THE ROLE OF HISTONE DURING AGING *IN VITRO*

by

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INTRODUCTION

A characteristic property of all matter which comprises the universe is that it undergoes a process referred to as aging. This process is an irreversible one, therefore investigations in this area are concerned primarily with changes induced by time, or studies which try to prolong the life of the material. Prevention of this process is impossible; only the achievement of slowing the effects of aging by artifical means (e.g., cryogenics), or understanding the cause and result of this process can be accomplished at present. It is hoped that elimination of adverse effects of aging may become possible.

The study of aging has great importance in biology for obvious reasons. Studies involving animal or human subjects are directed along two main routes: the in vivo and in vitro approaches. Since many factors interact in the animal, the simplest approach to the study of aging is studying changes in individual cells with time.

Tissue culture cells offer an ideal system for studying aging at the cellular level. The relatively definitive environment and cell population, ease of handling, and more important, the fact that cells in vitro undergo changes that have been suggested to parallel aging in vivo are some important considerations that make tissue culture an ideal tool for aging studies.

Senescence *in vitro* is characterized by a gradual loss of cellular division with increase in time in culture. Whether this is a reflection of suboptimal environmental conditions or inherent within the cell is debatable. The final result, that is cessation of division, and a possible explanation of this phenomenon is the direction to which this thesis is oriented.

LITERATURE REVIEW

Cultivation of animal cells in an artifical medium was initiated successfully in 1907 by Ross Harrison when he explanted fragments of tissue from the medullary tube region of frog embryos to lymph clots. When kept under aseptic conditions the tissue survived for many weeks and axons grew out from the cells.

The "traditional" techniques of cell culture were then established. Burrows (1910), studying with Harrison, introduced the use of a plasma clot in place of a lymph clot and made the discovery that embryo extract had a strong growth promoting effect. Further development of cell culture technique was very slow, however.

In the early 1940's, addition of antibiotics to tissue cell culture media in concentrations high enough to eliminate contaminating microorganisms obviated the traditional aseptic procedures and allowed many investigators to enter the field. Development of more complex media, initiation of new cell cultures, introduction of new techniques such as cloning, methods for detection of plating efficiency, karyology and many more advancements allowed the field to expand to other areas of interest. Application of cell culture to virology, vaccine production and cell biology has enhanced advancement of knowledge in these areas.

From the initiation of cell culture to the present time however, a serious drawback to many applications has been the finite life span of diploid cells in vitro. This limitation was first noted by Carrel and Ebeling (1921) who observed that the rate of cell multiplication varied inversely with the age of the plasma added to the medium. Since that time, limited proliferation in vitro has been observed in chick cells (Cohn and Murray, 1925; Harris, 1957; Melendez and Hanson, 1964; Hay and Strehler, 1967), and in rabbit fibroblasts (Haff and Swim, 1956), Chinese hamster fibroblasts (Ryan, 1967), mouse embryo cells (Todaro and Green, 1963) and various tissues of human origin (Erlichman, 1935; Bell and Johnson, 1956; Henle and Deinhardt, 1957; Chang, 1961; Hayflick and Moorhead, 1961).

Hayflick and Moorhead (1961) have divided the life span of diploid cells in vitro into three phases. Phase I, or the primary culture, terminates with formation of the first monolayer. Phase II is characterized by luxurient growth requiring many subcultivations, whereas during Phase III, cell division gradually subsides and the culture is eventually lost or becomes transformed to a cell line capable of indefinite cultivation.

Transformation, conversion (Koprowski et al., 1966) or cell alteration (Miles, 1964) of a diploid tissue in vitro is not a universal characteristic of cultivated cells. Indeed, this characteristic has been confined primarily to human

tissues (Jorden, 1956; Henle and Deinhardt, 1957; Zitcher and Dunnebacks, 1957; Chang, 1961; Katz, 1969) and mouse embryo cells (Todaro and Green, 1963). Transformation of chick embryo cells and many other cell types has not been reported in the literature. These cell types appear to be limited to their growth potential.

The ability of a cell to transform to a permanent cell line or be eventually lost might be determined by cellular alterations that occur during Phase III. Changes in morphology (Chang, 1961; Simons, 1967), karyology (Hsu and Moorhead, 1957; Moser, 1960; Saksela and Moorhead, 1963; Todaro et al., 1963), carbohydrate metabolism (Chang, 1961; Ryan, 1967), enzyme activities (Cristofalo, 1970), intracellular viral multiplication (Frothingham, 1959) and RNA/DNA, lipid/DNA (Hay and Strehler, 1967) and DNA/cell (Chang, 1962; Hay and Strehler, 1967) ratios have been noted. Hay and Strehler (1967) concluded that the proliferative cycle of Phase III chick cells is specifically inhibited in the G1 phase of the mitotic cycle.

