikh, 1969) and in many malignant tissues, such as leukemic cells (Maurer et al., 1968). Mendelsohn (1962) has operationally defined the growth fraction as the proportion of cells which proliferate in a population. Some representative growth fractions are: bone marrow stem cells, 0.8; liver parenchyma, 0.001; adult neurons, 0; and mammary tumors of C3H mice, 0.40. In the case of tumors, it is clearly of paramount importance to know whether the arrest of the nondividing cells is reversible.

Nondividing cells may be classified into two populations on the basis of the reversibility of the nondividing state. Lajtha (1963) and Quastler (1963) have termed reversibly arrested cells (cells which reenter the proliferating fraction by an appropriate stimulus) as G_0 cells (e.g., liver). Nonreversible cells lose their capacity to divide as they mature. Some, such as nerve cells, remain in the nonproliferative state for the lifespan of the organism. At the other extreme, others, such as neutrophilic granulocytes, survive for only a matter of days.

An aging WI-38 culture is considered to be a "mixed population" with respect to cell cycle kinetics and biochemistry. The simplest kinetic model would consist of two compartments, proliferating and nonproliferating. With age, the proliferating compartment shows

marked heterogeneity of cell cycle times (Absher et al., 1974; Maciera-Coelho et al., 1966). A number of biochemical parameters also show diversity. Lysosomes (Robbins et al., 1970; Lipetz and Cristofalo, 1972), and lipofuchsin, or "age-pigment" granules (Deamer and Gonzales, 1974), increase progressively with culture age, but they may be absent, or exist in widely varied amounts in single cells of aging cultures. Most research of aging WI-38 cultures has been performed on mass populations. The wide diversity of kinetic and biochemical parameters among individual cells must be considered in the interpretation of data from mass populations, where individual variations are lost.

The decrease in growth rate in aging WI-38 cultures appears to be due to both lengthening of the average cell cycle times (Macieira-Coelho et al., 1966; Absher et al., 1974) and to a progressive increase in the nonproliferating fraction of the population. The increase in the nonproliferating fraction is shown by the decreasing ability of single cell isolates to divide and form clones (Merz and Ross, 1969), and by an increase in the percent of cells unable to incorporate the radioactively labeled (tritium) deoxyribonucleoside thymidine (^{3}HdT) (Cristofalo and Sharf, 1973). Both of these responses increase exponentially with the age of the culture. Cristofalo and Sharf (1973) showed, by statistical analysis of their data, that the percent of nondividing cells was a more accurate criterion of culture age (percent of life-span completed) than passage number.

For the experiments reported here age was defined by the percent of nondividing cells. Nondividing cells were cells which did not incorporate ^{3}HdT for 48 hours (approximately two generations) prior to fixation. DNA synthesis and cell division are almost always correlated and incorporation of a DNA precursor during continuous exposure is a well accepted, convenient indication of whether a cell is actively involved in the mitotic cycle (e.g., Cleaver, 1967). It is believed that the majority of WI-38 cells designated nondividing are destined to die without dividing again. Further consideration is given to these concepts in the Discussion.

Pinpointing the phase in the cell cycle where cells arrest is just a step in understanding the sensitive events which led to that arrest. Knowledge of events specific to the phases of the cell cycle is scant. In the most simplistic view, G_1 is the period during which cells prepare for the initiation of DNA synthesis and G_2 is the period during which cells prepare for the initiation of mitosis. Cells may cease progression through the cell cycle in either the G_1 phase (e.g., Mueller, et al., 1962; Lajtha, 1963; Nilhausen and Green, 1965) or in the G_2 phase (e.g., Gelfant, 1963; Owen and MacPherson, 1963; Pederson and Gelfant, 1970) of the mammalian cell cycle, although G_1 arrest is, by far, more common (see review by Prescott, 1975, for an extensive discussion; also reviews by Baserga, 1965, 1968, and Epifanova and Terskikh, 1969). The cell kinetic data of Macieira-Coelho et al., (1966) showed that in aging WI-38 cultures the increase in average

cell cycle time was due primarily to a lengthening of the G_1 phase. In addition, there were cells with very long G_2 periods. This suggested that WI-38 cells may cease cycling in G_1 and G_2. Gelfant and Smith (1972) proposed that aging in vivo and in vitro can be explained by the progressive accumulation of nondividing cells in G_1 and G_2. The work presented here is evidence that the loss of proliferative capacity in human cell cultures occurs primarily in the G_1 phase.

To identify the phase(s) of the cell cycle wherein aging WI-38 cells arrest, biophysical and cytological techniques were utilized, in combination, on the same individual cells. Analysis of more than one parameter for individual cells, although time consuming, is essential to effectively study a "mixed population", such as aging WI-38 cultures. The approach was first to expose cells to ^{3}HdT for approximately two cell generations, then to determine the cycle phase in individual cells by one of two methods described below, and finally, for the same cells to score their proliferative status by autoradiographic evidence of uptake of ^{3}HdT.

The methods to identify the cycle phase of the nondividing WI-38 cells were 1) determination of the DNA stain content by Feulgen cytophotometry and 2) determination of chromosome constitution of interphase cells by the technique of premature chromosome condensation (PCC). Cycle phase may be determined in fixed, single cells by DNA cytophotometry (Swift, 1950; Walker and Yates, 1952; Dendy and

Cleaver, 1964). A comprehensive review of this subject has been written by Vendrely (1971). DNA content doubles during the S phase as two copies of the genetic material are made in anticipation of the partitioning of a full complement into the two daughter cells. If C is defined as the DNA content of a haploid cell, a diploid cell in G_1 has the 2C amount of DNA, and a cell in G_2 has the 4C amount. A cell in S phase has a DNA content between 2C and 4C.

A cell in G_1 may also be distinguished from a cell in G_2 by the presence of single or double chromatids, respectively. However, chromatids are not visible as entities during interphase; the chromosomes uncoil their DNA so that chromatin in interphase cells is, in the context of this discussion, amorphous. Only during the periods of mitosis or meiosis of mammalian cells does chromatin contract into structures recognizable as chromosomes and, traditionally, visualization and analysis of chromosomes has been confined to these periods. Johnson and Rao (1970a, 1970b) discovered an ingenious way to visualize the elusive interphase chromatin. To investigate the regulation of DNA synthesis and mitosis they fused various combinations of heterophasic cells. They noted a very interesting phenomenon when mitotic cells were fused with G_1, S or G_2 phase cells; the mitotic cell induced the interphase cell to condense its chromatin into discrete chromosomes. Johnson and Rao termed this phenomenon premature chromosome condensation or PCC. Because of the morphological similarity between PCC induction and prophase, this phenomenon has also been termed "prophasing" (Matsui, Weinfeld and

Sandberg, 1972). In comparison to metaphase chromosomes the PCC chromosomes are quite extended in length. Their morphology reflects the position of the interphase cell in the cycle at the time of fusion; cells in G_1 phase have single chromatids, cells in G_2 phase have paired chromatids, and cells in S phase have unevenly condensed chromatin. The experiments reported here utilized PCC to identify, in addition to the cycle phase, the "ploidy level" of nondividing cells. A cell was classified as either "diploid" or "tetraploid" by determination of the chromosome number.

At present, other techniques, such as determination of centriole position and number by transmission electron microscopy (Robbins et al., 1968), and metachromatic staining with safranine and indigo picarmine (Alvarez and Valladares, 1972) lack the precision and reliability needed to identify the cell cycle phase.

The research reported in this dissertation is organized into three sections. The cytophotometric-autoradiographic experiments presented in Section I gave initial results which indicated the cycle phase wherein nondividing WI-38 cells blocked, and raised further questions which were investigated in the other sections. Aneuploid DNA content, as a possible cause of cell arrest, and the effect of continuous exposure to ^{3}HdT on cell growth were investigated in the cytophotometric-autoradiographic experiments and the growth kinetic experiments presented in Section II. Finally, in Section III, the chromosomes of nondividing WI-38 cells were examined by the technique

of PCC, to define both the ploidy level and the cycle phase of the nondividing cells.

MATERIALS AND METHODS

The Materials and Methods are organized as follows: A. procedures to maintain stock cultures, B. theory and procedure of staining DNA with Feulgen dye, and quantitation of the DNA stain content by cytophotometry, C. experimental protocol of cytophotometric-autoradiographic studies (two approaches to these studies were utilized and, therefore, divided into Sections I and II), and D. experimental protocol for premature chromosome condensation-autoradiographic studies, or Section III.

A. General Maintenance of Cell Cultures

Cultures were maintained according to established cell culture techniques (e.g., Hayflick and Moorhead, 1961) and are presented in brief. Cells utilized were: diploid human cells, WI-38; heteroploid human cells, HeLa-S_3; and heteroploid Chinese hamster ovary cells, CHO. WI-38 cells were obtained from Dr. Vincent J. Cristofalo at the Wistar Institute, Philadelphia, Pennsylvania and from Dr. Leonard Hayflick of Stanford University, Palo Alto, California. HeLa-S_3 cells and CHO cells were obtained from Dr. L.H. Thompson of the Lawrence Livermore Laboratory, Livermore, California.

Wl-38 cells utilized in Section I were cultivated in autoclavable Eagle's Minimal Essential Media (MEM) with Earle's salts (Auto-Pow, Flow Laboratories, Rockville, Maryland) and supplemented with an additional mixture of 100X vitamins as formulated for Eagle's Basal

Media (BME) (1% V/V) (Eagle, 1955, 1959). Before use, the medium was supplemented with L-glutamine (2mM), $NaHCO_3$ (20 mM), aureomycin (50 µg/ml) and fetal calf serum (10% V/V). WI-38, CHO and HeLa cells utilized in Sections II and III were cultivated in α-MEM (Stanners et al., 1971) lacking nucleosides (Flow Laboratories). Before use, the medium was supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) and fetal calf serum (10% V/V).

Monolayer cultures were subcultivated when confluent. Cells were released from the glass surface by trypsin (0.25%) in Ca^{+2}- and Mg^{+2}-free phosphate buffered saline solution (PBS). After suspension of the cells in media containing 10% fetal calf serum, cells were innoculated into vessels at the appropriate density, and incubated at 37°C in an atmosphere of 5% CO_2: 95% air. Nonsenescent WI-38 cultures generally became confluent one week after subcultivation at a split ratio of 1:10. Senescent WI-38 cultures were subcultivated at a split ratio of 1:2, and were sustained by weekly feeding until becoming confluent. CHO cells were maintained in monolayer culture and were subcultured at a split ratio of 1:8. HeLa cells were maintained in suspension culture and were subcultivated at a split ratio of 1:8 when they reached a density of 2×10^4 to 4×10^5 cells/ml. Cells were electronically counted using a Coulter Counter.

Stock cultures were maintained frozen at -85°C in α-MEM containing fetal calf serum (20% V/V) and Dimethylsulfoxide (20% V/V). Cultures were periodically monitored for the presence of mycoplasma by Dr. Leonard Hayflick of Stanford University.

B. DNA Cytophotometry

This technique involved staining the DNA stoichiometrically and measuring the DNA stain content with a scanning cytophotometer. The DNA was stained with basic fuchsin in a Feulgen reaction. The integrated absorbance of the Feulgen stained cells was measured with the mechanical scanner of CYDAC (Cytophotometric Data Conversion), a scanning digital integrating cytophotometer (Mayall and Mendelsohn, 1970).

1. Feulgen cytochemistry

a. General theory

Accurate quantitative cytochemistry requires specific and stoichiometric chromophore binding to the molecule in question so that the stain content is directly proportional to the substrate. The specificity of the Feulgen stain for DNA is no longer in question (see review by Deitch, 1966). The Feulgen stain binds stoichiometrically to DNA, provided that precaution is observed during specimen preparation and staining, and provided that the specimen behaves in a reproducible manner (Swift, 1950). Occasional nonstoichiometric responses have been traced to the size of the nucleus and the degree of compaction of the DNA (Garcia, 1969, 1970). For example, the highly clumped and dense chromatin of small lymphocytes and neutrophils measures about 5% lower than the DNA of monocytes, which have large nuclei and fine chromatin patterns (Mayall, 1969). WI-38 cells have very large nuclei and such compaction effects are not to be

expected. Furthermore, results obtained from the cytophotometric measurements of Sections I and II suggest a well behaved stoichiometry on the basis of the precise doubling of 2C to 4C to 8C peaks, and the similar modal DNA value of labeled and unlabeled cells.

In the Feulgen reaction DNA is first depurinated by acid hydrolysis. This unmasks the aldehydes of deoxyribofuranose sugars. The aldehydes react with a decolorized or leuco Shiff reagent, which is then converted into its colored form (Deitch, 1966). The mass of the chromophore, and therefore the amount of DNA, may be determined cytophotometrically.

The Feulgen procedure used (Mayall, 1969), was modified from a protocol given by Deitch (1966), and is described in the staining recipe.

b. Staining recipes

The Feulgen stain was prepared in the following manner. Twenty-five grams of basic fuchsin (96% pure, National Aniline Dye, Allied Chemical Corp., New York) was added to 50 ml distilled water and boiled for 30 minutes. The solution was shaken, cooled to 50°C, and filtered through number 1 Whatman paper into a jar covered with aluminum foil. Five ml of 1N HCl and 1.0 g of $K_2S_2O_5$ were added to the filtrate and shaken to dissolve. The amber-colored solution was stored for 72 hours at 4°C. Before use, 0.125 g activated charcoal was added, the solution was shaken well and filtered through number 1 Whatman paper. Five grams of $K_2S_2O_5$ was added to the colorless filtrate. The optimum pH for the Feulgen reaction was 2.3 to 2.9.

Slides were stained in the following manner. The slides were hydrolyzed in 5N HCl at room temperature for 40 minutes. This method is preferable to the conventional Feulgen hydrolysis in 1N HCL at 60°C (Deitch, 1966), because it achieves a Feulgen intensity which is higher and is less criticaliy dependent on time and temperature (DeCosse and Aiello, 1966; Deitch et al., 1968). After hydrolysis, the slides were placed in the Feulgen dye for two hours. To wash the slides they were dipped, three times for five minutes each, into freshly made wash solution (7.5 ml 10% anhydrous $K_2S_2O_5$, 7.5 ml 1N HCl, and 135.0 ml distilled water); then the slides were dipped, successively, into distilled water for five minutes, absolute ethanol for two minutes, and xylene for 15 minutes.

For measurements the stained cells were mounted in oil of refractive index 1.552. An interference filter of 566 mm defined a spectral band close to the spectral absorbance peak of the Feulgen stain.

2. Scanning cytophotometry

a. General theory

The Beer Lambert Law, the basis of absorption photometry, describes the relationship between optical density and light transmission by the following formula:

$$OD = -\log T = kcl$$

where:

OD = optical density

T = light transmission

k = extinction coefficient of the chromophore

c = concentration of the chromophore

l = pathlength of the chromophore

The optical density of a stained specimen is directly proportional to the mass of the chromophore per unit area, since c = m/v, where m is the mass of the chromophore and v is the volume of the chromophore. Then,

$$OD = k(m/v)l = km/A$$

where A is the area of the chromophore.

When applied to biological specimens this relationship frequently does not hold. This is primarily due to inhomogeneous distribution of the chromophore, or the so-called distributional error. Simple photometric measurement of a field averages the transmissions of all the fractional elements of that field. If the elements are heterogeneous, the negative logarithm of the mean of the light transmitted through the whole field, will be less than the mean of the negative logarithms of the light transmitted through all the fractional elements [i.e., $(-\log \Sigma^{A} T) < (-\Sigma^{A} \log T)$]. Scanning cytophotometers are designed to correct for distributional error in the following ways. First, the field to be measured is divided into a large number of descrete elements. These elements are small, relative to the resolution of the optics, to reduce heterogeneity within each element. Second, the sample elements are taken uniformly from the

field and integrated. The integrated optical density is directly proportional to the total amount of chromophore: Σ^A OD = Σ^A km/A = km.

Scanning cytophotometry was first used for quantitative cytochemistry by Caspersson (1962).

b. CYDAC instrumentation

The mechanical scanner of CYDAC consists of: (1) a microscope for specimen viewing; (2) a dual beam photometer with a Nipkow disc scanning in the image plane; (3) a system for transforming and recording the photometric measurements into digital form; and (4) operation controls and displays for visual monitoring. The field containing the cell to be scanned is selected by the operator, who activates the recording process. Light, which is emitted from a ribbon filament tungsten lamp, passes through an interference filter of appropriate wavelength (566 nm for the Feulgen stain) and is split into two beams. One beam passes through the reference optics and is detected by a photomultiplier. The other beam passes through the specimen, and the light transmitted is scanned by a Nipkow disc and detected as discrete sampling elements by a second photomultiplier. The signals from the reference and data photomultipliers are logarithmically converted, the reference signal is subtracted from the data signal to correct for fluctuations in the light source, and finally this difference undergoes analogue to digital conversion. The operator selects an adjacent clear field and reactivates the recording process, which causes the clear field (background) values to be subtracted from the previous total. The final number displayed on the

accumulator is proportional to the integrated optical density of the object and is represented by the following formulas:

$$IOD_{CV} = - \Sigma^{A} \log T$$

where IOD CV is the CYDAC value of the integrated optical density. Since:

$$T = I/I_{o}$$

where:

I = intensity of the specimen field

I_{o} = intensity of the clear field

then:

$$IOD_{CV} = -G \Sigma^{A} \log \frac{i(r) - i(d)}{i(r)_{o} - i(d)_{o}}$$

where:

$i(r)$ = reference intensity of the specimen field

$i(d)$ = data intensity of the specimen field

$i(r)_{o}$ = reference intensity in the clear field

$i(d)_{o}$ = data intensity of the clear field

G = gain

CYDAC values may be converted to absolute integrated optical density (IOD_{ABS}):

$$IOD_{ABS} = \frac{IOD_{CV}}{\rho G}$$

where ρ is the pixel density in μ^{-2}.

C. Cytophotometric-Autoradiographic Studies

DNA cytophotometry was used to determine the cycle phase, at the time of fixation of the sample, of individual WI-38 cells from

cultures of various ages. Diploid cells in G_1 and G_2 phase had 2C and 4C DNA contents. Cells in S phase had DNA contents intermediate between 2C and 4C. Autoradiography, used in combination with cytophotometry, allowed the cells to be further classified as nondividing (no incorporation of ^{3}HdT at 48 hours of exposure to the label) or dividing.

A general description of the cytophotometric-autoradiographic procedure is as follows. Tritiated thymidine was added to the culture media for an appropriate time interval. Microscope slides of the cells were fixed and the DNA was stained with Feulgen. The DNA content of approximately 100 previously mapped interphase cells for each culture was determined cytophotometrically. The slides were dipped in emulsion for autoradiographic determination of the proliferative status of the same cells. These experiments are presented in Section I.

Section II presents a refinement of the techniques used in Section I. Measurements were made on cells whose growth kinetics were unperturbed. Slides were prepared to diminish cellular debris. In addition, the cells were double-labeled with ^{3}HdT to determine the precise boundary of cells in G_1, S, and G_2 for analysis of the coefficient of variation of DNA values. Section II also describes the growth kinetics of cells exposed to various levels of ^{3}HdT.

1. Section I procedure

   a. Cell cultivation and labeling

WI-38 cells were seeded at 1.3×10^4 cells/cm^2 in Lab-Tek microscope slide chambers (Miles Laboratories, Westmont, Ill.) and incubated at 37°C. Twenty four hours later, ^{3}HdT (specific activity, S.A., 2.0 Ci/mmole) was added to the medium to a final concentration of 0.1 μCi/ml and left in the culture for 48 hours (approximately two cell generations).

b. Slide preparation

At the end of the labeling period the growth chambers were removed from the microscope slides, and the cells attached to the slides were fixed with neutral formalin, rinsed in distilled water for one-half hour, and air-dried for further processing.

c. DNA cytophotometry

The fixed cells were Feulgen stained and cytophotometry was performed as described (Materials and Methods: B). The ocular had a magnification of 5X. The objective was either a 40X oil immersion apochromat, numerical aperture (NA) = 1.00, or a 25X planachromat, NA = 0.45, and the condensor aperture was set to 0.3, or 0.24, respectively. Each cell was scanned three times successively.

d. Autoradiography

The slides were dipped in Kodak NTB2 liquid emulsion and exposed in the dark at 4°C for four days, after which they were placed for five minutes each in D19 developer and acid fixer. Nuclei were scored as labeled (dividing) if they contained five or more silver grains. A review of autoradiographic techniques is given by Baserga (1967).

2. Section II procedure

a. Cell cultivation and labeling

(1) Effect of ^{3}HdT on WI-38 cell growth

Each 25 cm^2 Falcon flask received approximately 3×10^5 WI-38 cells. The appropriate amount of ^{3}HdT was added to half the flasks, and the rest of the cultures were controls. At 24 hour intervals the media was removed, the monolayers were washed with Ca^{2+}- and Mg^{2+}free PBS, and trypsinized as described (Materials and Methods: A). The cells were pelleted at 500g, and cell number and volume distribution was determined using the Coulter counter.

(2) Double labeling with ^{3}HdT

To determine the optimum labeling conditions two criteria had to be satisfied. First, the growth kinetics of the culture must be unperturbed during a 48 hour exposure to ^{3}HdT. Second, three types of cells must be easily discernable by autoradiography: (1) nondividing cells, (2) cells in S, and (3) all other dividing cells (i.e., cells in G_1 or G_2 phase). The first criterion was satisfied by the procedure described above in (1), "Effect of ^{3}HdT on WI-38 cell growth". The second criterion was satisfied by a double label technique. Continuous exposure to a very low level of ^{3}HdT labeled the dividing cells. At the end of the labeling period a pulse exposure to a very high level of ^{3}HdT revealed which cells of the dividing population synthesized DNA during the pulse. Preliminary experiments tested various combinations of a continuous exposure to

^{3}HdT (S.A. 2.0 Ci/mM) ranging from 0.1 to 0.0005 μCi/ml, with a pulse of the same S.A. ^{3}HdT ranging from 1.0 to 100.0 μCi/ml. The criteria were optimized when the cells were continuously exposed to 0.003 μCi/ml ^{3}HdT for 48 hours and pulsed with 100.0 μCi/ml ^{3}HdT for 30 minutes.

b. Slide preparation

(1) WI-38 cells

For cytophotometric measurements slides were prepared to minimize cellular debris. At the end of the labeling period the radioactive media was decanted from the T_{25} flasks. The cell monolayer was washed two times in Ca^{2+}- and Mg^{2+}free PBS, and trypsinized as described (Materials and Methods: A). The cells were pelleted at 500g and suspended in 0.075 M KCl at room temperature for nine minutes. Finally, the cells were pelleted, decanted, fixed in three changes of methanol: acetic acid (3:1) and dropped on wet, acid-cleaned microscope slides.

The procedure for cleaning slides is as follows. Soak the slides in chloroform: methanol (3:1) or in a 5% NaOH-methanol solution for one hour. Place the slides in HNO_3: H_2SO_4 (1:1) for 24 hours. Finally, soak the slides in dilute base for 30 minutes. A 30 minute rinse in distilled water follows each step.

(2) White blood cell standard

Heparinized blood, obtained by venipuncture, was layered over cold flotation media [2 parts 1% methylcellulose in 0.8% saline, 1 part 50% W/V sodium Hypaque (Winthrop Labs, New York)], and allowed to settle

for 1 hour at room temperature. The erythrocytes aggregated and sedimented out leaving an upper layer of leukocyte-rich serum, which was decanted and spun in a clinical centrifuge for five minutes at 800 rpm. The leukocyte pellet was resuspended in one tenth the volume of the serum supernatent. A small drop of leukocytes was placed on one end of a microscope slide, which contained previously fixed WI-38 cells, and was gently spread with the short edge of a second slide held at a 45^{0} angle. The slide containing the WI-38 and white blood cells was air-dried, and fixed in neutral formalin for 30 minutes.

c. DNA cytophotometry

Measurements were made as described (Materials and Methods: B). The ocular had a magnification of 5X. The objective was a 25X planachromat, NA = 0.45, and the condensor aperture was set to 0.3. Each cell was scanned once, using two different clear fields. The standard deviation of the replicate measurements of all WI-38 nuclei measured gave an average of 2.6%. Approximately 100 cells per culture were measured, 50 cells on each of two days, and the mean was computed. The data from the two days was normalized using white blood cells as a standard, and the measurements were combined to yield a single histogram.

c. Autoradiography

Autoradiography was performed as described for Section I with the modification that slides from cells double labeled with ^{3}HdT were exposed to the emulsion for 18 days. Preliminary experiments in which the exposure time was varied from four to 29 days revealed that 18

days gave the best resolution among nuclei which incorporated high levels of ^{3}HdT (S cells), nuclei which incorporated low levels of ^{3}HdT (dividing G_1 or G_2 cells) and unlabeled nuclei (nondividing cells).

D. Premature Chromosome Condensation-Autoradiographic Studies

The technique of PCC allowed visualization of the chromosomes of nondividing WI-38 cells. The chromosome morphology and number revealed the cell cycle phase and ploidy level of the cells, at the time of fixation of the sample. Cells in G_1 had single chromatids and cells in G_2 had paired chromatids. Diploid cells had 46 chromatids and tetraploid cells had 92 chromatids. Autoradiography, used in combination with PCC, allowed the cells to be further classified as nondividing (no incorporation of ^{3}HdT at 48 hours of exposure to the label) or dividing.

A general description of the PCC-autoradiographic procedure is as follows. Tritiated thymidine was added to the WI-38 culture media for 48 hours. Colcemid-blocked mitotic HeLa or CHO cells were fused to the WI-38 cells. Chromosome spreads were made of the fusion mixture. Approximately 100 mapped cells with PCC for each culture were located. The chromosome constitution was analyzed to identify the cycle phase and ploidy level of the cells. Finally, the slides were dipped in emulsion for autoradiographic detection of ^{3}HdT uptake of the same cells. These experiments are presented in Section III.

1. Section III procedure

a. Cell cultivation and labeling

(1) WI-38 cells

WI-38 cells were seeded at a density of 1.3×10^4 cells/cm^2 in Falcon flasks and incubated at 37°C. Twentyfour hours later, 0.1 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) was added to the cultures and left for 48 hours.

(2) CHO cells

Mitotic CHO cells were prepared by the method of Terasima and Tolmach (1963). When cells cultured in monolayer enter mitosis they round up and adhere much less firmly to the surface than interphase cells. Gentle agitation will selectively detach mitotic cells. Twenty- four hours after CHO cells were seeded in a roller bottle at a density of 4×10^4 cells/cm^2, the media was removed and the monolayer washed twice to remove dead cells and debris. Fresh media containing Colcemid (0.06 μg/ml) was added to accumulate cells in mitosis (Stubblefield and Klevecz, 1965). At appropriate times, the bottle was rolled gently on a horizontal surface, the media containing detached cells was decanted and placed on ice, and fresh media with Colcemid was added to the culture. Samples were analyzed for cell number and volume using a Coulter counter with a volume spectrometer, and for mitotic index. The mitotic index of the pooled aliquots was 91%. CHO cells did not produce PCC, therefore, HeLa cells were used for further experiments.

(3) HeLa cells

HeLa cultures were grown in suspension culture, until transferred to monolayer culture one week prior to use for cell fusion. The cells were labeled with 0.1 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) for 26 hours and for the final 20 hours of labeling, Colcemid (final concentration 0.25 μg/ml) was added to block mitosis. The unattached, arrested mitotic cells were collected by decanting and centrifugation. The mitotic index of these cells averaged 99.9% and the percentage of mitoses that were labeled was 99.7.

b. Cell fusion

(1) Fusion in monolayer

The method described by Harris et al., (1966) for cell fusion was utilized with minor modifications. WI-38 cells (5×10^4) were seeded per Lab-Tek microscope slide chamber (4 cm^2). Twenty-four hours later the media was removed and replaced with cold α-MEM, without serum, buffered at pH 7.0 with HEPES (N'-2-Hydroxymethylpiperazine-N'-Ethanesulfonic Acid). The monolayers were chilled on ice for five minutes. β-propiolactone-inactivated Sendai virus was added to the media to a final concentration of 10,000 hemagglutinating units (HAUs) per ml, and allowed to absorb to the monolayer for 30 minutes at 4°C. (The virus was kindly supplied by Dr. Larry Thompson, Lawrence Livermore Laboratory, Livermore, California.) The excess virus was removed and 5×10^5 mitotic HeLa cells in 0.5 ml medium, without serum, were added to each chamber. The slide chambers were incubated

at 37°C and after 30 minutes, one hour, four hours and six hours of incubation, samples were washed with media and fixed in Carnoys. No cells with PCC were observed, therefore, cells were fused in suspension for all further experiments.

(2) Fusion in suspension

The method described by Johnson and Rao for PCC (Johnson and Rao, 1970; Rao and Johnson, 1972) was utilized with minor modifications. Preliminary experiments were performed to test the effects of pH, virus and cell concentration. The highest percentage of PCC was obtained when 0.5×10^6 cells of each type (WI-38 and mitotic HeLa) were placed in a total volume of 0.5 ml of medium, without serum, buffered at pH 7.3 with HEPES (N'-2-Hydroxyethylpiperazine-N'-Ethanesulfonic Acid) containing 250 hemagglutinating units of b-propiolactone-inactivated Sendai virus. (The Sendai virus was generously donated by Dr. Barbara B. Knowles, Wistar Institute, Philadelphia, Pennsylvania.) The virus-cell mixutre was kept at 4°C for 20 minutes and then at 37°C for 45 minutes.

c. Slide preparation and chromosome identification

Chromosome preparations were made by exposing the cell mixture to hypotonic KCl (0.075M) at room temperature for 10 minutes, fixing and washing in three changes of methanol: acetic acid (3:1), and dropping the cells on wet microscope slides. Cells showing PCC were first located with phase optics, or with bright field after staining with 2% aceto-orcein. Chromosome complements were identified as G_1, if the

condensed chromatin contained a single chromatid, or G_2, if paired chromatids were present. Those in S were identified by their unevenly condensed chromatin, but since few could be unambiguously discerned they were not included in this study. The prematurely condensed chromosomes were counted and their complements were further classified as either "diploid" (2N) or "tetraploid" (4N) (where N is the number of chromosomes in a haploid nucleus, in this case 23). In some cases the counts could only be estimated to ± 20%, due to overlapping of the highly extended chromosomes.

d. Autoradiography

The slides were dipped in Kodak NTB2 liquid emulsion, exposed for four days, developed, fixed, and stained in a 7.5% Giemsa solution. The preclassified PCC cells were relocated and identified as labeled (dividing) or unlabeled (nondividing).

RESULTS

A. Cytophotometric-Autoradiographic Studies

DNA cytophotometry-^{3}HdT autoradiography was utilized in Section I to determine the position in the cycle of nondividing WI-38 cells from cultures of various ages. Section II presents a refinement of the techniques used in Section I. Measurements were made on cells whose growth kinetics were unperturbed. Slides were prepared to diminish cellular debris. In addition, the cells were double labeled with ^{3}HdT to determine the precise boundary of cells in G_1, S and G_2 for analysis of coefficient of variation (standard deviation/mean) of DNA values. Section II also describes the growth kinetics of cells exposed to various levels of ^{3}HdT.

1. Section I

Initial experiments to identify the cell cycle stage of nondividing WI-38 cells are presented in this section. Feulgen DNA stain content was quantified in fixed, single cells following continuous exposure to ^{3}HdT (0.1 μCi/ml, S.A. 2.0 Ci/mM) for 48 hours. Autoradiographic detection of ^{3}HdT uptake (proliferative status) subsequently was correlated with DNA stain content.

a. DNA cytophotometry of dividing and nondividing cells

Cytophotometric-autoradiographic measurements made on six WI-38 cultures of various ages are presented in the form of histograms (frequency distributions) in figure 1. Each histogram is divided into labeled (dividing) and unlabeled (nondividing) cells. The histograms

Figure 1. Feulgen DNA stain content of aging WI-38 cultures. The histograms are arranged according to increasing age (percent unlabeled nuclei). The stippled bars represent the labeled cells (0.1 μCi/ml ^{3}HdT, S.A. 2.0 Ci/mM for 48 hours) and the clear bars represent the unlabeled cells. DNA content is given in arbitrary CYDAC units (ACU) of C where 2C is the diploid DNA content, operationally defined in terms of the mode of first peak.

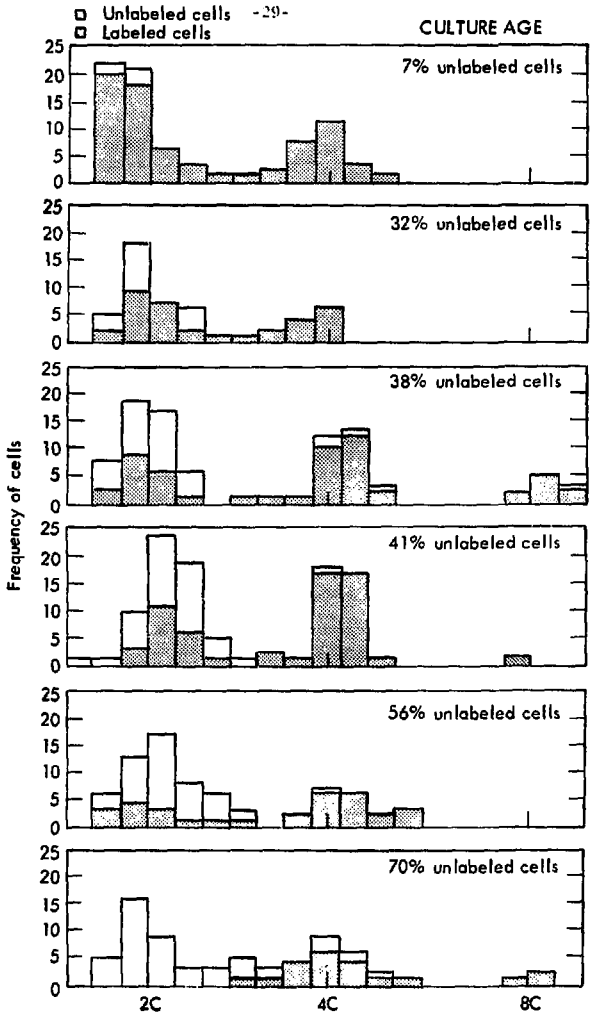

Unlabeled cells
Labeled cells
CULTURE AGE
7% unlabeled cells
32% unlabeled cells
38% unlabeled cells
41% unlabeled cells
56% unlabeled cells
70% unlabeled cells
Frequency of cells
0
5
10
15
20
25
2C
4C
8C
Log DNA stain content (ACU)

Figure 1.

are arranged according to increasing culture age, as defined by percent unlabeled cells (Cristofalo and Sharf, 1973).

The histogram of the youngest culture (7% unlabeled cells) is characteristic of a growing diploid population. The Feulgen DNA values are distributed into two peaks with an intervening plateau. The first peak, called the 2C peak, has a DNA content similar to a single pole of a cell in anaphase, while the second peak, called the 4C peak, has a DNA content similar to a cell in metaphase. As expected, the DNA content of the 2C peak is approximately half that of the 4C peak. These observations indicate that the 2C and 4C peaks represent diploid G_1 and diploid G_2 cells, and that the intervening plateau represents S cells. There are more labeled cells in the 2C peak than in the 4C peak since each G_2 cell becomes two G_1 cells as it passes through the point of division.

The majority of the cells in the youngest culture incorporated ^{3}HdT. The few cells that did not incorporate label had 2C DNA contents, and therefore, were blocked in the diploid G_1 phase.

The histogram of the culture containing 32% unlabeled cells has a similar profile to the youngest culture, except that there are fewer labeled cells in G_1, S and G_2. The histogram is bimodal and the culture is presumed to be diploid. The unlabeled cells have 2C DNA contents.

The histogram of the culture with 38% unlabeled cells has a third peak of DNA values at 8C, which indicates that the culture contains

polyploid cells. In contrast to the two younger cultures, this culture has more labeled 4C cells than labeled 2C cells, which suggests that the growth kinetics of the cycling cells are disturbed. Similar to the younger cultures, most unlabeled cells have 2C DNA contents. However, a few unlabeled cells have 4C DNA contents and one cell has an 8C DNA content. The occurance of polyploid cells complicates the interpretation of the histogram because DNA cytophotometry cannot determine whether the unlabeled cells with 4C DNA contents are G_2 cells from a diploid population or are G_1 cells from a tetraploid population. (This problem is addressed in Section III.) Analogously, the unlabeled 8C cells could be either G_1-blocked octaploid cells or G_2-blocked tetraploid cells.

With further increasing culture age there is progressive deviation from the DNA distribution of the vigorously growing young culture. Cultures containing 41%, 56% and 70% unlabeled cells have polyploid cells, and few or no proliferating S cells. The fraction of labeled cells with 2C DNA content declines with age, while the fraction of labeled cells with 4C DNA content increases. The most extreme example is the oldest culture (70% unlabeled cells) where, for this sample, there are no labeled 2C cells; labeled cells have either 4C DNA or 8C DNA. In this senescent culture, it is possible that the entire proliferating subpopulation is either in the G_2 phase or is polyploid. In all cultures the majority of unlabeled cells have 2C DNA contents but with increasing culture age there is an increased frequency of unlabeled 4C cells.

An interesting age-correlated observation is the progressive dispersion of DNA stain content in the two peaks, 2C and 4C, especially for nondividing cells. "Noisy" DNA stain content could reflect aneuploid DNA content, which could lead to cell arrest. One would expect, therefore, that the dispersion of DNA values would be greater for old cultures as compared to young cultures, and for nondividing cells as compared to dividing cells. It is difficult to quantitate the peak dispersion (i.e., calculate the coefficient of variation or CV) because the boundary between the peaks and the plateau is arbitrary. Experiments in Section II were designed to identify cells in the plateau (i.e., cells in S phase) in order to permit calculation of the CV.

In summary, most unlabeled cells from aging WI-38 cultures have 2C DNA values. Unlabeled 4C cells appear increasingly with the age of the culture. These may be cells arrested in diploid G_2 or tetraploid G_1 phase. Experiments described in Section III were designed to resolve this enigma. Most labeled cells in aging cultures have 4C DNA values, whereas most labeled cells in young cultures have 2C DNA values. Experiments in Section II suggest an explanation for the increase in labeled 4C cells. In addition, old cultures have labeled and unlabeled polyploid (8C) cells. Finally, there may be an increased dispersion of DNA values for 2C or 4C cells of older cultures. This may be more pronounced for nondividing cells. A quantitative approach to determine the dispersion of DNA values is exploited in Section II.

2. Section II

Experiments in Section I indicated that old WI-38 cultures have a greater dispersion of DNA contents at the 2C and 4C levels than young cultures. Experiments in this section involved cytophotometric-autoradiographic methods which allow identifcation of S phase cells and thus permits a quantitative evaluation of the dispersion.

An increased dispersion of DNA values could be due to aneuploid DNA content. Several lines of evidence (e.g., Levisohn and Thompson, 1973; Prescott, 1975) suggest that somatic cell division, as well as the aging process (e.g., Crowley and Curtis, 1963; Curtis, 1963) are regulated at the chromosomal level. It is conceivable that at mitosis, uneven partitioning of chromosomes which carry genes involved in cell reproduction, could cause cell arrest. One would expect, therefore, that old cells would have a greater CV of DNA values than young cells (i.e., would be more aneuploid), and that nondividing cells would have a greater CV of DNA values than dividing cells.