Cellular function in the G1 phase of mitosis is associated with formation of ribonucleic acid (RNA) and protein. Synthesis of RNA occurs off a deoxyribonucleic acid (DNA) template whereas protein synthesis involves formation of a messenger RNA (mRNA) (containing a base sequence complementary to DNA) off a DNA template, movement of the mRNA to the ribosomal level at

which peptide elongation and subsequent protein formation occurs. In vivo inhibition of protein synthesis has been suggested to occur via histone mediation (Stedman and Stedman, 1950; 1951).

Association of basic proteins with cellular nucleic acid was first observed by Meischer in 1864. He proposed the name "protamine" for protein isolated from salmon sperm nuclei (Kossel, 1928). Kossel (1884) isolated nucleoprotein from goose erythrocytes and gave it the name "histone". The significant differences (and reason for different names) between these two types of protein was the degree of heterogeneity in amino acid composition and distribution in cell types. Protamines were thought to be composed of only arginine, histidine and lysine, whereas histones contained approximately 20 different amino acids. Also, protamines were considered to be confined to the nucleus of cells composing the testicles and spermatozoa of fish whereas histones were widely dispersed in nature (Kossel, 1928). It is currently thought, however, that the distinction between these proteins is not so sharpely defined (Murray, 1964).

Lilienfeld in 1894 introduced the term "nucleohistone" to apply to basic proteins extracted from thymus gland nuclei. Investigations concerning nucleohistone until 1928 dealt mainly with amino acid composition and quantity, total nitrogen values, and methods of extraction and classification of histones

according to the above characteristics. Biological sources of nucleohistone were confined to the thymus gland of various animals, nucleated red blood cells and spermatozoa of fish (Kossel, 1928). Mirsky and Pollister (1942) were able to extend the biological source of nucleohistone by extracting this protein from mammalian liver, kidney, pancreas, spleen, thymus and brain. Extractions were also obtained from the liver, spleen and blood cells of the dogfish. Amino acid composition of these nucleohistones confirmed previous observations of a high arginine content (Kossel, 1928), but indicated the absence of tryptophan.

Because nucleohistone was available from a variety of sources, intensive studies into the macromolecular composition of this protein were initiated by many investigators. Large proteins may be resolved into smaller fractions by several procedures: The use of cation-exchange chromatography (e.g., Murray, 1964), electrophoresis (Cruft et al., 1954) and fractional extraction (Johns, 1964) provided convenient means for fractionation of histones. Use of these methods indicated that although all tissues contained nucleohistone, the histone from any one tissue was not a homogeneous product, but rather a mixture that could be separated into several fractions. If one can generalize about the fractions composing nucleohistone, the protein may be divided into five major groups for calf thymus histone (Phillips and Johns, 1965; Gurley and Hardin,

1968) and five (Beeson and Triplett, 1967) or seven (Neelin and Butler, 1961; Kischer and Hnilica, 1967) components for nucleohistone derived from chick tissues. These fractions are based on electrophoresis or chromatographic separation and are referred to as the lysine-rich histone, F1 (Johns, 1964), slightly lysine-rich histones, F2b and F2a2 (Phillips and Johns, 1965), arginine-rich histones, F3 (Johns, 1964) and F2a1 (Phillips and Johns, 1965).

Investigations into the biological function of nucleohistones were absent from the literature except for a brief statement by Kossel (1928) that nucleohistone "might be closely connected with the process of cell division". Stedman and Stedman (1943) initiated investigations into the possible function of nucleohistones by noting that histone content in nuclei of carcinoma tissue and embryonic tissue was markedly lower than in nuclei from normal tissue. They concluded that a high content of histone in the nucleus of a cell produced an inhibition of processes leading to mitosis. Inhibitory effects of histone on mouse carcinoma cells (Stedman et al., 1944) confirmed earlier observations that histones served to inhibit cellular division. Their results were compiled to serve as a basis for their proposal in 1950 (Stedman and Stedman) and 1951 (Stedman and Stedman) that the biological function of histones was to act as gene suppressors.