DNA histograms in Section I could not be analyzed for CV because of uncertainty in the boundaries between the peaks and the plateau. Experiments in this section were designed to identify cells in the plateau (i.e., cells in S) and thus identify the boundaries of the peaks. Cultures were exposed for 48 hours to a low concentration of ^{3}HdT (0.003 μCi/ml, S.A. 2.0 Ci/mM) in order to label cycling cells. This was followed by a 30 minute pulse with high concentrations of

^{3}HdT (100 μCi/ml, S.A. 2.0 Ci/mM) to label cells in the S phase. DNA cytophotometry was performed on fixed, single nuclei followed by autoradiographic detection of ^{3}HdT uptake. S phase nuclei were heavily labeled, cycling G_1 and G_2 nuclei were lightly labeled, and noncycling nuclei were unlabeled. The S phase nuclei were subtracted from the DNA histograms of the total nuclei to yield 2C and 4C peaks. Finally, the CV of the 2C and 4C peaks was calculated and compared for old and young cells, and for dividing and nondividing cells.

a. Effect of ^{3}HdT on WI-38 cell growth

Preliminary to the cytophotometric experiments, the effect of continuous exposure to varied levels of ^{3}HdT on cell growth was examined. Cells were exposed to the various ^{3}HdT concentrations that were used for cytophotometric experiments. Cell growth was monitored during continuous exposure to low levels of ^{3}HdT (0.1 μCi/ml or 0.003 μCi/ml, S.A. 2.0 Ci/mm), and to high levels of ^{3}HdT (1.0 μCi/ml or 5.0 μCi/ml, S.A. 20.0 Ci/mM). Use of each concentration of ^{3}HdT is described in brief. Uptake of 0.1 μCi/ml ^{3}HdT defined dividing cells in Section I. Uptake of 0.003 μCi/ml ^{3}HdT defined dividing cells in Section II. This low concentration of ^{3}HdT was used so that: (1) the growth kinetics of cells which incorporate label were unperturbed and thus did not distort the data analysis; (2) cells which incorporated low concentrations of ^{3}HdT during continuous exposure were easy to distinguish autoradiographically from cells which were pulse labeled with high concentrations of ^{3}HdT. Other cultures were grown in the

presence of 1.0 μCi/ml ^{3}HdT or 5.0 μCi/ml ^{3}HdT and thereby were enriched for nondividing cells.

(1) Labeling with low levels of ^{3}HdT

In section I nondividing WI-38 cells were cells which did not incorporate 0.1 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) at 48 hours of exposure. Cristofalo and Sharf (1973) reported that the LI of cells exposed to this ^{3}HdT concentration reached a plateau at 24-30 hours. In a well behaved system the LI should not plateau, but should asymptote toward 100% as the dividing (labeled) population increases by proliferation. The behavior of cells grown with and without label (0.1 μCi/ml ^{3}HdT or 0.003 μCi/ml ^{3}HdT, S.A. 2.0 Ci/mM) was monitored by cell number and Coulter volume.

Figure 2 shows that cellular proliferation is inhibited by 0.1 μCi/ml ^{3}HdT and is not inhibited by 0.003 μCi/ml ^{3}HdT. The cell number of the control (no ^{3}HdT) increases linearly with time after seeding the culture, until 72 hours. At this time the cell number reaches a plateau as the result of density dependent inhibition. The culture with 0.003 μCi/ml ^{3}HdT shows no significant difference in cell number from the control culture for the duration of the experiment. The culture exposed to 0.1 μCi/ml ^{3}HdT has the same cell number as the control for the first 24 hours of labeling. At 48 hours of ^{3}HdT exposure (0.1 μCi/ml ^{3}HdT) there are significantly fewer cells than in the control culture. The cell number is unchanged during the remainder of the labeling period. These results may be explained by inhibition of cell division. In addition, cell death may occur.

Figure 2. Effect of continuous exposure to low levels of ^{3}HdT (2.0 Ci/mM) on WI-38 cell growth. The boldface arrow indicates the time of addition of 0.1 μCi/ml ^{3}HdT or of 0.003 μCi/ml ^{3}HdT.

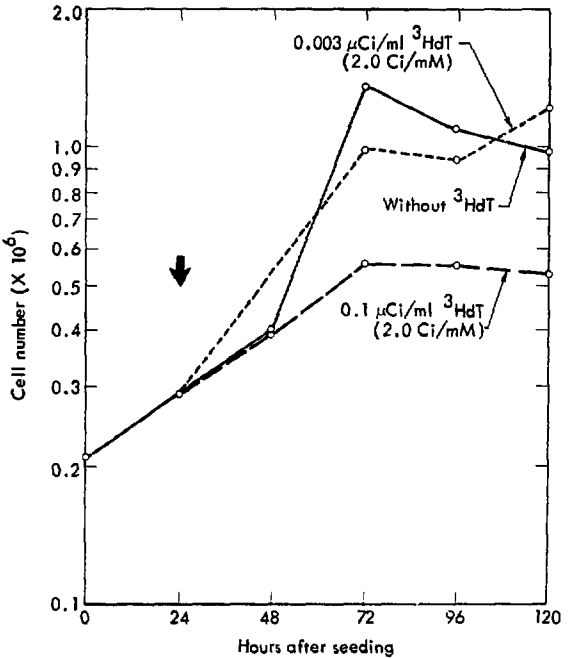

2.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Cell number (X 10^6)
0
24
48
72
96
120
Hours after seeding
0.003 μCi/ml 3HdT
(2.0 Ci/mM)
Without 3HdT
0.1 μCi/ml 3HdT
(2.0 Ci/mM)

Figure 2.

Figure 3 shows that LI is inhibited by continuous exposure to 0.1 μCi/ml ^{3}HdT. The LI reaches a plateau at 48 hours of ^{3}HdT exposure.

Coulter volume distributions are shown in figure 4. The control culture has a distribution which is skewed toward the right. Small cells, which predominate, appear on the left side, and the larger cells tail off toward the right. This distribution is typical of an asynchronous log-phase population, in which the age distribution decreases exponentially from birth (small cells) to mitosis (large cells). At the extreme left, a slight rise in the curve occurs due to the presence of cellular or other debris. Cells which were exposed to 0.003 μCi/ml ^{3}HdT for four days have a volume distribution similar to the control culture (figure 4; day 5). Cells which were exposed to 0.1 μCi/ml ^{3}HdT for four days have a broad volume distribution which is shifted to the right, indicating that these cells are larger than the control cells. The increase in cellular volume may be due to cells which are blocked in diploid G_2 or tetraploid G_1, and which may have unbalanced growth.

Table 1 shows the modal channel numbers of the Coulter volume of cells grown with and without ^{3}HdT. The modal channel number of the control and of the culture with 0.003 μCi/ml ^{3}HdT shows no significant change with time. The modal channel number of the culture with 0.1 μCi/ml ^{3}HdT increases to approximately one and one-half times the control culture, at four days of labeling (or day 5).

To determine if 0.003 μCi/ml ^{3}HdT affects the growth of cultures of various ages, cells were grown in the presence and absence of ^{3}HdT.

Figure 3. Labeling index of WI-38 cells continuously exposed to low levels of ^{3}HdT (2.0 Ci/mM). The boldface arrow indicates the time of addition of 0.1 μCi/ml ^{3}HdT.

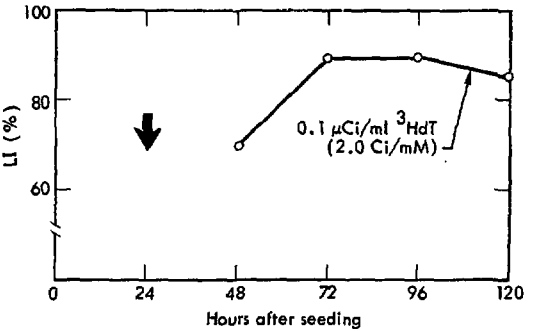

100
80
60
LI (%)
0.1 μCi/ml ^{3}HdT
(2.0 Ci/mM)
0
24
48
72
96
120
Hours after seeding

Figure 3.

Figure 4. Coulter volume distribution of WI-38 cells continuously exposed to low levels of ^{3}HdT (2.0 Ci/mM). The label (0.1 μCi/ml ^{3}HdT or 0.003 μCi/ml ^{3}HdT) was added on day 1.

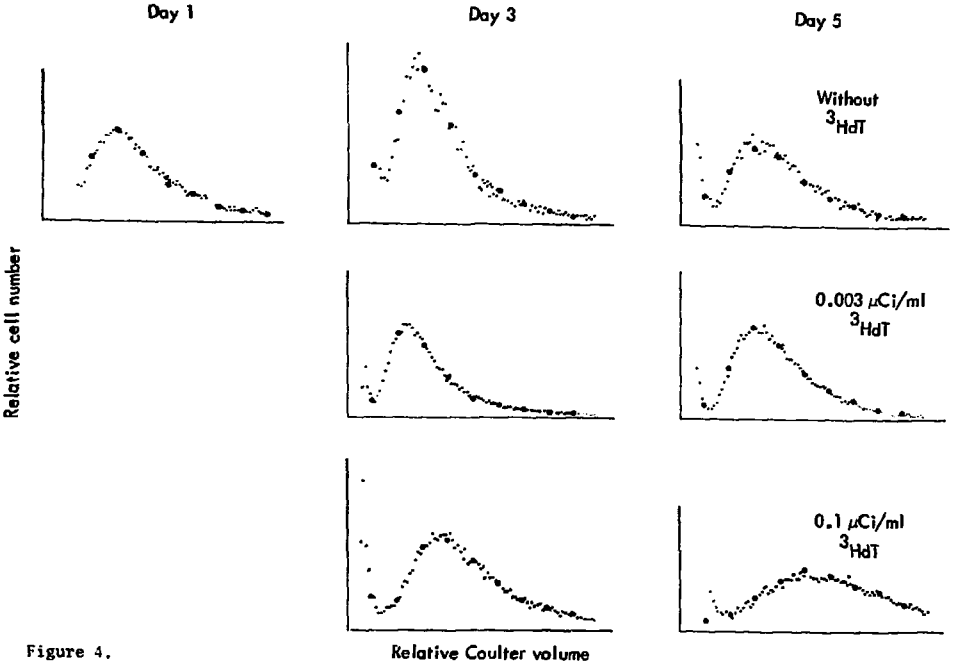

Day 1
Day 3
Day 5
Without ^{3}HdT
0.003 μCi/ml ^{3}HdT
0.1 μCi/ml ^{3}HdT
Relative cell number
Relative Coulter volume

Figure 4.

MODAL CHANNEL OF COULTER VOLUMES OF WI-38 CELLS GROWN WITH AND WITHOUT 2.0 Ci/mM ^{3}HdT

Day[a]	Modal channel number		
	0.1 μCi/ml ^{3}HdT	0.003 μCi/ml ^{3}HdT	Without ^{3}HdT
0	-	-	37
1[b]	-	-	40
2	40	41	39
3	49	34	37
5	60	43	39

a. Day after seeding the culture
b. Time of addition of ^{3}HdT

Table 1.

Growth curves of young, middle-aged, and old cultures (figures 5, 6 and 7 respectively) show an initial lag period followed by a rise in cell number which is, as expected, steeper for the young and middle-aged cultures than for the old culture. At all ages, the control and ^{3}HdT-labeled curves show identical growth kinetics, within 95% confidence limits, therefore this ^{3}HdT concentration does not retard growth.

In summary, these data show that cellular growth is inhibited by continuous exposure to 0.1 μCi/ml (S.A. 2.0 Ci/mM). During the first 24 hours of labeling the cell number increases at a rate similar to the control. At 48 hours of labeling the cell number is significantly less than the control. After 48 hours the cell number reaches a plateau. This indicates that the plateau of LI after 48 hours of ^{3}HdT exposure may be explained by the inhibition of division. By 120 hours of labeling the cell volume is approximately one and one-half times the control. This may be due to cells which are blocked in diploid G_2 or tetraploid G_1 and which may undergo unbalanced growth. On the other hand, cellular growth is not affected by continuous exposure to 0.003 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM). There is no difference in cell number for control cells and cells exposed to 0.003 μCi/ml, regardless of the age of the culture.

(2) Labeling with high levels of ^{3}HdT

Cellular growth was examined for WI-38 cultures grown in the presence and absence of 1.0 μCi/ml ^{3}HdT (S.A. 20.0 Ci/mM) or 5.0 μCi/ml

Figure 5. Effect of continuous exposure to low levels of ^{3}HdT (2.0 Ci/mM) on young WI-38 culture growth. The PDL of this culture was 30. The boldface arrow indicates the time of addition of 0.003 μCi/ml ^{3}HdT.

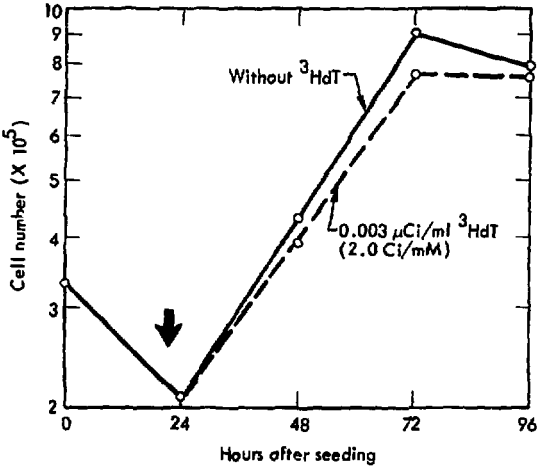

Cell number (X 10^5)
10
9
8
7
6
5
4
3
2
0
24
48
72
96
Hours after seeding
Without ^{3}HdT
0.003 μCi/ml ^{3}HdT
(2.0 Ci/mM)

Figure 5.

Figure 6. Effect of continuous exposure to low levels of ^{3}HdT (2.0 Ci/mM) on middle-aged WI-38 culture growth. The PDL of this culture was 47. The boldface arrow indicates the time of addition of 0.003 μCi/ml ^{3}HdT.

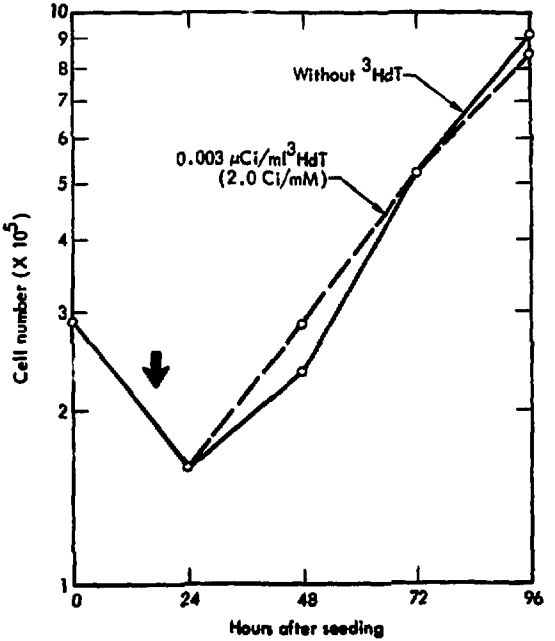

Without ^{3}HdT
0.003 μCi/ml ^{3}HdT
(2.0 Ci/mM)
Cell number (X 10^5)
Hours after seeding
10
9
8
7
6
5
4
3
2
1
0
24
48
72
96

Figure 6.

Figure 7. Effect of continuous exposure to low levels of ^{3}HdT (2.0 Ci/mM) on old WI-38 culture growth. The PDL of this culture was 53. The boldface arrow indicates the time of addition of 0.003 μCi/ml ^{3}HdT.

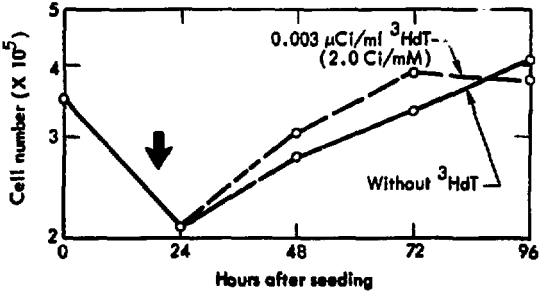

Cell number (X 10^5)
5
4
3
2
0
24
48
72
96
Hours after seeding
0.003 μCi/ml ³HdT-
(2.0 Ci/mM)
Without ³HdT

Figure 7.

^{3}HdT (S.A. 20.0 Ci/mM). Cells were monitored for cell number, Coulter volume and LI.

Figure 8 shows that cellular proliferation is inhibited by continuous exposure to 1.0 μCi/ml ^{3}HdT. The cell number of the control (no ^{3}HdT) increases linearly with time after seeding the culture. The cell number of the cul[illegible] with ^{3}HdT increases at a linear rate, which is slightly less [illegible] the control, for the first 24 hours of labeling. The cell number is unchanged during the following 24 hours of labeling.

Figure 9 shows that LI is inhibited by continuous exposure to 1.0 μCi/ml ^{3}HdT. The LI does not increase significantly from 24 hours to 72 hours after the addition of ^{3}HdT.

Table 2 shows that cellular volume is increased by exposure to the ^{3}HdT. The modal channel of the culture with 1.0 μCi/ml ^{3}HdT increases by approximately two times the control at 48 hours of exposure or day 3.

Figure 10 shows that 5.0 μCi/ml ^{3}HdT also inhibits cellular proliferation. The cell number of the control increases linearly with time after seeding the culture. The labeled culture has the same cell number as the control at 24 hours of exposure to ^{3}HdT. There is no further increase in cell number for the labeled culture f[illegible] the duration of the experiment.

Figure 11 shows that LI is inhibited by continuous exposure to 5.0 μCi/ml ^{3}HdT. During the first 24 hours of labeling, 84% of the cells

Figure 8. Effect of continuous exposure to high levels of ^{3}HdT (20.0 Ci/mM) on old WI-38 culture growth. The PDL of this culture was 53. The boldface arrow indicates the time of addition of 1.0 μCi/ml ^{3}HdT.

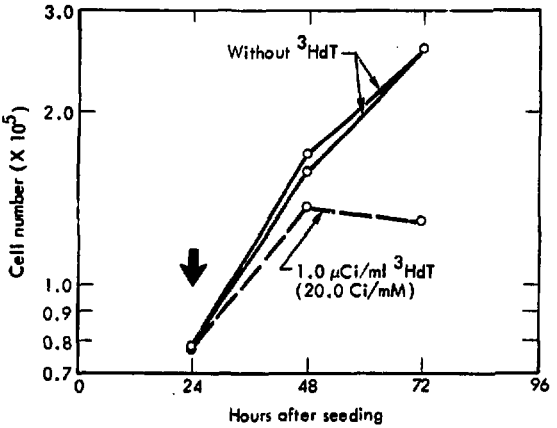

3.0
2.0
1.0
0.9
0.8
0.7
Cell number (X 10^5)
Without 3HdT
1.0 μCi/ml 3HdT
(20.0 Ci/mM)
0
24
48
72
96
Hours after seeding

Figure 8.

Figure 9. Labeling index of WI-38 cells continuously exposed to high levels of ^{3}HdT (20.0 Ci/mM). The PDL of this culture was 42. The boldface arrow indicates the time of addition of 1.0 Ci/ml ^{3}HdT.

Figure 11. Labeling index of WI-38 cells continuously exposed to high levels of ^{3}HdT (20.0 Ci/mM). The PDL of this culture was 46. The boldface arrow indicates the time of addition of 5.0 µCi/ml ^{3}HdT.

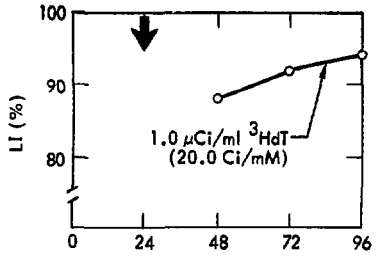


Figure 9.

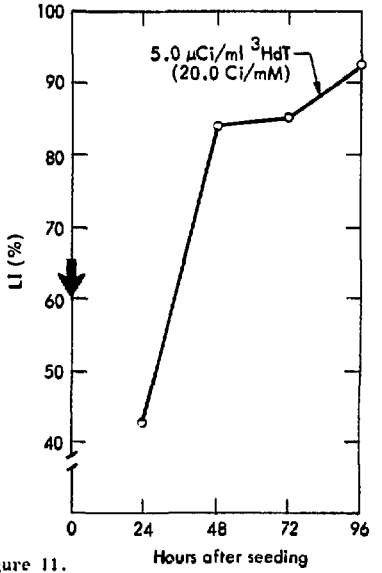


Figure 11.

MODAL CHANNEL OF COULTER VOLUMES OF WI-38 CELLS GROWN WITH AND WITHOUT 20.0 Ci/mM ^{3}HdT

Day[a]	Modal channel	
	Without ^{3}HdT	1.0 μCi/ml ^{3}HdT
1[b]	-	-
2	31	40
3	26	54

a. Days after seeding the culture
b. Time of addition of ^{3}HdT

Table 2.