Indirect, but not conclusive support of the above hypothesis was demonstrated by Cruft et al. (1954) who also demonstrated that histones might function as gene inhibitors. Direct support for regulation of gene activity by histone-DNA interaction came from the results of Huang and Bonner (1962). They demonstrated that chromatin (DNA complexed with histone and non-histone protein) from pea embryos was less effective in the support of DNA-dependent RNA synthesis than an equal amount of pure pea DNA in the presence of soluble RNA polymerase. Furthermore, they reconstituted chromatin from its purified components and noted an inhibition of the RNA synthesizing ability of the system. Combination of histone with DNA was shown to be reversible, satisfying a possible regulatory function. Decreased ability of DNA to serve as a template for RNA synthesis when complexed to histone has been confirmed by several other workers (Barr and Butler, 1963; Hurwitz et al., 1963), as well as has a decreased ability for DNA synthesis (Billen and Hnilica, 1963; Gurley et al., 1964) under these conditions.

Extending the above results, Marushige and Bonner (1966) demonstrated that template activity of rat liver chromatin was five times less than that of deproteinized DNA. If chromatin was deproteinized using techniques that selectively removed histone but not non-histone protein, template activity was comparable to deproteinized DNA. They also showed that RNA

polymerase bound equally well to DNA and to chromatin, but with chromatin, histone prevented the transcription process.

Combining knowledge of procedures for fractionation of histone and understanding of its biological function, Allfrey et al., (1963) supplemented Huang and Bonners' findings by noting the arginine-rich histone fractions (F3, F2a1) were strongly inhibitory to RNA synthesis, whereas the lysine-rich fraction (F1) was comparatively ineffectual in this regard. Bonner et al. (1968b) also suggested that arginine-rich histones play an important part in regulation of template activity. Additional support for the action of arginine-rich histones in regulating template activity has been demonstrated by Spelsberg et al. (1969a) who found that arginine-rich histone (F3) interacted with RNA polymerase and inhibited RNA synthesis.

Conflicting reports concerning the above reported specificity have been noted, however. Georgiev et al. (1966) concluded that lysine-rich (F1) histone served as the in vivo repressor of mRNA synthesis. Stellwagen and Cole (1969) are also of the opinion that lysine-rich histone is functional in template repression. A careful study by Spelsberg et al. (1969b), however, may provide a bridge for resolving such conflicting reports. They found that lysine-rich histone (F1) exhibited a greater inhibition of RNA synthesis than did arginine-rich histones (F3, F2a1) when they were allowed to interact first with the DNA template before addition of RNA

polymerase. However, arginine-rich histones showed even greater inhibition when both histone types were allowed to interact with the polymerase prior to addition of DNA. When RNA polymerase was allowed to interact initially with DNA, addition of histone types indicated greater inhibition by lysine-rich histones. The question as to which fraction is active in the cell is not settled at present.

Combining the following data that (1) cells in Phase III gradually decline from a state of active cell division to a non-dividing state; (2) histones play an important role in genetic regulation; (3) a new histone type occurs in non-dividing tissues in vivo (Panyim and Chalkley, 1969), and (4) histone protein has not been investigated for diploid cells in vitro, this project was undertaken to test the following ideas. Do histones, as a class of proteins, differ quantitatively in Phase II compared to Phase III? Is any specific histone fraction affected when cells enter Phase III? Does a new histone type appear when cells enter Phase III?

MATERIALS AND METHODS

Preparation of Monolayers

Ten-day-old Leghorn chick embryos obtained from the Veterinary Medical Research Institute, Ames, Iowa, were removed from the eggs aseptically and transferred to a sterile petri dish. All subsequent work was done under aseptic conditions. The embryos (usually 8-10) were decapitated, delimbed, thoroughly washed in Earles' balanced salt solution (EBSS) (Earle et al., 1943) and forced through a 20 ml syringe into a trypsinizing flask containing 0.05% trypsin and 0.06% ethylene-diaminetetraacetic acid (EDTA) in calcium and magnesium free EBSS. Trypsinization was allowed to proceed for 20 minutes at room temperature. The cell suspension was poured through four layers of gauze, transferred to 50 ml centrifuge tubes and centrifuged at 4 C for 10 minutes (164 x g). The pellet was suspended in Eagles minimal essential medium[1] containing 4% newborn calf serum[1], 1% chicken serum[1], 10% tryptose phosphate broth,[2] 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were inoculated into a roller bottle (1410 cm^2 surface area)[3], gassed for 1 minute with 95% air-5% CO_2 and placed on a cell production apparatus.[3] Bottles were rotated at 0.6 rpm

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[1]Grand Island Biological Co., Grand Island, New York.