MODAL CHANNEL OF COULTER VOLUMES OF WI-38 CELLS GROWN WITH AND WITHOUT 20.0 Ci/mM ^{3}HdT

Day[a]	Modal channel	
	Without ^{3}HdT	5.0 μCi/ml ^{3}HdT
0[b]	24	23
1	29	28
2	30	56
3	28	78
4	26	117

a. Days after seeding the culture
b. Time of addition of ^{3}HdT

Table 3.

Figure 10. Effect of continuous exposure to high levels of ^{3}HdT (20.0 Ci/mM) on WI-38 cell growth. The PDL of this culture was 46. The boldface arrow indicates the time of addition of 5.0 μCi/ml ^{3}HdT.

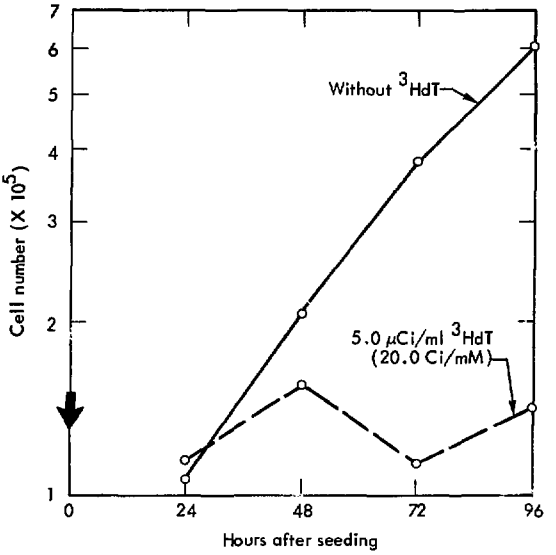

7
6
5
4
3
2
1
Cell number (X 10^5)
Without 3HdT
5.0 μCi/ml 3HdT
(20.0 Ci/mM)
0
24
48
72
96
Hours after seeding

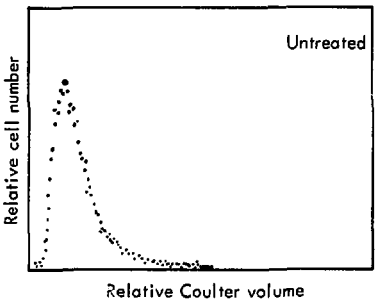
Figure 10.

incorporate ^{3}HdT. At 72 hours of labeling there is no change in LI and at 96 hours there is a slight, but statistically insignificant, rise in LI. The LI approximately doubles from 24 to 48 hours of ^{3}HdT exposure, whereas the cell number (figure 10) remains constant. These results may be explained by the occurence of cells which incorporate ^{3}HdT and then do not proceed through division (G_2-blocked cells). Also, cell death from ^{3}HdT exposure could contribute to the plateau of cell number.

Figure 12 is an example of volume distribution for cells grown with and without 5.0 μCi/ml ^{3}HdT. The control culture has a volume distribution compatible with an asynchronous growing population. The distribution is skewed toward the right. Small cells, which predominate, are on the left side and large cells tail off toward the right. The culture labeled for 72 hours has a volume distribution which is displaced to the right and which has a wide dispersion, indicating that these cells are larger than the control cells. The increase in cellular volume may be due to cells which are blocked in diploid G_2 or tetraploid G_1 followed by unbalanced growth or an increase in ploidy level.

Table 3 shows that there is a continuous increase in cellular volume during exposure to the ^{3}HdT. For the control culture, the modal channel number shows no change with time. The modal channel number of the culture with 5.0 μCi/ml ^{3}HdT increases by approximately two times the control at 48 hours of exposure (or day 2). The modal

Figure 12. Coulter volume distribution of WI-38 cells continuously exposed to high levels of ^{3}HdT (20.0 Ci/mM). The upper panel represents the untreated cells. The lower panel represents the cells which were labeled with 5.0 μCi/ml ^{3}HdT for 72 hours.


Untreated
Relative cell number
Relative Coulter volume

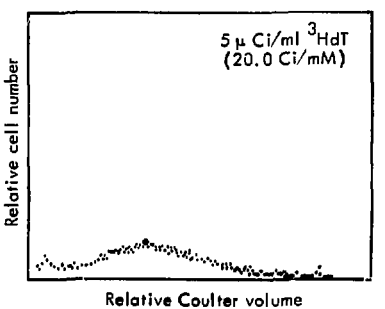

5 μ Ci/ml ^{3}HdT
(20.0 Ci/mM)
Relative cell number
Relative Coulter volume

Figure 12.

channel number continues to increase with time, and is four and one-half times larger than the control at 96 hours of exposure (or day 4).

In summary, these data show that cellular growth is inhibited by continuous exposure to 1.0 μCi/ml ^{3}HdT (S.A. 20.0 Ci/mM) or 5.0 μCi/ml ^{3}HdT (S.A. 20.0 Ci/mM). A direct comparison of the growth kinetics of cells exposed to these two levels of ^{3}HdT should not be made, since 1.0 μCi/ml ^{3}HdT was added 24 hours after seeding the culture (exponential phase), whereas 5.0 μCi/ml ^{3}HdT was added at the same time the culture was seeded (lag phase). Nevertheless, data from both experiments are consistent with the interpretation that cell division may proceed during the first 24 hours of labeling. At 48 hours or longer periods of labeling the cell number and LI are constant, which indicates that cell division is inhibited. In addition, at 48 hours ^{3}HdT exposure the cell volume is approximately twice the control, which suggests that the cells block in diploid G_2 or tetraploid G_1 phase. Further exposure to ^{3}HdT results in further volume increases, due to unbalanced growth or an increase in ploidy level. Cytophotometric-autoradiographic experiments, reported in Section II, show that virtually all cells which incorporate ^{3}HdT have 4C DNA, which strengthens the hypothesis that high levels of ^{3}HdT block cells in diploid G_2 or tetraploid G_1 phase.

b. Effect of different methods of slide preparation on labeling index

An increase in the CV of DNA values for old cultures could arise from a high slide background. Aging WI-38 cultures have increased amounts of "cellular debris". DNA contents reported in Section I were determined on nuclei of intact whole cells which were fixed, stained and measured while remaining attached to the slides on which they were grown (see Materials and Methods: C,1b). Utilizing this method of slide preparation, a large proportion of the debris also remained attached to the slides. DNA contents reported in Section II were determined on nuclei devoid of cytoplasm, and fixed to freshly cleaned slides. To prepare these slides, cells were subjected to trypsin (which lyses a good deal of the debris), hypotonic KCl and centrifugation, and were dropped on wet microscope slides (see Materials and Methods: C, 2b). Selective loss of either dividing or nondividing cells during treatment was tested by measuring the LI of cells treated according to Section I (method 1) compared to the labeling index of cells treated according to Section II (method II). Cultures of various population doubling levels (PDL) were utilized.

In all the experiments the labeling index was higher for method II (see table 4). A simple T test revealed that in only one instance (experiment 4) was the difference between methods significant. A paired T test (Snedecor and Cochran, 1967), using a arcsin transformation of the labeling indices in order to stabilize the

EFFECT OF DIFFERENT METHODS OF SLIDE PREPARATION ON LABELING INDEX

Experiment	PDL[a]	LI (%)[b]	
		Method I[c]	Method II[d]
1	44	91.8	94.8
2	46	91.8	95.0
3	46	87.8	91.0
4	59	53.5	61.3
5	61	18.3	21.5

a. Different strains of WI-38 cultures were used, therefore a direct comparison of PDL to determine "age" cannot be made.
b. 400 cells per culture were counted.
c. Cells remained attached to slides on which they were grown.
d. Cells were trypsinized and devoid of cytoplasm.

Table 4.

variance, showed that the labeling index of cells treated by method II is significantly higher than the labeling index from cells treated by method I. This indicated that nondividing cells were selectively lost by the harsh treatment of method II.

This hypothesis was tested further by measuring the labeling index of cells that resisted release from the plastic by trypsin (experiment 5). The labeling index of cells remaining on the plastic after trypsin treatment (4.0%) was much lower than the labeling index of cells subjected to method I (18.3%) or method II (21.5%). Nondividing cells appear to resist the releasing action of trypsin more than dividing cells. This does not rule out the possibility of further loss of dividing and/or nondividing cells due to cellular lysis during method II.

In conclusion, nondividing WI-38 cells are selectively lost when treated by method II. The effect is a small one and can only be shown by involved analysis on experimentally paired data. However, DNA measurements were made on only 100 cells per culture (based on a pragmatic time limit). This small sample size could introduce a bias, and should be kept in mind in interpretation of the data.

c. DNA cytophotometry of dividing and nondividing WI-38 cells

(1) Double labeling with ^{3}HdT

Experiments were performed to compare the dispersion of DNA values

in old and young WI-38 cultures, and in dividing and nondividing cells. Cells were double labeled with ^{3}HdT. Exposure to 0.003 μCi/ml ^{3}HdT for 48 hours labeled the dividing cells but did not affect their growth kinetics (Results: 2, a(1)) and at the end of this period, exposure to 100 μCi/ml ^{3}HdT for 30 minutes labeled the cells in S phase. Cytophotometric measurements were made on fixed, stained nuclei, followed by autoradiographic detection of ^{3}HdT uptake.

Figure 13 shows histograms of cytophotometric-autoradiographic data from young population doubling level 24 (PDL 24) and old (PDL 55 and PDL 62) cultures. Each histogram is divided into lightly labeled (cycling G_1 or G_2 phase) nuclei, heavily labeled (cycling S phase) nuclei and unlabeled (noncycling) nuclei.

The youngest culture (PDL 24) has a bimodal distribution typical of an asynchronous proliferating population. There are no unlabeled nuclei. Lightly labeled nuclei are distributed in well defined peaks at 2C and 4C, and are, presumably, in diploid G_1 and G_2 phase. Thirty percent of the nuclei are in G_1 phase; 22% of the nuclei are in G_2 phase. Heavily labeled (S phase) nuclei have DNA values intermediate between 2C and 4C, and comprise 47% of the nuclei. There is one nucleus with a hyperdiploid DNA content, and it has a labeling pattern and DNA content consistent with being in S phase.

An older culture (PDL 55) has polyploid nuclei with 8C and 16C DNA. Unlabeled nuclei have well demarcated peaks of 2C, 4C and 8C DNA contents. The 2C peak contains 40% of the unlabeled nuclei and the 4C

Figure 13. Feulgen DNA stain content of aging WI-38 cultures double labeled with ^{3}HdT. The histograms are arranged according to increasing age (PDL). The stippled bars represent cells which incorporated 0.003 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) at 48 hours of exposure and which were lightly labeled in autoradiograms. The hatched bars represent cells which incorporated 100.0 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) at a 30 minute pulse and which were heavily labeled in autoradiograms. The clear bars represent the unlabeled cells. DNA content is given in arbitrary CYDAC units (ACU) of C where 2C is the diploid DNA content, operationally defined in terms of the mean of the first peak. Included in the data of the PDL 62 culture are the DNA values of the unlabeled cells from a sister culture labeled for 48 hours with 5 μCi/ml ^{3}HdT (S.A. 20.0 Ci/mM) (see figure 14, bottom).

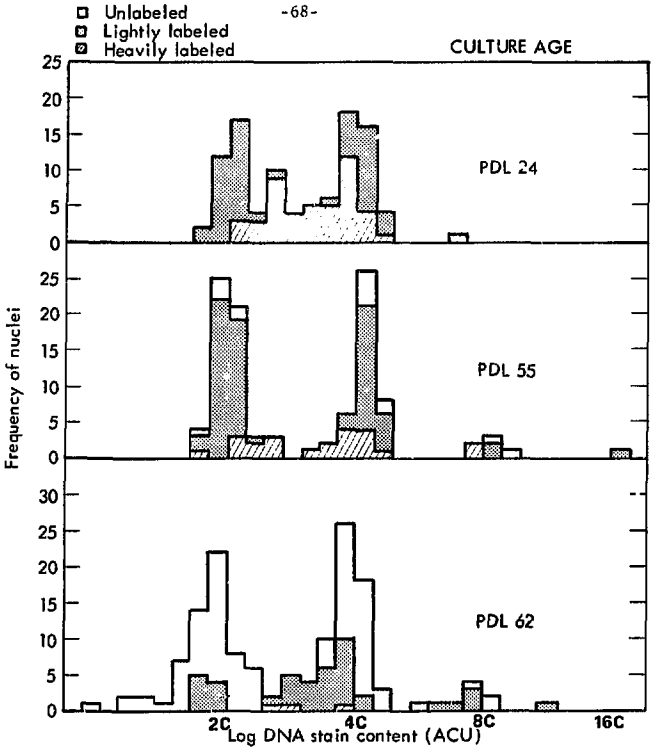

Unlabeled
Lightly labeled
Heavily labeled
CULTURE AGE
PDL 24
PDL 55
PDL 62
Frequency of nuclei
2C
4C
8C
16C
Log DNA stain content (ACU)

Figure 13.

peak contains 47%. Once again, the heavily labeled nuclei (S phase) are intermediate in DNA value, and the lightly labeled nuclei define sharp peaks at the 2C and 4C levels. There are fewer heavily labeled (S phase) nuclei than in the young culture: 25% of the labeled nuclei are in S phase here, whereas 47% were in S phase in the younger culture. The 2C and 4C peaks contain, respectively, 45% and 26% of the labeled nuclei. There are two nuclei with hyperdiploid DNA contents and they are presumed to be in S phase.

Interpretation of the histogram of the oldest culture (PDL 62) is not as definitive as the other cultures. There are polyploid labeled and unlabeled nuclei with 8C DNA. Heavily labeled nuclei have intermediate DNA values. Only 7% of the labeled nuclei have a labeling pattern consistent with being in the S phase. Lightly labeled nuclei have 2C, 4C, 8C and intermediate DNA values. Only 20% of the labeled nuclei are in the 2C peak, and 59% have either 4C or intermediate DNA values. The lightly labeled nuclei with intermediate DNA values cause a left skew to the 4C peak. These nuclei may be aneuploid, or they may be lightly labeled S phase nuclei (i.e., cells in S phase which incorporated low levels of ^{3}HdT). The few lightly labeled 8C nuclei appear to be skewed to the left. Unlabeled nuclei have 2C, 4C, 8C and intermediate DNA values. The 2C peak contains 53% of the unlabeled nuclei. Nuclei with hypodiploid DNA contents cause the unlabeled 2C peak to skew to the left. The unlabeled 4C and 8C peaks contain, respectively, 44% and 3% of the unlabeled nuclei.

Quantitative data analysis is shown in table 5. S phase nuclei were subtracted from the DNA histograms in figure 13 to yield 2C and 4C peaks of DNA values. Then the CV of the 2C and 4C peaks, and the ratio of 4C to 2C DNA values were calculated.

In the PDL 24 culture, the peaks of labeled 2C and 4C nuclei have a small CV (6.9% and 6.6%, respectively), which indicates that there is little dispersion in the DNA values. In addition, the ratio of 4C to 2C mean DNA values is approximately two. This indicates that the specimen, the Feulgen stain, and the cytophotometric measurements behaved extremely well.

In the PDL 55 culture, the CV is similar for labeled and unlabeled nuclei, in both the 2C and 4C peaks of DNA values. Like the young culture, the CV is small (approximately 6.0%), and the ratio of 4C to 2C mean DNA values is approximately two.

The PDL 62 culture has a labeled 2C CV which is similar to the younger cultures. In this old culture, however, the unlabeled 2C CV (17.4%) is much larger than the labeled 2C CV (5.5%). The CV of the unlabeled 4C peak (11.0%) is similar to the CV of the labeled 4C peak (12.6%). With the exception of the labeled 2C nuclei, the PDL 62 culture has a CV of peak DNA values which is more than two fold larger than the PDL 55 or PDL 24 cultures. The significance of the increased CV in the oldest culture is uncertain because the peaks are skewed. Possible explanations for the skewed data are given in the Discussion. Deviation from the expected 2 to 1 ratio of 4C to 2C mean DNA values

COEFFICIENT OF VARIATION OF DNA VALUES AND RATIO OF 4C TO 2C DNA VALUES WITH AGE

Culture age	Coefficient of variation (%)				Ratio of mean DNA values of 4C to 2C peaks	
	2C peak		4C peak			
	Labeled	Unlabeled	Labeled	Unlabeled	Labeled	Unlabeled
PDL 24	6.9	-	6.6	-	1.97	-
PDL 55	5.6	6.2	5.7	5.2	2.08	2.13
PDL 62	5.5	17.4	12.6	11.0	1.85	2.30

Table 5.

is probably due to the skewed data. The labeled 4C peak is more skewed than the labeled 2C peak, and the ratio of their mean DNA values is significantly less than two. The opposite is true for unlabeled nuclei. The unlabeled 4C peak is less skewed than the unlabeled 2C peak, and the ratio of the 4C to 2C mean DNA values is significantly greater than two.

Generally speaking, the oldest WI-38 culture has an increased CV of 2C and 4C DNA values. In addition, for cultures of all ages, unlabeled nuclei do not have a greater CV than labeled nuclei. The combination of double label ^{3}HdT autoradiography and DNA cytophotometry proved to be an expedient approach to identify cells in S, and thereby define the boundaries of 2C and 4C peaks. However, the data from the oldest culture was more complex than anticipated, and is difficult to interpret.

(2) Labeling with high levels of ^{3}HdT

Cytophotometric-autoradiographic experiments were performed on fixed, stained nuclei from WI-38 cultures continuously labeled with 5.0 μCi/ml ^{3}HdT (S.A. 20.0 Ci/mM). Cells grown in this concentration of ^{3}HdT cease to divide and increase in volume (Results: 2, a(2)). The DNA content of the cells blocked by high levels of ^{3}HdT was determined, and the results are shown in figure 14.

Figure 14 shows histograms of the data from young (PDL 24) and old (PDL 62) cultures which are sister cultures to those utilized in the double labeling experiment. Nuclei which are labeled ceased cycling

Figure 14. Feulgen DNA stain content of aging WI-38 cultures exposed to high levels of ^{3}HdT (20.0 Ci/mM). The histograms are arranged according to increasing age (PDL). The solid bars represent cells which incorporated 5 μCi/ml ^{3}HdT during a 48 hour exposure. The clear bars represent the unlabeled cells. DNA content is given in arbitrary CYDAC units (ACU) of C where 2C is the diploid DNA content, operationally defined in terms of the mean of the first peak.

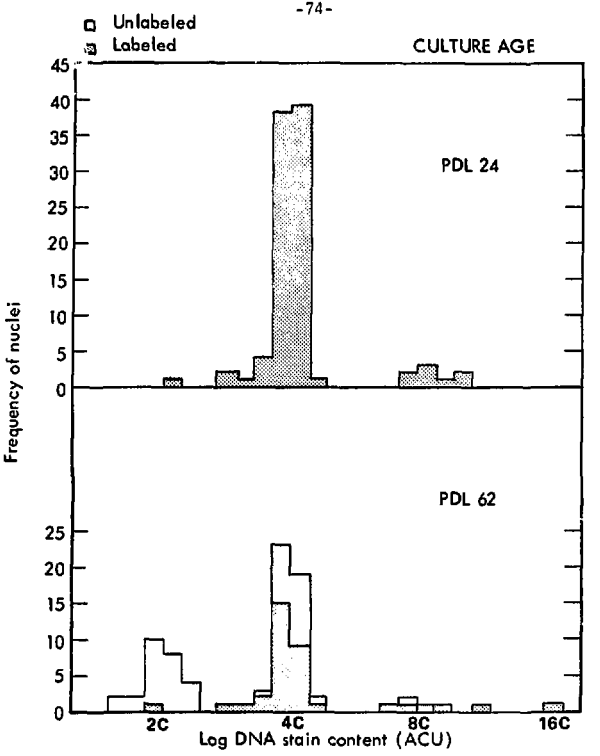

Unlabeled
Labeled
CULTURE AGE
PDL 24
PDL 62
Frequency of nuclei
45
40
35
30
25
20
15
10
5
0
25
20
15
10
5
0
2C
4C
8C
16C
Log DNA stain content (ACU)

Figure 14.

after the addition of ^{3}HdT, and are termed blocked dividing cells. Unlabeled nuclei ceased cycling before the addition of ^{3}HdT, and are termed nondividing cells.

In the PDL 24 culture, there is a major peak of DNA values (90% of the nuclei) at the 4C level. One percent of the nuclei have 2C DNA values and 9% of the nuclei have 8C DNA values. All the nuclei are labeled; there are no nondividing cells.

The labeled nuclei of the PDL 62 culture have a distribution similar to the PDL 24 culture. There is a major peak of DNA values (83% of the labeled nuclei) at the 4C level. Three percent of the labeled nuclei have 2C DNA values and 14% have between 18C and 16C DNA values. The majority (54%) of the unlabeled nuclei have 2C DNA values and 4% have 8C DNA values.

In summary, WI-38 cells which label during 48 hours exposure to high levels of ^{3}HdT have, almost exclusively, 4C DNA. These 4C blocked dividing cells may be diploids in G_2 phase or tetraploids in G_1 phase.

B. Premature Chromosome Condensation-Autoradiographic Studies

1. Section III

Combined DNA cytophotometry and autoradiography, reported in Sections I and II, demonstrated that the majority of nondividing WI-38 cells have 2C DNA contents, consistent with the interpretation that they are diploid cells arrested in G_1. Increasing culture age was

associated with increasing numbers of nondividing cells with 4C DNA contents as well as with proliferating and nonproliferating polyploid cells. It was uncertain whether the nondividing 4C cells were arrested as diploid G_2 or as tetraploid G_1 cells. To continue the investigation of aging nondividing cells premature chromosome condensation, (PCC) was utilized (1) to determine whether nondividing cells could condense their chromatin; (2) to determine the stage of the cell cycle in which these cells are arrested; and (3) to determine the ploidy level of the nondividing cells.

The premature chromosome condensation-autoradiograph technique was performed as follows. Middle-aged and old cultures of WI-38 cells were labeled for 48 hours with 0.1 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM). WI-38 cells were fused with mitotic cells, which induced interphase chromatin to condense into prematurely condensed chromosomes. The chromosomes were examined to determine cycle stage and ploidy level; cells were classified as either G_1 if they contained single chromatids or G_2 if they contained double chromatids, and, as either diploid if they contained 46 chromosomes or tetraploid if they contained 92 chromosomes. Autoradiographic detection of ^{3}HdT uptake further classified the cells as dividing (labeled) or nondividing (unlabeled).

a. Fusion of WI-38 cells with mitotic CHO cells

Mitotic CHO cells were chosen to induce PCC because the diploid chromosome number (21) is easy to distinguish from the diploid chromosome number of WI-38 cells (46). Mitotic CHO cells were collected by a shake - synchrony method (Materials and Methods:

D,1a(2)). At appropriate time intervals, CHO cells blocked in mitosis by Colcemid, were collected from the monolayer by gentle shaking. After 45 minutes in Colcemid (sample B) loose cells and debris come off the monolayer along with mitotic cells. The mitotic index (MI) is 41% (table 6). The MI rises to 78% one hour later (sample C). Samples D, E and F, collected over the next few hours, have a MI from 91 to 92%. Each sample was monitored for Coulter volume distribution. The modal channel number is approximately two times greater for mitotic cells than for cells without Colcemid (figure 15). Pooled CHO cells from samples D, E and F were suspended, according to the fusion procedure (Materials and Methods: D,1b(2)), with WI-38 cells, but did not induce PCC. A likely explanation is that CHO cells and WI-38 cells do not fuse readily.

b. Fusion of WI-38 with mitotic HeLa cells

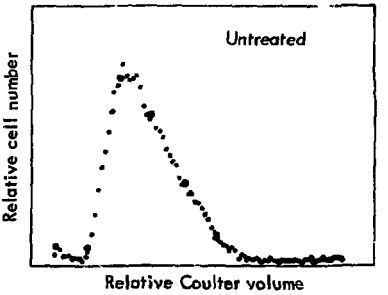
HeLa cells are known to fuse readily with WI-38 cells and, therefore, were chosen to induce PCC. The modal chromosome number of HeLa cells is sufficiently large (approximately 100) to distinguish them from WI-38 cells. To further aid identification the HeLa cells were labeled with ^{3}HdT (0.1 μCi/ml ^{3}HdT, S.A. 2.0 Ci/mM). Colcemid was added to the HeLa monolayer to block mitosis and the mitotic cells were collected by decanting (Materials and Methods: D,1a(3)). The MI was 99.9% and 99.7% of the mitoses labeled. The modal volume of the mitotic HeLa cells was approximately 2.5 times the untreated HeLa cells (figure 16). This is larger than twice the volume of a cell in

MITOTIC INDEX OF CHO CELLS COLLECTED IN COLCEMID (0.06 μg/ml)

Sample	Time in Colcemid (min)	Mitotic Index (%)
A	0	-
B	45	41
C	105	78
D	165	91
E	225	92
F	390	91

Table 6.

Figure 15. Coulter volume distribution of mitotic CHO cells collected in Colcemid by the shake-synchrony method. The upper panel represents the untreated cells. The lower panel represents the cells treated with 0.06 µg/ml Colcemid; the cells were pooled from samples D, E and F (see table 6), and had a MI of approximately 92%.


Untreated
Relative cell number
Relative Coulter volume

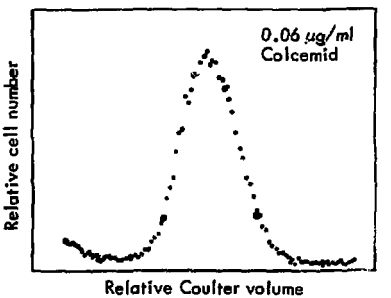

0.06 μg/ml
Colcemid
Relative cell number
Relative Coulter volume

Figure 15.

Figure 16. Coulter volume distribution of labeled mitotic HeLa cells collected in Colcemid. The upper panel represents the untreated cells. The lower panel represents the cells exposed to 0.1 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) for 26 hours. For the final 26 hours of labeling 0.25 μg/ml Colcemid was added. The mitotic index of this culture was 99.9% and 99.7% of the mitoses were labeled.

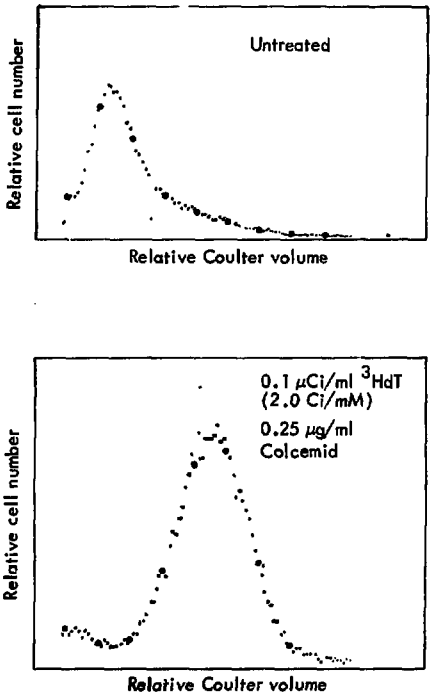

Untreated
Relative cell number
Relative Coulter volume
0.1 μCi/ml ^{3}HdT
(2.0 Ci/mM)
0.25 μg/ml
Colcemid
Relative cell number
Relative Coulter volume

Figure 16.

G_1. A likely explanation is that Colcemid blocked cells still synthesize protein, which results in increased volume.

Two cultures, a middle-aged culture containing 34% nondividing cells (culture A) and an old culture with 76% nondividing cells (culture B), were fused to mitotic HeLa cells to induce PCC. The frequency of PCC (WI-38) per total cells (WI-38, HeLa, heterokaryons) was 1.3% for culture A and 1.0% for culture B. The difference is not significant ($p = 0.37$).

Typical chromosome condensation patterns observed are shown in figures 17-20. Since the chromosomes of the mitotic HeLa cells were highly contracted and were always labeled, they were easy to distinguish from the less condensed interphase WI-38 chromosomes. Out of 178 G_1 diploid PCC configurations (figure 17) examined from the two cultures, 98 were unlabeled (table 7). In 19 PCC cells, the WI-38 chromosomes exhibited the G_2 diploid pattern (figure 18). All of these cells were labeled (figure 18, inset). The WI-38 G_1 tetraploid PCC configuration shown in figure 19 was observed in 15 cells of which 10 were unlabeled. This typical polyploid cell has approximately 92 unpaired chromatids. Such a configuration is distinct from that observed in figure 20, where the 92 chromatids show apparent homologous pairing at the centromere regions and exhibit wide separation along the arms. This anomalous pairing was observed in three cells, all unlabeled. Additionally, two unlabeled cells with approximately a diploid chromosome number also demonstrated this unexpected pairing. Unfortunately the chromatids were highly clumped

Figure 17. Autoradiograph of an unlabeled WI-38 G_1 diploid PCC and labeled mitotic HeLa chromosomes.

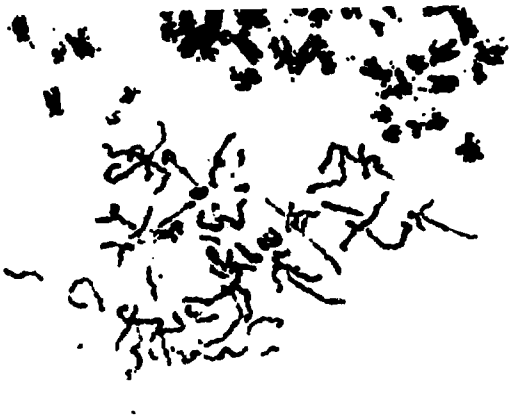
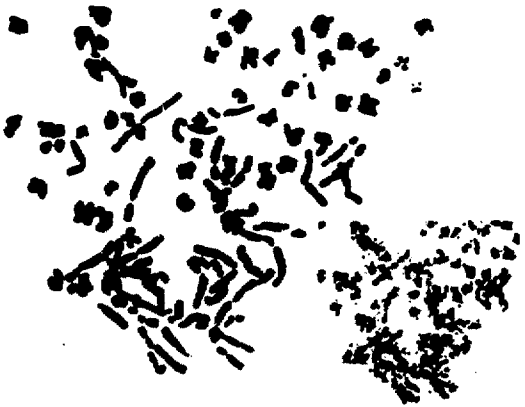


Figure 17.
R. Yanishevsky

Figure 18. WI-38 G_2 diploid PCC and mitotic HeLa chromosomes. Inset = the same cell after autoradiography.



Figur 18.
R. Yanishevsky

Figure 19. WI-38 G_1 tetraploid PCC with approximately 92 single chromatids and mitotic HeLa chromosomes.

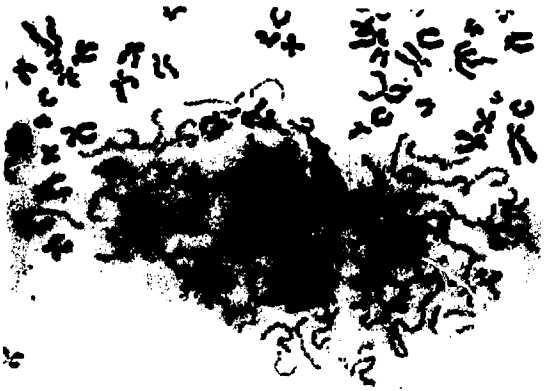


Figure 19.
R. Yanishevsky

Figure 20. Autoradiograph of an unlabeled WI-38 PCC containing approximately 92 paired chromatids and labeled mitotic HeLa chromosomes.

Figure 20.
R. Yanishevsky

PERCENTAGE OF NONDIVIDING CELLS IN PARENT CULTURES, FREQUENCY OF PCC, AND CELL CYCLE DISTRIBUTION OF UNLABELED CELLS WITH PCC.

Numbers in parentheses refer to actual cell counts.

	Nondividing cells in parent cultures (%)	Cells with PCC (%)	Unlabeled cells with PCC (%)			
			Total	"Diploid" G_1	"Diploid" G_2	"Tetraploid" G_1
Culture A	34[a] (175/519)	1.3[b] (20/1549)	32[c] (33/104)	37[d] (30/81)	0 (0/16)	43 (3/7)
Culture B	76 (996/1308)	1.0 (5/504)	69 (75/108)	70 (68/97)	0 (0/3)	88 (7/8)

a. Expressed as 100 × unlabeled cells/total cells.
b. Expressed as 100 × WI-38 cells with PCC/heterokaryons, WI-38 and Hela cells.
c. Expressed as 100 × (unlabeled G_1 and G_2 cells with PCC)/(G_1 and G_2 cells with PCC).
d. Expressed as 100 × unlabeled G_1 diploids/G_1 diploids,
100 × unlabeled G_2 diploids/G_2 diploids,
or 100 × unlabeled G_1 tetraploids/G_1 tetraploids

Table 7.

and thus we could only roughly estimate them. These five cells were not included in the analysis of the quantitative data but will be considered in the Discussion.

As shown in table 7, a total of 104 PCC configurations was scored in culture A and 108 in culture B. Thirty-two percent of these in culture A and 69% in culture B were unlabeled. This is similar to 34% and 76% nondividing cells observed in parent cultures A and B, respectively. In the calculation of the percent of cells with PCC the S phase cells were excluded, whereas in the calculation of the percent of nondividing cells in the parent cultures they were included. The effect of labeled S phase PCC configurations would be to decrease the percentage of unlabeled cells with PCC observed. If the data in table 7 is expressed as the ratio of culture B to culture A, the aging effect on cells in G_1 and G_2 is shown independently of the S phase cells. This ratio is equal to 2.2 for both the percent of unlabeled cells with PCC and the percent of nondividing cells in the parent cultures. This approximate two-fold increase in the frequency of nondividing cells correlates with the increase in unlabeled G_1 diploid cells with PCC. The frequency of all G_1 cells with PCC (labeled and unlabeled) increases significantly ($p = 0.03$) from culture A to culture B, while the frequency of labeled G_2 cells with PCC decreases significantly ($p = 0.002$). The number of unlabeled tetraploid cells observed is small and there is no significant increase in this class with culture age ($p = 0.11$).

The expected proportion of G_1 and G_2 cells showing PCC in culture A (34% nondividing cells) was estimated using cytophotometric-autoradiographic data presented in Section I of a culture (here called culture C) containing 32% nondividing cells. The DNA histogram of the dividing population of culture C was partitioned into G_1 (2C) and G_2 (4C) cells by subtracting out the cells with intermediate DNA contents (an approximation of the cells in S). In the nondividing population the DNA histogram consisted only of cells with 2C DNA contents. This information gave the percentages of dividing and nondividing cells with 2C and 4C DNA in a culture of this age. To calculate the expected partitioning of cells with PCC in culture A, the percentage of cells in the different phases was multiplied by the total number of observed labeled or unlabeled cells with PCC. The observed and expected values for culture A are given in table 8. There is no significant difference within the various classes, indicating that, with the statistical limitations of the data, PCC is induced with equal efficiency in dividing and nondividing cells as well as in G_1 and G_2 cells. A comparable analysis with culture B was not possible with the data at hand. In interpretation of this data it should be kept in mind that the sample size was small, and that the PCC and cytophotometric experiments utilized different methods of slide preparations, which may bias the data.

In conclusion, PCC of middle-aged and old WI-38 cultures revealed (1) that nondividing cells condense their chromatin as readily as

NUMBER OF OBSERVED AND EXPECTED WI-38 CELLS WITH PCC IN CULTURE A

The labeled and unlabeled cells are categorized as either "diploid", (2C) or (4C), or "tetraploid" (4C).

	Total No. cells	"Diploid" cells G_1 (2C)	"Diploid" cells G_2 (4C)	"Tetraploid" cells G_1 (4C)
Labeled cells with PCC:				
Observed	71	51	16	4
Expected[a]	–	50	21[b]	
Unlabeled cells with PCC:				
Observed	33	30	0	3
Expected	–	33	0[b]	

a. Calculated from cytophotometric autoradiographic data of Section I.

b. DNA cytophotometry cannot distinguish between these two classes because their DNA contents are the same.

Table 8.

dividing cells; (2) that for the middle-aged culture the efficiency of PCC induction is independent of phase in the cell cycle; (3) that 96% of the nondividing cells, diploid or tetraploid, induced into PCC have single chromatids and are therefore blocked in the G_1 phase; (4) that the remaining 4% of the nondividing cells have anomalous centromeric pairing; (5) that typical G_2 configurations (double chromatids) were observed only among nondividing cells.

These data further suggest that nondividing cells with 4C DNA (see DNA histograms of figures 1 and 13) are arrested in tetraploid G_1 phase, rather than in diploid G_2 phase.

DISCUSSION

As human diploid WI-38 cultures age, there is a progressive increase in the nondividing fraction of the population. Biophysical and cytological techniques were employed to identify the cycle phase of the nondividing cells. These experiments revealed that most nondividing cells were diploid, and that nondividing cells, diploid or tetraploid, predominated in the G_1 phase of the cell cycle. Additional key findings were that older cultures had an increased frequency of proliferating and nonproliferating polyploid cells, that G_1 and G_2 cells in older cultures had a wider dispersion of DNA values and that some nondividing cells in aging cultures had atypical chromosome configurations. These data are discussed in Sections I and II (Cytophotometric-Autoradiographic Studies) and Section III (Premature Chromosome Condensation-Autoradiographic Studies). The following is a discussion on the limited proliferative capacity of cells in vivo and a brief overview of some current aging theories.

A. Cytophotometric-Autoradiographic Studies

1. Section I

a. DNA distribution of dividing and nondividing WI-38 cells

The cycle phase wherein aging nondividing cells were blocked was initially identified by correlation of DNA stain content and proliferative status for single cells of labeled (0.1 μCi/ml ^{3}HdT,

S.A. 2.0 Ci/mM, for 48 hours) WI-38 cultures. Histograms of figure 1 showed progressive age related changes in DNA content of dividing and nondividing cells.

Young cultures (7% and 32% unlabeled cells) had a DNA distribution typical of an asynchronous uniploid population. Most of the cells were proliferating. As expected, since each G_2 cell becomes two G_1 cells at the point of division, 2C (diploid G_1) cells predominate over 4C (diploid G_2) cells. Cells with intermediate DNA values were presumed to be S phase. The few nondividing cells had 2C DNA and were in diploid G_1 phase.

Older cultures (38% through 70% unlabeled cells) had a DNA distribution which indicated deviant kinetic behavior. In contrast to the younger cultures, there were more proliferating 4C cells than proliferating 2C cells. Few proliferating cells were in S phase. In addition, there were proliferating and nonproliferating 8C cells. Nondividing cells predominantly had 2C DNA contents and, occasionally, had 4C DNA contents.

The restriction of the majority of the unlabeled cells to the 2C DNA content is consistent with the interpretation that most WI-38 cells are in the diploid G_1 phase when they cease cycling. In older cultures, the appearance of unlabeled 4C cells can be interpreted in the same way provided that these 4C cells are actually stopping in a tetraploid G_1. The simultaneous appearance of labeled 8C cells supports the tetraploid interpretation for at least some of these

cells. Alternatively, the results of Macieira-Coelho et al. (1966), show that aging WI-38 cultures contain cells with very long G_2 periods; hence unlabeled 4C cells could be diploids arrested in or slowly traversing G_2. Further experiments were designed to identify nondividing diploid G_2 and tetraploid G_1 cells by the technique of PCC (Section III).

An interesting age-correlated observation is the progressive increase in dispersion of DNA stain content in the 2C and 4C peaks, especially for nondividing cells. Several interpretations of this age-associated effect have been considered: (1) aging cells may be increasingly arrested in S phase; (2) changes in chromatin organization are known to alter the stoichiometry of the staining reaction (Garcia, 1969, 1970); (3) increased "debris" associated with aging cultures may lead to errors in the cytophotometry; (4) aneuploid DNA content may increase in noncycling older cells.

With respect to the first interpretation, to my knowledge, there are no documented reports that cells cease to cycle in S phase (unless they are chemically blocked). In fact, a number of previous studies (e.g., see reviews by Prescott, 1975; Baserga, 1965, 1968; and Epifanova and Terskikh, 1969) have led to the basic tenet of cell cycle control theories that cells cease to cycle in either G_1 phase or G_2 phase. Furthermore, if cells arrested in S phase, the DNA data would be skewed; this did not occur. Although we cannot rule out noncycling S phase cells, the weight of evidence makes it improbable.