[2]Difco Laboratories Inc., Detroit, Michigan.

[3]Bellco Glass Inc., Vineland, New Jersey.

at 38 C ± 1.0 C.

Subcultivation of Confluent Cultures

The spent medium was poured off the monolayer cultures and 25-35 ml of 0.05% trypsin-0.06% EDTA in calcium and magnesium free EBSS were added to each culture bottle. Cells came off the surface of the glass within 15-30 seconds and were then transferred to sterile centrifuge tubes. The cell suspension was centrifuged (164 x g) for 10 minutes at 4 C. The cellular pellet was suspended in 200 ml of culture medium and cells were inoculated into roller bottles at an initial cell population of 27.9 to 31.0 x 10^6 cells/bottle. The cultures were gassed as previously described, prior to incubation at 38 C. The total amount of medium per bottle was approximately 320 ml.

The cell cultures were examined daily for extent of growth and typically reached confluency in 3-4 days during Phase II. During Phase III, medium was renewed every third or fourth day of incubation until confluency was reached.

Preservation and Reconstitution of Cell Cultures

At various passages, aliquots of cells were frozen and stored in liquid nitrogen; the procedure followed was that of Dougherty (1962). Briefly, a confluent monolayer of cells was trypsinized and the monodispersed suspension of cells was centrifuged for 10 minutes at 4 C (164 x g). The cellular

pellet was suspended in 10 ml of Eagles minimal essential medium containing 15% newborn calf serum and 10% dimethyl sulfoxide. The cell suspension was inoculated into two 5 ml freeze-drying ampules (Thermovac Industries Corp., Copiague, New York) and the ampules were sealed. Cells were refrigerated at a rate of 1 C/minute until the temperature dropped to -95 C. The ampules then were transferred to a liquid nitrogen container where they were stored.

The procedure employed for thawing of liquid nitrogen frozen cells was similar to that of Porterfield and Ashwood-Smith (1962). Two ampules were taken from liquid nitrogen, placed at 37 C and agitated in the waterbath to enhance rapid thawing. Growth medium was added drop by drop to the thawed cell suspension until the dimethyl sulfoxide concentration was diluted below 1%. Cells were then inoculated into a roller bottle, gassed and placed at 38 C, as before.

Test for Mycoplasmas

To establish that cultures were free of contamination by mycoplasmas, the spent medium and cells from a culture in the 14th or 15th passage were tested for the presence of these organisms. Spent medium was poured into sterile centrifuge tubes and was centrifuged for 20 minutes at 4 C (4080 x g). The pellet was suspended in 5 ml of culture medium and a portion of the cellular suspension was streaked onto the

surface of two "Mycoplasma test agar" plates (gifts from the Veterinary Research Institute) containing mycoplasmas on one sector, to serve as positive controls.

Inoculation of chick cells onto mycoplasma test plates was carried out in the same fashion as for spent medium, except that the initial centrifugation was at 164 x g for 10 minutes, rather than at 4080 x g. Mycoplasma test plates were incubated at 37 C in a humidified atmosphere of 95% air-5% CO_2 for 10 days, when plates were examined by microscopy for growth of mycoplasma.

Treatment of Glassware and Medium

Cultivation of chick cells on roller bottles initially was found to be impossible because of the inability of cells to attach to untreated glass. To offset this effect, roller bottles were cleaned by the following procedure: Roller culture bottles containing 10% aqueous NaOH were rotated on the roller apparatus at 38 C for a minimum of 1 hour, after which the NaOH solution was discarded and a solution of 10% $NaHCO_3$ + 1% EDTA was added. Roller bottles again were rotated at 38 C, as before. The alkaline chelating solution was discarded and bottles were rinsed three or four times with double-distilled, deionized water. Bottles were sterilized by dry heat (minimum of 190 C for a minimum of 1 hour). All other glassware was sterilized by autoclaving the material at 121 C,

15 lbs. pressure for 20 minutes.

Tissue cell culture medium was prepared in double-distilled, deionized water and filtered through a 0.22 μ millipore filter. Usually 24-26 liters were prepared at one time. All media were stored at 1 C, and prewarmed to 25 C just prior to use.

Isolation of DNA and Nucleohistone

Tissue culture cells were suspended in hypotonic trypsin (equal volumes of trypsin solution and double-distilled, deionized water) and were centrifuged at 4 C for 10 minutes (164 x g). The cellular pellet was suspended in cold hypotonic EBSS (equal volumes of EBSS and double-distilled, deionized water) and two 0.5 ml samples were taken for determination of cell population. The suspension was centrifuged as before and the cell samples were counted via a Coulter Counter Model B. All of the following operations were carried out at 1-4 C.