Alternatively, it is possible that the cells are cycling in S phase, even though they are unlabeled. For instance, cells could synthesize DNA at a rate too low to be detected by the autoradiographic methods used and would be misscored as unlabeled cells. Petes et al. (1974) used DNA fibre autoradiography to show a 25% reduction in the average rate of DNA chain elongation in senescent human diploid cells. This infers that aging cells have a decreased rate of DNA replication. Other experiments, however, showed that for middle-aged cultures the LI did not increase with increased ^{3}HdT concentration or with increased exposure to autoradiographic emulsion (Cristofalo and Sharf, 1973; Cristofalo, 1975). These experiments indicate that, within statistical limits, cells which incorporate ^{3}HdT will be detected by the autoradiographic methods used. Further experimentation is required for very old cultures.

The second interpretation of the age-associated increase in dispersion of DNA values is that DNA stains nonstoichiometrically in older cells. Nonstoichiometric staining has been traced to small nuclei and highly compacted DNA (e.g., Garcia, 1966; Mayall, 1969). WI-38 cells have very large nuclei and such compaction effects are not to be expected. In fact, cell size (Cristofalo and Kritchevsky, 1969) increases with age; although no systematic quantitative studies have been performed, we have observed that nuclear size also increases with age. Furthermore, the stoichiometry appears well behaved on the basis of both the doubling of 2C to 4C to 8C peaks and the lack of shift of

DNA modes when labeled and unlabeled cells are compared. Therefore, the possibility of nonstoichemtric staining seems unlikely.

The third interpretation is that "debis" leads to errors in cytophotometric measurements. Large amounts of debris occur in old cultures, probably from the lysis of dead cells. Debris causes a nonuniform slide background. This could cause inaccurate DNA measurements. Each DNA measurement requires two background readings: (1) the integrated optical density (IOD) is measured for a stained nucleus and the surrounding background contained in a defined area; (2) the IOD for an adjacent area of similar dimensions, subsequently is subtracted to yield the IOD of the nucleus. In Section I, DNA measurements were made on nuclei of intact whole cells which were fixed, stained and measured while remaining attached to the slides on which they were grown. A large amount of debris remained on the slides. To diminish debris, DNA measurements in Section II were determined on cells which were trypsinized from their growing surface, were devoid of their cytoplasm, and were fixed to freshly cleaned slides. The DNA histograms (figure 13) for cultures of all ages sharpened up, but the age-correlated increase in dispersion persisted.

The last interpretation considered is that aneuploid DNA content may increase in older noncycling cells. Aneuploidy occurs in proliferating cells of aging WI-38 cultures (e.g., Saksela and Moorhead, 1963). In vivo, aneuploidy also increases with age (e.g., Jacobs et al., 1963; Crowley and Curtis, 1963). It has been

postulated that chromosomal abberrations, especially for nondividing cells, could cause aging (Curtis, 1963). If aneuploid DNA content caused cell arrest, the dispersion of DNA values (i.e., the CV) of the 2C and 4C cells would be greater for old cultures as compared to young cultures, and for nondividing cells as compared to dividing cells. The CV could not be calculated because the boundary between 2C and 4C cells, and cells in S phase was uncertain. Experiments in Section II were designed to identify S phase cells and permit calculation of the CV.

In conclusion, the existing evidence strongly suggests that the age-associated increased dispersion of DNA values is not due to noncycling S phase cells or to altered stoichiochmetry of the staining reaction. The possibility that debris is the intrinsic cause of the increased dispersion of DNA values in older cultures is ruled out by the experiments of Section II. These experiments further infer that aneuploid DNA content correlates with culture age.

Figure 21 summarizes the relationships among aging, proliferative capacity and DNA content suggested by this data and by the work of others (Baserga et al., 1971; Cristofalo and Sharf, 1973; Macieira-Coelho and Berumen, 1973). We belive there are two kinds of quiescent (Q) WI-38 cells. The first is exemplified by those cells whose quiescence is induced by density-dependent inhibition of division through various kinds of nutritional depletion. This occurs at any age, and is characterized primarily by its ease of reversibility. These reversibly quiescent cells are referred to as

Figure 21. The changing relationships of the frequency of proliferating and quiescent cells with culture age. The latter are further divided into reversible (Qr) and appraent nonreversible (Qn) cells, and by DNA content (2C or 4C). The arrows indicate pathways and suggest that the cells transit to the Qn state either directly or via Qr.

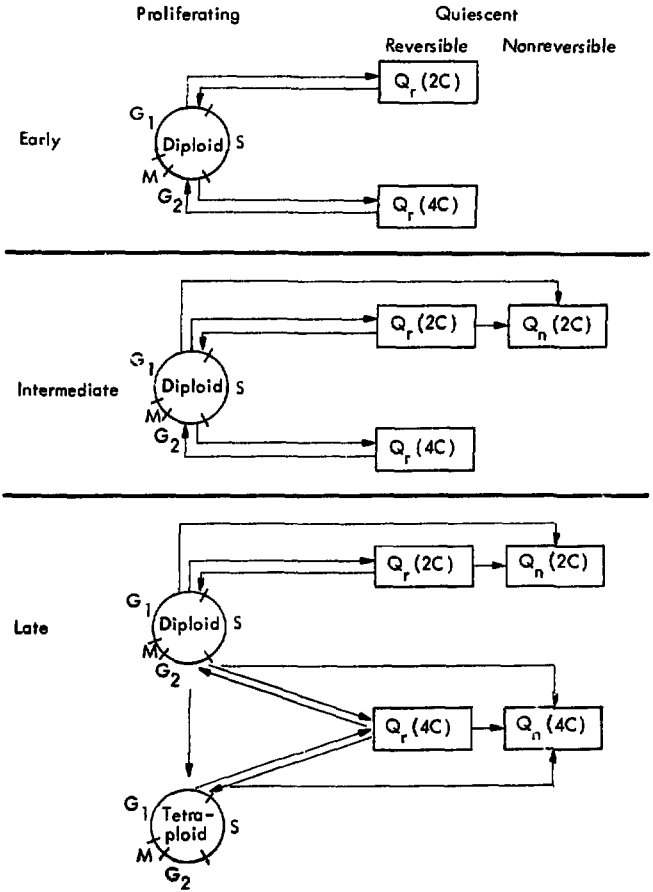

Proliferating
Quiescent
Reversible
Nonreversible
Early
G1
Diploid
S
M
G2
Qr (2C)
Qr (4C)
Intermediate
Qn (2C)
Late
Tetra-
ploid

Figure 21.

Qr. The second kind of Q cell cannot be readily reversed, appears to be nonreversibly quiescent and is designated as Qn. These, we belive, are the cells that remain unlabeled for 48 hours, that increase in incidence with the age of the culture, and that explain the finiteness of the population. In the aging culture, there is an increased frequency of 2C (diploid G_1) and 4C (either diploid G_2 or tetraploid G_1) Qn cells. Although not shown in figure 21, there is also an increase in proliferating and quiescent polyploid (8C) cells.

b. Are "nondividing" WI-38 cells truly out-of-cycle or are they simply cycling slowly?

Recently Macieria-Coelho (1974, 1975) questioned the existence of nondividing WI-38 cells. He cited an experiment which showed that very long ^{3}HdT-labeling periods (up to 40 days) reduce the percent of unlabeled cells from 34 to 8. On this basis he concluded that unlabeled cells are a manifestation of the increased length of the average cell cycle and the wide range of cell cycle times in older populations. This is a reasonable alternative to the notion of noncycling cells. We and others (Good, 1974) question, however, whether Macieria-Coelho's experiments adequately support this conclusion. After long labeling periods a decline in the apparent percent of unlabeled cells is, in fact, to be expected. Firstly, at least some unlabeled cells will die without dividing again. Further, all labeled cells which later become quiescent will be misscored as dividers. Finally, the unlabeled fraction of the population will be

diluted by division of the labeled fraction; for example, if initially 34% of the total cells are unlabeled, after two divisions the unlabeled fraction would comprise only 11% of the total. In spite of these considerations, Macieria-Coelho found that after 40 days of ^{3}HdT exposure a substantial fraction (8%) of the population was unlabeled. This, in fact, supports the notion of nondividing cells. At present, there is no unequivocal way to distinguish cells which are truly out-of-cycle from cells which are cycling very slowly (this is discussed by Mendelsohn, 1962).

In the context of these experiments we have defined a pragmatic point (48 hours) after which a cell is considered nondividing. To determine what fraction of potential dividers are labeled under these conditions, the cell kinetics data of Macieira-Coelho et al. (1966) were reanalyzed by a recently developed automated fitting procedure (Takahashi et al., 1971). The best computer fit showed the average cell cycle time to be 17.4 hours for young WI-38 cells (G_1 = 2.4 hours, S = 10.5 hours, G_2 = 3.3 hours, M = 1.2 hours) and 30.5 hours for old WI-38 cells (G_1 = 14.3 hours, S = 11.0 hours, G_2 = 3.8 hours, M = 1.5 hours). The standard deviation of the cell cycle time is 2.8 hours in young cultures, and 10.4 hours in old cultures. Taking this analysis literally, one would expect all but 1% of young dividing cells to have completed at least one cycle by 24 hours; by 48 hours no stragglers should remain. In old cultures 55 hours is required to commit all but 1% of the cells to cycle, and approximately 5% of dividing old cells have not yet finished their first cycle by 48

hours. Thus, after 48 hours a nonlabeling index of 50% in old cultures might represent 3% of tardy dividers and 47% of cells out of cycle.

This definition of nondividing cells permits a convenient way to quantify the age of the population by the LI, since there is a progressive increase in the nondividing fraction of the population with age (Cristofalo and Sharf, 1973). It is an operational definition and it does not imply that all cells defined as nondividing for this specific set of conditions would remain perpetually quiescent. On the other hand, sterility or self destruction of cells must occur or, with time, the slowly cycling cells would ultimately repopulate the culture, and the culture would not have a finite life-span; there are no reports to that effect.

2. Section II

In Section I an age-associated increase in dispersion of DNA values for 2C and 4C peaks was indicated. It was hypothesized that this could be due to aneuploid DNA content which could cause cell arrest. This hypothesis was examined in the experiments presented in Section II. A double labeling cytophotometric-autoradiographic technique permitted quantitation of the dispersion. The results indicated that there was a correlation, but not a causal relationship, for the increased dispersion of DNA values and cell arrest.

a. Effect of ^{3}HdT on WI-38 cell growth

Preliminary to the cytophotometric experiments, the effect of continuous exposure to varied levels of ^{3}HdT on cell growth was examined. The various levels of ^{3}HdT that were tested were used to label dividing cells for all experiments presented in this manuscript. The results showed that cellular growth was inhibited by 0.1 μCi/ml ^{3}HdT (2.0 Ci/mM) and higher concentrations (1.0 μCi/ml or 5.0 μCi/ml, 20.0 Ci/mM), most likely from radiation damage. Cytophotometric-autoradiographic experiments indicated that the cells blocked in the diploid G_2 or the tetraploid G_1 phase.

Cell growth was monitored during continuous exposure to low and high levels of ^{3}HdT. Growth was measured by cell number, Coulter volume and LI.

(1) Labeling with low levels of ^{3}HdT

Labeling with 0.1 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM)

Uptake of 0.1 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) at 48 hours of exposure to the label defined dividing cells in cytophotometric-autoradiographic experiments of Section I (Results: A,1a). Data in Section II (Results: A, 2a(1)) showed that this quantity of ^{3}HdT inhibited cellular proliferation.

At 48 hours of exposure, the LI reached a plateau (figure 3). One would predict that the LI would not plateau but would asymptote toward 100% as the dividing (labeled) population increased by proliferation. Cristofalo (Cristofalo and Sharf, 1973; Cristofalo, 1975) considered

several explanations for the plateau: (1) the presence of cycling cells which synthesized DNA at an undetectable rate; (2) the presence of G_2 delayed cells which ultimately entered the proliferating pool; (3) depleted ^{3}HdT or depleted essential nutrients; (4) inhibition of cell division by dT; (5) inhibition of cell division by ^{3}H damage.

With respect to the first explanation, cycling cells could by autoradiographically detected as unlabeled (noncycling) if, for example, the rate of DNA synthesis is very low. This possibility was considered in the discussion of Section I. It is unlikely that unlabeled cycling cells occur at a sufficient frequency to account for the LI plateau.

A similar argument applies to the second alternative, that G_2 delayed cells enter the proliferating pool. Even if all unlabeled 4C cells in older populations are delayed in G_2, they are quantitatively insufficient (figure 1) to account for the plateau in LI.

The third possibility is that cells did not label due to media depletion. Cristofalo (1975) used conditioned media, which contained ^{3}HdT and which was in contact with cells for 30 to 48 hours, to label another culture. The LI was the same as for a sister culture which was exposed to fresh media. Therefore, the third alternative is excluded.

The fourth possibility is eliminated because the low dT concentration (5×10^{-5} mM) does not inhibit cell division. Populations exposed to media supplemented with 5×10^{-8}M unlabeled dT,

and to media supplemented with no additional dT had similar growth rates, which were higher than the ^{3}HdT-labeled population (Cristofalo, 1975).

The final explanation considered is that the ^{3}H could inhibit cell division. Indeed, figure 2 showed that at 48 hours of labeling, the cell number was significantly lower than for the control (no ^{3}HdT), and at 72 hours the cell number reached a plateau. Similar results were found by Cristofalo (1975). These results indicate that cell division is inhibited, by radiation damage, which could account for the LI plateau. Subsequent cell death may also contribute to the LI plateau.

Continuous ^{3}HdT (0.1 μCi/ml, S.A. 2.0 Ci/mM) exposure resulted in increased cellular volume. The modal channel number of Coulter volumes (figure 4 and table 1) was greater for cells grown with ^{3}HdT than for cells grown without ^{3}HdT. This suggested that some cells block in diploid G_2 or tetraploid G_1 and/or had unbalanced growth.

The interpretation that ^{3}HdT blocks cells in diploid G_2 or tetraploid G_1 is supported by the cytophotometric-autoradiographic experiments (figure 1) of Section I. In comparison to the frequency of labeled 2C cells, older cultures had a disproportionate occurance of labeled 4C cells. This may be explained by cells which incorporated ^{3}HdT, and as a result of radiation damage subsequently blocked in diploid G_2 phase. The occurance of labeled 8C cells suggests that they also may be blocked in tetraploid G_1 phase.

Labeling with 0.003 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM)

Uptake of 0.003 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM) at 48 hours of exposure to the label, defined dividing cells in cytophotometric-autoradiographic experiments of Section II (Results: A,2c(1)). This quantity of ^{3}HdT did not perturb cellular growth (Results:A,2a(1)).

Cultures grown with 0.003 μCi/ml ^{3}HdT showed no significant difference in cell number from the control culture (figure 2), regardless of culture age (figures 5, 6 and 7). The Coulter volume distribution (figure 4) and modal channel number (table 1) also were similar for the labeled culture and for the control culture. These data showed that cellular growth was not inhibited by continuous exposure to 0.003 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM).

This ^{3}HdT concentration was used in double label cytophotometric-autoradiographic experiments designed to calculate the CV of DNA values so that. (1) the growth kinetics of cells which labeled were unperturbed and did not distort the data analysis; (2) cells which incorporated low levels of ^{3}HdT during continuous exposure were easy to distinguish autoradiographically from cells which were pulse labeled with high concentrations of ^{3}HdT.

(2) Labeling with high levels of ^{3}HdT

Labeling with 1.0 μCi/ml ^{3}HdT or 5.0 μCi/ml ^{3}HdT (S.A. 2.0 Ci/mM)

Cellular growth was inhibited by continuous exposure to high levels of ^{3}HdT (1.0 μCi/ml or 5.0 μCi/ml ^{3}HdT, S.A. 20.0 Ci/mM). Figures 8 and 10 showed that cell division proceeded for approximately the first 24 hours of labeling, and thereafter the cell number remained constant. At 48 to 72 hours of ^{3}HdT exposure the LI reached a plateau (figures 9 and 11), which further indicated that cell division was inhibited. Subsequent cell death also may have contributed to the LI plateau.

At 48 hours of ^{3}HdT exposure, cell volume (figure 12, tables 2 and 3) was approximately twice the control volume. Further ^{3}HdT exposure resulted in further volume increases. This indicated that cells blocked in diploid G_2 or tetraploid G_1, followed by unbalanced growth, or an increase in ploidy level.

An estimate of the maximum radiation dose to cells at 48 hours of exposure to 20.0 Ci/mM ^{3}HdT is 90rad/minute or a total of 260krad (see Appendix for calculation). This is quite a high dose, which could easily inhibit cell division and cause cell death.

The effects of acute irradiation on progress through the cell cycle are well documented (see review by Altman et al., 1970). The most striking effect in the first postirradiation cell cycle is a delay in G_2 phase. Also, there is a delay in S phase, and there is no effect on M or G_1 phase. The second postirradiation cell cycle is characterized by a decrease in the percent of dividing cells and an increase in the percent of dead and nondividing cells. With

increasing time and radiation dose there is an increase in the fraction of cells which grow without division, or so-called giant cells.

Little attention has been given to the formation and fate of radiation-induced giant cells. It is known, however, that they do not exhibit metaphase and telophase figures, and that they synthesize DNA, RNA and protein. This indicates that these cells do not divide and are endoreduplicated or are polyploid.

A likely sequence of events to have occurred during exposure to high levels of ^{3}HdT is as follows: the cells incorporated ^{3}HdT at S phase; most cells proceeded through the first division, as evidenced by cell number (figures 8 and 10); following a second round of DNA synthesis the cells blocked in G_2 and, as will be shown in figure 14, have 4C DNA contents; judging from Coulter volume distributions (figure 12, tables 2 and 3) the arrested cells continued to metabolize and grow in volume. Cell death was not followed, but at these doses of radiation one can predict essentially zero survival of clonogenic cells. Further experimentation would be needed to determine if this is the actual sequence of events of growth inhibition. For the purposes of this discussion it is sufficient to know that these concentrations of ^{3}HdT inhibit division, cause a plateau in the LI curve, distort DNA histograms, and generally interfere with kinetic analysis.

b. Effect of different methods of slide preparation on LI

The cytophotometric-autoradiographic experiments of Section II were designed to quantitatively evaluate the age-correlated increase in dispersion of DNA contents. Debris associated with aging cultures could lead to errors in cytophotometry and thus, increased dispersion of DNA contents. A method of slide preparation which diminished debris was utilized for the cytophotometric-autoradiographic experiments of Section II. The methods of slide preparation used in Sections I and II were compared for their effect on the LI. The debris most likely results from lysis of dead cells. To diminish debris, DNA measurements were determined on cells which were trypsinized from their growing surface, were devoid of their cytoplasm, and were fixed to freshly cleaned slides. This method of slide preparation effectively removed the debris, and the DNA histograms (figure 13) for cultures of all ages sharpened up; nevertheless, the age-correlated increase in dispersion persisted.

This method, however, is more harsh than the method of slide preparation used in Section I; in Section I DNA measurements were made on nuclei of intact whole cells which were fixed, stained and measured while remaining attached to the slides on which they were grown. Selective loss of either dividing or nondividing cells during treatment was tested by measuring the LI of cells treated according to Section I and Section II.

Table 4 shows that nondividing cells were selectively lost when treated by the method of Section II. Although the loss is slight, it

should be kept in mind in interpretation of the data.

In addition, the method of slide preparation might introduce bias to the data because of the distribution of cells on the slide: for Section I measurements, cells were distributed in the manner which they grew; for Section II measurements, cells were randomly distributed.

C. DNA distribution of dividing and nondividing WI-38 cells

(1) Double labeling with ^{3}HdT

These cytophotometric-autoradiographic experiments were designed to quantitatively evaluate the age-correlated increase in dispersion of DNA contents. One interpretation of the increased dispersion is that it represents aneuploid DNA content which could cause cell arrest. If this hypothesis were true the CV of DNA values would be larger for old cultures than for young cultures, and for nondividing cells than for dividing cells.

Cells were double labeled with ^{3}HdT to identify cycling G_1 and G_2 cells (by incorporation of low levels of ^{3}HdT after continuous exposure), cells in S phase (by incorporation of high levels of ^{3}HdT after a pulse), and noncycling cells (by no ^{3}HdT incorporation). DNA cytophotometry was performed for single, fixed and stained cells, followed by autoradiographic detection of ^{3}HdT uptake for the same cells. The S phase cells were subtracted from the DNA histograms to yield 2C and 4C peaks, and the CV of DNA values for the peaks was calculated.

DNA histograms of figure 13 show progressive changes in DNA content as WI-38 cultures age. The passage 24 culture had a bimodal DNA distribution characteristic of a proliferating diploid population. All cells were cycling. The well defined 2C and 4C peaks were diploid G_1 and G_2 nuclei and the intervening plateau contained S phase nuclei. As expected, the 2C peak was larger than the 4C peak; each G_2 cell becomes two G_1 cells as it passes through the point of division.

The DNA histogram of the passage 55 culture showed age-associated changes in DNA content and proliferative status. The culture was polyploid (8C and 16C nuclei). Nondividing nuclei with 2C DNA were blocked in diploid G_1; nondividing nuclei with 4C DNA were blocked in diploid G_2 or tetraploid G_1; nondividing nuclei with 8C DNA were blocked in tetraploid G_2 or octaploid G_1. The percent of cycling nuclei in S phase was about 50% less than for the young culture.

The passage 62 culture showed similar, but more severe age-associated changes in DNA content and proliferative status. The culture was polyploid. There was a greater fraction of nondividing cells than for the passage 54 culture. Like the passage 54 culture, nondividing cells had 2C, 4C and 8C DNA contents. The percent of cycling nuclei in S phase was only about 15% of the S phase nuclei in the young culture.

These data confirmed earlier cytophotometric-autoradiographic findings (figure 1) that with culture age there was an increased frequency of polyploid cells and of nondividing 2C, 4C and 8C cells. In addition, there was a decrease in the fraction of S phase cells.

The 2C and 4C nuclei of the passage 62 culture had a wider dispersion of DNA values than the passage 55 and passage 24 cultures (figure 13). This was particularly evident for the unlabeled 2C nuclei and the lightly labeled 4C nuclei; and, these peaks were skewed to the left. Possible explanations for the skewed data are: (1) cells synthesize DNA, but do not have a labeling pattern consistent with being in S phase; (2) cells with aneuploid DNA contents are selected for or against; (3) cells form micronuclei which are subsequently lost during the slide preparation.

The first alternative is compatible with recent evidence which suggests that there is a reduction in the rate of DNA replication in aging human fibroblast cultures (Petes et al., 1974). The rate may be low enough that after autoradiography some cells would appear lightly labeled or unlabeled. This could explain the left skew to the lightly labeled 4C peak. As pointed out in the Discussion of Section I, for young and middle-aged cultures, it is unlikely that a significant fraction of cells would go undetected by the autoradiographic methods employed (Cristofalo and Sharf, 1973; Cristofalo, 1975). Further experimentation is required for very old cultures, and lightly labeled or unlabeled S phase nuclei in the passage 62 culture cannot be completely ruled out with the data at hand.

The second possibility involving aneuploid DNA content is supported by karyotypic analysis which showed aneuploidy and other chromosome aberrations (e.g., fragments or translocations) in

proliferating cells of aging WI-38 cultures (e.g., Saksela and Moorhead, 1963). The aneuploid cells were predominantly hypodiploid (Saksela and Moorhead, 1963), which would explain the left skew to the unlabeled 2C and labeled 4C peaks. The predominance of hypodiploid cells infers that there is a selection mechanism operative; either there is selection for hypodiploid cells or there is selection against hyperdiploid cells.

Further evidence associates aneuploidy and other chromosomal aberrations with the aging process. For example, studies showed that the proportion of aneuploid cells in cultures of human leukocytes increased with age (Jacobs et al., 1963; Hamerton et al., 1965). Hypodiploidy, which was more common than hyperdiploidy, was highly correlated with the loss of the X chromosome from female leukocytes and the Y chromosome from male leukocytes. In addition, chromosome and chromatid aberrations were found in the white blood cells of aged persons.

In another study, Crowley and Curtis (1963) scored chromosome aberrations as a function of age in regenerating livers of long-lived and short-lived strains of mice. The development of aberrations was inversely proportional to life expectancy.

Thus, it is reasonable to postulate that one of the effects of aging is a lowering of the mitotic efficiency, leading to lagging of chromosomes or to non-disjunction. This could result in chromosome loss (hypodiploidy) or chromosome duplication (hyperdiploidy). If

these chromosomes were involved in the control of division the cell could cease to divide.

The final alternative considered is that senescent cells form micronuclei and subsequently lose them during slide preparation. A micronucleus is a small nucleus, separate from and additional to the main nucleus. It is formed by lagging chromosomes or chromosome fragments, during telophase.

Senescent WI-38 cultures have an increased frequency of nuclei with unusual shapes and protrusions, as well as nuclei with one or more relatively small DNA containing vesicles. Figure 22 shows an extreme example of a nucleus with such vesicles. For a future study, it would be of interest to quantitate the unusual nuclear shapes and structures with culture age.

Micronuclei could be released from association with the main nucleus during the slide preparation method; the cytoplasm was removed in the method used for Section II. The remaining nucleus would be hypodiploid and there would be a left skew to the DNA data (figure 13). In contrast, the slide preparation method used in Section I did not remove the cytoplasm. DNA measurements were made on intact cells, and the data (figure 1) was not skewed.

Other studies presented evidence that atypical nuclear structures, called nuclear blebs, were associated with imparied DNA synthesis and with aneuploidy. For instance, nuclear blebs were induced in the bone marrow cells of patients treated with 5-fluorouracil, a DNA-inhibiting

Figure 22. Photomicrograph of an aging WI-38 cell with micronuclei. The cell was stained with a 7.5% Giemsa solution.

Figur
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drug (Ahearn et al., 1967). Also, for the bone marrow of leukemic patients there was a positive correlation between nuclear blebs and aneuploidy (Ahearn et al., 1974).

The last two explanations for the skewed DNA data are the most strongly supported alternatives. In fact, it is extremely likely that the two explanations, that is, the occurance of aneuploidy and micronuclei, are directly related; the formation of micronuclei can lead to an aneuploid cell.

For quantitative analysis of the DNA histogram (figure 13), the S phase nuclei were subtracted from the DNA histogram, and the CV of DNA values and the ratio of mean DNA values for the 2C and the 4C nuclei were calculated (see table 5).

As shown in table 5, the young culture had very little dispersion of DNA values for 2C and 4C nuclei. Also, there was precise doubling of mean DNA values for 2C to 4C nuclei. These data suggested that this culture was euploid. Furthermore, these data indicated that the specimen was well behaved and that there was stoichiometry of staining.

The passage 54 culture had essentially the same CV of DNA values as the young culture. Once again, there was an approximate doubling of mean DNA values for 2C to 4C nuclei. Furthermore, nondividing cells had the same mean and CV of DNA values as dividing cells. These data indicated that the culture was euploid. In addition, the specimen was well behaved and there was stoichiometry of staining.

Furthermore, these data indicated that, for this culture, aneuploidy was not associated with cell arrest.

For the oldest culture, the quantitative changes in dispersion of DNA values are more difficult to interpret. Except for the labeled 2C nuclei, the passage 62 culture had a CV of DNA values which were more than two fold larger than the passage 55 and passage 24 cultures. The significance of the increased CV for the passage 62 culture is uncertain because the 2C and 4C peaks were skewed. Possible explanations for the skewed data were previously discussed.

In summary, cytophotometry-autoradiography (figures 1 and 13) revealed that the majority of nondividing cells in aging WI-38 cultures had 2C DNA contents and were in diploid G_1 phase. Nondividing cells also had 4C DNA and were in diploid G_2 or tetraploid G_1 phase. In addition, aging cultures had polyploid (8C and 16C) dividing and nondividing cells.

Quantitative analysis (table 5) of the cytophotometric-autoradiographic data showed that for aging cultures, G_1 and G_2 cells had a wider spread of DNA values than for young cultures. The most likely source of the age-associated increased CV of DNA values, is an increased frequency of aneuploid cells and of cells which lost micronuclei during slide preparation. In general, nondividing cells had the same CV of DNA values as dividing cells. These results suggested that aneuploid DNA content is associated with the aging process, rather than causally related to the loss of proliferative capacity.

(2) Labeling with high levels of ^{3}HdT

In these cytophotometric-autoradiographic experiments the cycle stage was identified for cells which were continuously exposed to high levels of ^{3}HdT (20.0 Ci/mM). Experiments already discussed showed that cellular growth (figures 8-12, tables 2 and 3) was inhibited at 48 hours ^{3}HdT exposure, consistent with a radiation effect. Labeled nuclei ceased cycling after the addition of ^{3}HdT, and were termed blocked dividing cells. Unlabeled nuclei ceased cycling before the addition of ^{3}HdT, and were termed nondividing cells.

DNA histograms of figure 14 showed that for the young culture all the nuclei were labeled and therefore were blocked dividing. There were no unlabeled or nondividing nuclei. Virtually all of the nuclei had 4C DNA; they could be G_2 diploid or G_1 tetraploid cells. As previously discussed, a striking effect of radiation is G_2 delay. These cells markedly increase in volume (figure 12, tables 2 and 3), and could also be radiation-induced giant cells; this is, cells which do not divide and which synthesize DNA, RNA and protein. However, S phase polyploid nuclei would be expected and this did not occur. The young culture had a relatively high frequency (9%) of polyploid 8C nuclei. There were no S phase polyploid nuclei, therefore it is unlikely that ^{3}HdT induced the formation of 8C nuclei. On the other hand, it is likely that ^{3}HdT blocked 8C cells and thereby enhanced their frequency, analogous to the way that Colcemid blocks mitotic cells and thereby enhances their frequency.

The DNA histogram of the labeled nuclei for the old culture is strikingly similar to the young culture. Virtually all of the labeled nuclei had 4C DNA. Also, there were labeled polyploid 8C and 16C nuclei. Most unlabeled nuclei had 2C DNA contents and were blocked as diploid G_1. In addition, there were unlabeled 4C nuclei, which were diploid G_2 or tetraploid G_1, and there were unlabeled 8C nuclei, which were tetraploid G_2 or octaploid G_1.

In summary, continuous exposure to high levels of ^{3}HdT inhibited progression through the cell cycle for young and old cultures. Nuclei which incorporated these high levels of ^{3}HdT had, almost exclusively, 4C DNA. It is likely that these cells accumulated in G_2 phase consistent with a radiation effect.

B. Premature Chromosome Condensation-Autoradiographic Studies (Section III)

Premature chromosome studies were utilized (1) to determine whether nondividing cells could condense their chromatin; (2) to determine the stage of the cell cycle in which these cells were arrested; (3) to determine the ploidy level of the nondividing cells.

These studies showed that senescent dividing and nondividing cells were able to condense their chromatin to form PCC. Furthermore, there was no difference in the percent of PCC induced for middle-aged or old WI-38 cultures, i.e., dividing cells appeared to induce as readily as nondividing cells.

The data in table 7 confirmed the cytophotometric-autoradiographic data (figures 1 and 13) which showed that the subpopulation of nondividing WI-38 cells increased with culture age and that the majority of these cells were diploids arrested in G_1. The cytophotometric data also revealed that with increasing age there was an increased fraction of nondividing cells with 4C DNA contents, i.e., either G_2 diploid or G_1 tetraploid cells. There is some evidence for the existence of a G_2 delayed subpopulation in aging WI-38 cultures (Macieira-Coelho et al., 1966) as well as in other aging cell systems (Gelfant and Smith, 1972); however, no unlabeled prematurely condensed chromosomes with typical G_2 configurations were observed in this study.

Table 7 further showed that the percentage of nondividing cells induced into PCC and the percentage of nondividing cells present in the parent population did not differ significantly. However, the elimination of S phase cells from this calculation in the PCC population could well be obscuring a deficit of unlabeled cells; hence, there is a possibility that unlabeled G_2 cells have gone undetected by this technique.

For example, the lack of unlabeled G_2 cells with PCC could be accounted for if G_2 cells were not induced into PCC with a frequency proportionate to G_1 cells. Johnson and Rao (1970) reported that in HeLa cells PCC was induced more readily in G_1 nuclei than in G_2 nuclei. Apparently this was not the case for dividing WI-38 cells in

culture A, since both labeled G_1 and G_2 cells with PCC were present in the frequencies expected (table 8). In culture B the total frequency of G_2 cells declined in comparison to culture A (table 7). Cytophotometric-autoradiographic evidence (figure 1) indicated that the number of unlabeled 4C cells present in aging cultures is small. Thus the probability of observing unlabeled G_2 cells in these cultures is low. However, the number of unlabeled G_1 cells induced into PCC was in agreement with the expected values (table 8). There is no reason to expect that unlabeled G_2 cells would differ unless they were blocked due to a defective mechanism for chromosome condensation.

The anomalous PCC configuration shown in figure 20 was observed in three unlabeled cells; approximately 92 chromatids appeared to be homologues paired at the centromere and widely separated along the arms. Two additional unlabeled cells with approximately a diploid number of prematurely condensed chromatids were paired at the centromere. It is possible that these two cells are, in some unexplained manner, G_1 diploids or G_2 haploids. The cells with 92 chromatids may represent arrested G_2 cells with atypical sister chromatid pairing or tetraploid G_1 cells with homologue pairing. This PCC homologue configuration is unlike that reported for G_2 cells by Johnson and Rao (1970) where typically the sister chromatids lie in juxtaposition along their entire length. The mechanism for the pairing that was observed presently eludes explanation. Such an anomalous configuration might be envisioned as a preliminary step to

endoreduplication. However, since it was observed in nondividing cells only, the pairing phenomenon is more likely the result of a faulty mechanism for chromosome contraction (which may result in a diploid G_2 block) or the result of an aborted mitosis (which may result in a tetraploid G_1 block.

In conclusion, all premature chromosome condensations from nondividing cells that were unambiguously classified according to descriptions given by Johnson and Rao (1970) were of the G_1 type. It is difficult to collect a large number of cells for good statistical analysis, but the technique of PCC proved to be powerful in that it revealed a cell type which heretofore could not be identified, the nondividing G_1 tetraploid. In addition, the chromosomes of nondividing cells may now be analyzed for structural and numerical aberrations and so provide a useful tool for the investigation of the senescence phenomenon in cell culture.

C. Is Finite Cellular Life-Span In Vitro Related to Aging In Vivo?

A salient feature of aging in vivo is the decreased ability to respond to stress or stimuli. One theory of aging is that the decline in adaptability renders the animal more suceptible to death as the result of loss of cellular proliferative capacity. This does not imply that finite cellular life-span is the single causative event responsible for aging. Senescence in vivo is a highly complex and poorly understood process. It involves intercellular and

intracellular factors, and it involves different kinds of cells, tissues, and organs, as well as various physiological processes. Most likely, the causative events responsible for aging are multiple and interrelated. I will provide evidence that there is a positive correlation between the loss of proliferative capacity and aging, both in vivo and in vitro. Whether this relationship is cause or effect remains unknown.

Tissue culture provides an opportunity to study isolated cells in a controlled environment. This is extremely useful in extricating the factors involved in cellular aging. Of course, it should be kept in mind that these cells are in an artificial environment and that direct extrapolation from in vitro studies to in vivo aging may be unwarranted. Nevertheless, in vitro studies can yield an immense amount of knowledge of aging at the cellular level.

Karyotypically normal mammalian cells, regardless of tissue origin, have a finite life-span in vitro. Rare exceptions (e.g., rodent cell cultures, Petursson et al., 1964; Krooth et al., 1964; and human lymphoblastoid cells, Moore and McLimans, 1968) occur, but the normality of these cells is questionable; for example, human lymphoblastoid cell lines typically contain herpes-type virus (Levy et al., 1968), and minor chromosome anomalies (Huang and Moore, 1969). Immortal cells are karyotypically abnormal and usually have cancer-like properties. Paradoxically, this suggests that for mammalian somatic cells to become biologically immortal, they first

must be induced to the neoplastic state. Human diploid cells *in vitro*, strain WI-38, have been transformed to immortal heteroploid cells after infection with the tumor virus, SV_{40} (Giradi *et al.*, 1965). This suggests that the normal diploid karyotype, rather than the artificial environment of tissue culture, places a limit on cellular life-span.

To date, Hayflick and Moorhead (1961) have presented the most convincing evidence that the finite life-span of human diploid cells *in vitro* is not due to inadequate nutrition, technical difficulties, toxic metabolic products or microcontaminants. Mixtures of young and old populations (male and female), distinguishable by karyotypic markers, (Barr bodies) were grown in the same culture vessel. The older population ceased to divide when it reached approximately 50 doublings. The younger population continued to proliferate until it too reached approximately 50 doublings. This indicated that the finite life-span of human diploid cells *in vitro* is intrinsically controlled.

The loss of proliferative capacity *in vitro* declines with age *in vivo*. Hayflick (1965), Goldstein *et al.* (1969) and Martin *et al.* (1970) showed a negative regression for the number of population doublings the culture attained as a function of the age of the donor.

In other experiments, cells cultured from patients with progeria or with Werner's syndrome (which are diseases associated with premature aging) showed a striking reduction in proliferative capacity

(Goldstein et al., 1969; Martin et al., 1970). For example, skin fibroblasts from a nine year old boy with progeria underwent only two population doublings in vitro. On the other hand, age matched control cultures were capable of 20-30 population doublings (Goldstein, 1969). Thus, proliferative capacity in vitro reflects both chronological and biological age in vivo.

As mentioned, the aging organism has a decreased response to stress or stimuli. A decreased response to stimulation of cell division with age, has been demonstrated for tissues as different as salivary glands (Adelman et al., 1972) and the immune system (Williamson and Askonas, 1972; Price and Makinodan, 1972).

Adelman et al., (1972) administered isoproterenol to various aged rats to initiate a tissue specific burst of DNA synthesis and cell division for salivary glands. From two to at least 24 months of age, the time required to initiate DNA synthesis (measured by ^{3}HdT incorporation) increased linearly. For example, for two month old rats, DNA synthesis initiated at 30 hours after isoproterenol administration, whereas for 12 month old rats DNA synthesis initiated at 42 hours. In addition, there was a marked reduction with age in the magnitude of DNA synthesis and cell proliferation.

The immune system is less responsive to stress or stimuli with age. For instance, older animals are more vulnerable to infections and are more difficult to immunize effectively. The decline in the immune response is related to a decline in proliferative capacity of

the "bursa"-derived B-cells and the thymus-derived T-cells. The T-cells are antigen specific helper cells which stimulate the B-cells to synthesize antibody.

Williamson and Askonas (1972) indicated that there was a limit to the number of divisions of antibody-forming cell clones under antigenic stimulation. They serially transferred single clones of antibody-producing cells to mice whose immune system had been destroyed by heavy irradiation. At each transfer, antigen was injected and clonal activity was recognized by the production of characteristic antibody. Continuing serial transfer showed a loss of antibody production, which indicated that the antibody-producing cell clones had a finite life-span.

By measuring the amount of antigen necessary for maximum antibody production, Price and Makinodan (1972) determined that the immune response declined by about 10 fold in old mice, when compared to young mice. The kinetic details from such dose-response experiments further indicated that this effect was due to a marked decline in proliferative capacity of T- and B-cells from old mice.

Serial transplanation experiments gave additional evidence that cells in vivo have decreased proliferative capacity with age. These experiments also approached the question of whether the loss of proliferative capacity is truly intrinsic to the cell, or is dependent on environmental influences of the old animal. Designated somatic tissues were serially transplanted into new, young, inbred hosts each time the recipient approached old age. In general, normal somatic

cells (e.g., hematopoietic cells, Siminovitch et al., 1964 and Cudkowicz et al., 1964; mammary cells, Daniel, 1972) serially transplanted to young hosts clearly showed a decline in proliferative capacity with cell age. This indicated that the loss of proliferative capacity is intrinsic to the cell.

Of these studies, the most comprehensive serial transplantation study was of mouse mammary tissue (Daniel, 1972). Mammary tissue was transplanted to inguinal fat pads of three week old mice, from which the mammary rudiment was surgically excised. Primary transplants grew for two to three months, until they filled the fat pad. With serial transplantation of the mammary tissue there was an approximately linear decline in growth potential, measured as the percentage of the fat pad filled in a fixed time interval (Daniel et al., 1968). This decline in growth potential could not be due to the trauma of subcultivation because the number of successful transplants did not decline with increasing subcultivation.

In contrast, precancerous mouse mammary cells, transformed spontaneously or by chemicals, showed unlimited growth potential when serially transplanted in vivo (Daniel et al., 1975). This contrast parallels the finite life-span of normal diploid WI-38 cells and the unlimited growth potential of SV_{40} transformed WI-38 cells.

Further studies of mouse mammary tissue presented more evidence for intrinsic control of proliferation capacity (Daniel and Young, 1971). As mentioned, a primary transplant of mammary tissue, fills

the fat pad in approximately two to three months. Subsequently growth ceases, but the cells still metabolize. Mammary tissue was transplanted at two subcultivation intervals. One line was transplanted at three month intervals, and the tissue proliferated almost continuously. The other line was transplanted at 12 month intervals. During most of the 12 month interval the tissue was metabolically active and did not proliferate. The tissue transplanted at three month intervals declined more rapidly in growth potential than the tissue transplanted at 12 month intervals. This indicated that proliferative potential is intrinsically controlled, since the number of cell divisions, rather than the passage of "metabolic" or calendar time, places the limit upon cellular life-span.

Further support for these concepts came from a different approach (Daniel and Young, 1971). Ductal outgrowths arising from mammary transplants proliferate by apical addition; the center of growth experiences relatively few divisions, whereas the periphery of the outgrowth is the site of intense mitotic activity. Two transplants lines, one from the center of the outgrowth and the other from the periphery, were serially propagated. The transplant from the periphery of the outgrowth, which had more cumulative cell divisions, declined more rapidly in growth potential than the transplant from the center of the outgrowth. This is further proof that there is a cellular program which places an ultimate limit on the possible number of cell divisions in vivo, and hence on cellular life-span.

This concept is supported by comparable data obtained in vitro (Dell' Orco et al., 1974; Dell' Orco, 1974). WI-38 cells were maintained in a metabolically active, nonmitotic state by reducing the serum concentration of the growh medium from 10% to 0.5%. At various time periods, the nonmitotic cells were returned to the proliferating state by subcultivation with growth medium containing 10% serum. These cells were capable of an equivalent number of cell divisions, in a proportionately longer calendar time, as controls that had been continuously cultured on growth medium. Thus, the number of divisions, and not the "metabolic" or calendar time, places the limit upon cellular life-span.

In summary, these experiments showed that with age there is a loss of proliferative capacity in vitro and in vivo. Furthermore, the loss of proliferative capacity appears to be intrinsically controlled. The role of specific tissues in this phenomenon will now be discussed.

D. Loss of Proliferative Capacity for Different Tissue Types

Senescence in vivo is a complex process which involves different tissue types. In terms of proliferative capacity, Messier and Leblond (1960) designated adult mammalian tissues as renewing, expanding, or static, according to whether they undergo cell division to replenish lost cells, resort to mitosis only to augment a population of permanent cells, or lack the capacity of mitosis altogether. I will provide evidence that with age, there is a decline of proliferative capacity or of cell number for all types of tissue.

Interpretation of the literature in this area is complicated by several factors: 1. The literature is scarce and few studies are well documented. 2. All tissues within a specific classification (e.g., renewing tissue) did not exhibit a decline of proliferative capacity or of cell number with age. 3. Some tissues (e.g., nervous tissue) declined in proliferative capacity or in cell number for some animals and not others. Of course, the last two factors do not discredit age-correlated proliferative loss. Some authors (e.g., Kohn, 1975), however, have rejected aging theories which involve loss of proliferative capacity because of the negative examples. Nevertheless, even a conservative assessment of the existing literature clearly indicates that there is a loss of proliferative capacity and cell number for some cells in all tissue types

1. Renewing tissue

Renewing tissues (e.g., intestinal and skin epithelium, hematopoietic tissues) have a proliferating compartment of immature stem cells which gives rise to nonproliferating differentiated cells. With time, the differentiated cells die. The stem cells replenish the dead cells and maintain the population in a steady state.

The population dynamics of WI-38 cells are most similar to renewing tissues in vivo. The major difference is that WI-38 cells are without in vivo feedback controls and are subcultivated repeatedly; hence, they continuously proliferate rather than maintain a steady state.

With age, the stem cells of some renewing tissues (see below) have an increased cell cycle time and the number of stem cells declines. Decline in cell renewal would seem to lead to impaired tissue function and a decreased response to stress or stimuli. There is not a complete loss of proliferative activity as with WI-38 cells. This may be because cells in vivo are under homeostatic controls and do not realize their full potential for division and, therefore, age slower. This implies that if an organism lived long enough, the stem cells of renewing tissues would have a complete loss of proliferative capacity.

Few studies exist for humans but there are numerous animal studies showing a decline in proliferative capacity with age for various renewing tissues (see review by Buetow, 1971, and Gelfant and Smith, 1972). The mouse was the most extensively studied animal. Cell cycle kinetics of mouse tissues that were examined showed that virtually all renewing tissues had an increased cell cycle time, primarily due to lengthening of the G_1 phase. In addition, there was a decreased number of cells in the proliferating compartment. Examples of mouse tissues that were examined are: crypt cells of the colon (Thrasher, 1971) and of the duodenum (Lesher and Sacher, 1968); the esophogeal ephithelium (Thrasher, 1971); the epidermis of the ear and hindpaw (Cameron, 1972); the epithelium on the ventral surface of the tongue (Cameron, 1972). Ear epidermis also had a progressive increase of noncycling G_2-blocked cells, from 2.5% for two week old mice to 12.0% for 30 month old mice (Pederson and Gelfant, 1966).

As discussed previously, there is a decline in the immune response with age. In addition to the studies mentioned (Williamson and Askonas, 1972; Price and Makinodan, 1972), Davis and coworkers (1971) showed that there was a progressive decline of functional stem cells of mouse bone marrow from middle-age to death. Quantitation of functional stem cells was determined by the ability of bone marrow to give rise to spleen modules of hemopoiesis when transplanted into lethally irradiated, young adult, syngeneic recipients.

2. Expanding tissue

Expanding tissue (e.g., liver tissue, kidney tissue, many endocrine and exocrine tissues) primarily consists of G_0 cells; typically the cells are nonproliferating, but the retain the capacity for division. Such G_0 cells in expanding tissues are long-lived and turnover slowly, whereas the bulk of parenchymal cells of renewing tissues are short-lived and are constantly being replaced.

Some investigators (e.g., Curtis, 1963) have proposed that the aging phenomenon is most likely caused by loss of functional capacity of organs containing cells which either rarely divide (expanding tissue) or are incapable of division (static tissue), since the defective cells will be retained indefinitely.

Liver is readily induced to proliferate after partial hepatectomy. Marshak and Byron (1945) found that age increasingly delayed the initiation of mitosis in regenerating liver. Bucher and her coworkers (1964) discovered that the increasing delay in mitosis was related to an increasing delay in the initiation of DNA synthesis. For weanling

rats, the rate of DNA synthesis reached a peak approximately 21 hours after partial hepatectomy. Young adults (4 months old) had maximum synthesis 3 hours later than the weanlings, and older rats (12-15 months) lagged 8 to 12 hours behind the weanlings. At 40 to 50 hours there was no difference in DNA synthesis for old rats and young adults. With age, there was also a loss of synchrony of DNA synthesis. These results showed that the initial proliferative response after hepatectomy was progressively delayed with increasing age.

For the kidney, the only way growth can occur is by increasing the lengths and diameters of nephrons. This involves both hypertrophy and hyperplasia of the tubular cells. Unilateral nephrectomy stimulates hypertrophy and hyperplasia in cells of the proximal convoluted tubules of the remaining kidney. McCreight and Sulkin (1959) showed that for control and nephrectomized rats, the MI varied inversely with age. Control senile (38 month old) rats had a three fold lower MI than control young (4 month old) rats. Nephrectomized senile rats had a five fold lower MI than nephrectomized young rats.

The lung is an organ capable of partial growth. To some degree, the ability to form new functional units, or alveoli, persists into adult life, but this ability disappears with age. In compensatory hypertrophy following removal of lung tissue there some evidence for new alveolar formation in young animals. In contrast, older animals simply showed dilatation of the existing functional units (Bremer,

1937; Longacre et al., 1940). It is probable that the decline in the capacity of older animals to develop new alveoli would restrict their ability to respond to increased respiratory demand.

3. Static tissue

Static tissues (e.g., nervous tissue, striated muscle fibers) are so fully differentiated, they can no longer divide. The cells have the potential to live as long as the organism survives. However, cells which are lost are irreplacable and loss of essential cells may render the animal susceptible to death.

There are numerous reports of an age-correlated decline in the number of nerve cells and fibers for humans, but only a few reports for other animals (see review by Buetow, 1971). For example, with age there is a marked loss of cells from the superior temporal gyrus and the post central gyrus of the human cerebral cortex (Brody, 1955). Further documentation of these experiments by new counting techniques would be desired, because there are wide variations in the number of nerve cells and of fibers under normal conditions.

The regeneration of nerve fibers declines with age. Drahota and Gutmann (1961) showed that there was marked delay in axonal growth of crushed motor nerves for old rats. Loss of nerve cells or decline in regenerative ability could clearly lead to decreased responsiveness for the aging organism.

In summary, for some cells of all tissue types there is a positive correlation for loss of proliferative capacity with age. It is

important to remember that biochemical, vascular and other physiological decrements which occur well before loss of proliferative capacity, could be the salient variables that cause aging. Clearly, further experimentation is required to determine whether proliferative loss is an etiological or a pathogenic factor of aging in vivo.

E. Possible Mechanisms of Finite Cellular Life-Span In Vitro

There are two basic schools of thought, programmed senescence and random senescence, to account for the finite proliferative capacity of cells in vitro. The first school of thought infers that there is a program or a "biological clock" with a fixed number of cell divisions. The second school of thought infers that the accumulation of damage from random events limits cellular life-span.

From the two schools of thought, branch a myriad of aging hypothesis, tantamount to a review of contempory molecular biology. For this discussion it would not be feasible, nor appropriate, to review the literature on aging theories. I will briefly discuss the most popular theory of each school of thought; (1) that there is a genetic program with a fixed number of cell divisions (programmed senescence) (Hayflick and Moorhead, 1961; Hayflick, 1965), (2) that random errors in protein synthesis lead to, after many divisions, a lethal "error catastrophy" (random senescence) (Orgel, 1963).

Programmed senescence. Since different species have characteristic and inheritible life-spans, it follows that aging is under genetic

control. At the cellular level there is some evidence for a positive correlation between the mean life-span of a species and the number of population doublings the embryonic fibroblasts can attain in vitro (Hayflick, 1975).

Programmed cell death is the usual method of eliminating organs and tissues that are useful only in the larval or embryonic stages of many animals, including mammals [e.g., the pronephros and mesonephros of higher vertebrates, the tail and gills of tadpoles, and larval insect organs (Saunders, 1966)]. A similar kind of programmed cell death may explain aging in vitro.

Numerous well documented experiments already described support the theory that cellular life-span is programmed. These experiments provided evidence that the life-span of diploid cells is related to the number of cell divisions rather than to chronological time (e.g. Hayflick and Moorhead, 1961; Hayflick, 1965; Daniel et al., 1968; Daniel, 1972; Daniel and Young, 1971; Dell'Orco et al., 1974; Dell'Orco, 1974).

Random senescence. In 1963, Orgel proposed the "error catastrophe" theory for cellular aging. He suggested that random mistakes in amino acid sequences of proteins, especially enzymes concerned with the processing of genetic information (e.g., polymerases), will result in further mistakes in protein synthesis. Consequently, there is a self-propagating accumulation of errors which leads to cellular deterioration and death. Recently, the original theory was modified

to include the possibility that DNA damage also may account for the initial error frequency (Holliday and Tarr , 1972; Orgel, 1973).

The error theory is supported by experiments which showed that certain environmental treatments altered the life-span of diploid cells. Fetal lung fibroblasts, strain MRC-5, were grown at 40°C. The growth rate was normal, nevertheless, there was a 50% reduction in life-span (Thompson and Holliday, 1973). Shifting the cultures to 37°C did not lead to recovery. In another experiment (Holliday and Tarrant, 1972), MCR-5 cells were grown in nontoxic concentrations of 5-fluorouracil, which incorporates into RNA and reduces the fidelity of transcription and/or translation. These cultures also had a significantly shortened life-span.

Chronic administration of low doses of ionizing irradiation shortened the life-span of chick embryo fibroblasts in vitro (Lima et al., 1972) and whole animals (see review by Curtis, 1963). It has been suggested that irradiation induced chromosomal aberrations and accelerates the accumulation of errors. The interpretation of such experiments, however, is complicated by accompanying disease due to radiation. In addition, shortening the life-span by environmental treatments does not prove the error theory; such treatments also may damage the clock mechanism.

The error catastrophe theory is further supported by experiments which showed that certain proteins [glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Holliday and Tarrant, 1972), and lactic dehydrogenase (Lewis and Tarrant, 1972)] were altered in aging

MRC-5 cultures. These data further showed a decreased enzyme specificity in aging cultures. However, this does not establish that protein synthesis is inaccurate. It also could reflect accumulation of post-syntheticaly modified proteins in old cells.

A major prediction of the error theory has not been fulfilled; viruses grew in senescent WI-38 cells as effectively as in young cells, and they did not contain defective proteins or have an increased frequency of mutations (Holland et al., 1973; Tomkins et al., 1974). Viruses use the cells own machinery for synthesizing proteins, therefore these experiments infered that old cells maintained accuracy of their protein synthesizing machinery.

Recently, Ryan et al. (1974) also presented evidence against the error theory. WI-38 cells grown in the presence of nontoxic concentrations of amino acid analogs, p-fluorophenylalanine and ethionine, had a life-span similar to the control. Higher concentrations did inhibit proliferation, but if the cells were placed in fresh analog free medium, they recovered and achieved the same number of population doublings as the control.

Clearly further studies are needed before either programmed senescence or random senescence can be proved or disproved. I believe that the existing evidence is in favor of programmed aging, but I also believe that further experimentation will prove that the two schools of thought are inextricably interdependent.

F. A Profile of the Nondividing Cell in Aging WI-38 Cultures

Knowledge of the biochemistry and etiology of nondividing cells in aging human diploid cultures is nearly nonexistent. The existing biochemical information (see review by Cristofalo, 1972) for mass populations of aging WI-38 cultures cannot be directly applied to nondividing cells, since the cultures are "mixed populations" which contain dividing and nondividing cells. The approach utilized in this investigation does allow one to study nondividing cells directly. The only other individual cell study reported (Deamer and Gonzales, 1974) showed that nondividing cells accumulate lysosomes containing "age pigment" or "lipofuchsin".

Individual cell studies reported in this dissertation defined the following properties of nondividing cells: (1) Diploid and tetraploid cells cease to cycle in the G_1 phase. (2) Most nondividing cells are diploid. (3) With age, there is an increased frequency of nondividing cells with polyploid (tetraploid) and aneuploid DNA contents. (4) Most likely, measurable aneuploidy does not cause cell arrest. (5) Nondividing cells have the capacity to condense their chromatin and form chromosomes as readily as dividing cells. (6) The chromosomes of some nondividing cells in aging cultures have anomalous chromosome configurations; chromatids show apparent homologous pairing at the centromere regions and exhibit wide separation along the arms.

The work presented in this dissertation was undertaken to identify the cell cycle phase of nondividing aging human cells in culture.

Biophysical and cytological techniques were utilized, in combination, on individual cells. The results show that nondividing cells, diploid or tetraploid, are in the G_1 phase. This is a step in the expansion of our understanding of the senescence phenomenon in cell culture.

CONCLUSIONS

Cytophotometric-Autoradiographic Studies, Section I

(1) With age, there is a progressive increase in nondividing WI-38 cells relative to dividing cells.

(2) The majority of nondividing cells have 2C DNA contents and therefore are arrested in diploid [illegible] se.

(3) Nondividing 4C cells appear increasingly with the age of the culture. These may be cells arrested in diploid G_2 or tetraploid G_1 phase.

(4) The frequency of dividing and nondividing polyploid (8C) cells increases with culture age.

(5) Older cultures have a wider dispersion of DNA values than younger cultures.

Cytophotometric-Autoradiographic Studies, Section II

(1) Continuous exposure to high levels of ^{3}HdT inhibits WI-38 cell cycle traverse: the cell number and LI curves reach a plateau; the cell volume increases; the cells accumulate with 4C DN[illegible]ontents and it is likely that they block in G_2 phase. This patter[illegible]s consistent with a radiation effect.

(2) Cytophotometric-autoradiographic experiments using double labeling to identify S phase cells, confirm the age-correlated observations of Section I: there is a progressive increase in the

frequency of nondividing cells, which predominate in the diploid G_1 phase; there is an increased frequency of nondividing 4C cells; there is an increased frequency of dividing and nondividing polyploid (8C and 16C) cells. In addition, there is a decrease in the fraction of S phase cells.

(3) These double label cytophotometric-autoradiographic experiments also permit quantitation of the dispersion of DNA values. These data show that the CV of DNA values for 2C and 4C cells increases with age, and, in general, that the CV of DNA values is similar for dividing and nondividing cells. Thus, there is a positive correlation with increased CV of DNA values and cell age. Furthermore, it appears that there is not a causal relationship for measurable CV of DNA values and cell arrest. The increased CV of DNA values is consistent with the interpretation that there is an increased frequency of aneuploid dividing and nondividing cells in aging cultures.

Premature Chromosome Condensation-Autoradiographic Studies, Section III

(1) Nondividing WI-38 cells appear to condense their chromatin to form PCC as readily as dividing cells.

(2) The efficiency of PCC induction is independent of phase in the cell cycle (G_1 or G_2) as shown by comparison of observed frequencies with expected frequences for middle-aged cultures.

(3) Ninety-six percent of the nondividing cells, diploid or tetraploid, induced into PCC have single chromatids and are therefore

blocked in the G_1 phase.

(4) The remaining 4% of the nondividing cells have anomalous PCC configurations; chromatids show apparent homologous pairing at the centromere regions and exhibit wide separation along the arms. These may be G_2 cells with atypical sister chromatid pairing or G_1 cells with homologue pairing.

(5) Typical G_2 configurations (double chromatids which pair along the entire arm length) were observed only among dividing cells.

(6) These data indicate that nondividing cells with 4C DNA contents shown in the cytophotometric-autoradiographic experiments of Sections I and II are arrested in tetraploid G_1 phase, rather than in diploid G_2 phase.

APPENDIX

Calculation of Dose (Rad/Cell) from ^{3}HdT

Cleaver and his coworkers (1972 and personal communication) have calculated the equivalent dose of rad/cell from ^{3}HdT incorporation:

Assume that the diploid DNA content is approximately 6×10^{-6} µg/cell, therefore, in exponential gorwth the average DNA content is 9×10^{-6} µg/cell; and that the number of thymines per cell in exponential growth is approximately 3.6×10^{9} t/cell.

Let the S.A. of the thymidine supplied equal S Ci/mM. Since 30 Ci/mM equals 1 ^{3}H atom/thymidine, then the number of ^{3}H atoms per thymidine molecule is $(S/30)^{3}$H/T.

If all the DNA thymidine comes from exogenously supplied thymidine (i.e., when the concentration of thymidine in the medium exceeds approximately 10^{-6}M), then after one cycle of DNA replication (one strand substitution) the number of ^{3}H atoms per cell is $(3.6 \times 10^{9}/2)$t/cell $\cdot$ $(S/30)^{3}$H/T
$= 6S \times 10^{7}$ ^{3}H/cell

The ^{3}H decay rate is 10^{-7} per ^{3}H atom per minute, therefore the decay rate in cells labeled with ^{3}HdT is 65 dpm/cell. One ^{3}H decay is equivalent to 0.5rad x-rays (Cleaver et al., 1972) therefore the dose rate delivered to cells equals 3S rad/min.

After two cell cycles (one and one-half strand substitution) the dose rate delivered to the cells is 4.5(S)rad/min. Thus, at 48 hours of exposure to 20 Ci/mM ^{3}HdT the dose rate is 90rad/min. This dose rate may be an overestimate, since the thymidine concentration approximately equals $0.2 \times 10^{-6}M$, and it is estimated that all the DNA thymidine comes from exogeneously supplied thymidine when the concentration exceeds approximately $10^{-6}M$.