The cellular pellet was resuspended in hypotonic buffer (5×10^{-4} M tris(hydroxymethyl)aminomethane + 8×10^{-4} M $MgCl_2$, pH 7.0)(HB) and centrifuged at 650 x g for 20 minutes. Cells were again suspended in fresh HB and allowed to sit in an ice bath for 15 minutes. Every 5 minutes the suspension was touched to a Vortex mixer (Scientific Products, Evanston, Ill.) for about 10 seconds. After 15 minutes, the cell suspension was homogenized (about 20 strokes) in a 40 ml Dounce homogenizer (Lab Glass, Inc., Vineland, New Jersey) with a type B (tight

fitting) pestle. Nuclei observed under phase microscopy (1000 x) were usually noted to be free from adhering cytoplasm. They were centrifuged at 650 x g for 20 minutes, and the pellet of nuclei was resuspended in HB and the above procedure repeated, except that homogenization was reduced to 10 strokes. The nuclear suspension was centrifuged as before, resuspended in 5 x 10^{-3} M Tris + 8 x 10^{-3} M $MgCl_2$, pH 7.0 (5-8 TM) and an equal volume of 64.5% sucrose (sucrose concentration employed here was determined by refractometry) in 5-8 TM was added. The suspension was layered over 50.8% sucrose in 5-8 TM and was centrifuged in the SW-27 swinging bucket at 80,900 x g for 45 minutes. The pellet was washed once in 5-8 TM and was resuspended in 5-8 TM by brief homogenization. An aliquot was taken for microscopic observation. The remainder of the nuclear suspension was centrifuged at 650 x g for 20 minutes. The resulting pellet was resuspended in 0.5 N H_2SO_4, sonicated 20-30 seconds with a Biosonic sonicator (Bronwill Scientific Co., Rochester, New York) and allowed to stand for 30 minutes at 1 C. Insoluble protein and nucleic acids were sedimented at 10,000 x g for 30 minutes. The supernatent was removed and trichloroacetic acid was added to give a final concentration of 20% trichloroacetic acid. Histone was allowed to precipitate at 1 C for 30 minutes. The pellet (insoluble non-histone protein and nucleic acid) was suspended in 5 ml of 0.5 N H_2SO_4 and placed in a boiling water bath for 15 minutes to hydrolyze

the nucleic acids. Both tubes (i.e., the histone fraction and non-histone protein-nucleic acid fraction) were centrifuged at 10,000 x g for 30 minutes. Histone was suspended in 5 ml of 1 N NaOH and was placed at 1 C overnight. The supernatent (containing the hydrolyzed nucleic acids) was frozen immediately. Isolation of histone and DNA was usually completed within 8 hours.

Qualitative and Quantitative Determinations

Histones were characterized by their solubility in dilute acid (0.5 N H_2SO_4) and base (1 N NaOH) and insolubility in strong acid (20% trichloroacetic acid). Histone fractions were also subjected to a spectrophotometric scan from 340 nm to 210 nm on a DB Beckman spectrophotometer.

Protein concentration was determined according to the procedure of Lowry and Rosebrough (1951) using bovine serum albumin fraction V as a standard. DNA was assayed by the method of Ceriotti (1952) with calf thymus DNA as a standard. All DNA and protein determinations were made in duplicate.

RESULTS AND DISCUSSION

Characterization of Cells in Phase II and Phase III

In order to effectively study changes that might occur in the histone content in young and old cells, it was necessary to conduct preliminary studies to determine when Phase III began, and to characterize how Phase III cells differed from Phase II cells.

A main difference associated with cells in Phase III, as compared to Phase II cells, was the alteration of cellular morphology and cell density. A comparison of cellular morphology and cell density in Phase II and Phase III is illustrated in Figures 1-6. Considerable intracellular granulation and extracellular debris is associated with cells in Phase III. The amount of cellular granulation and extracellular debris was noted to increase if cultures were maintained for several months after the last subcultivation (Figure 6). These results are similar to observations made by Hayflick and Moorhead (1961) and many other investigators (Haff and Swim, 1956; Harris, 1957; Swim and Parker, 1957; Simons, 1967). Indeed, Hayflick and Moorhead (1961) consider the appearance of cellular debris to be the first indication of entry of cells into Phase III.

Figure 1. Cells in Phase II fixed with 95% ethanol and stained with 0.5% methylene blue. 55x

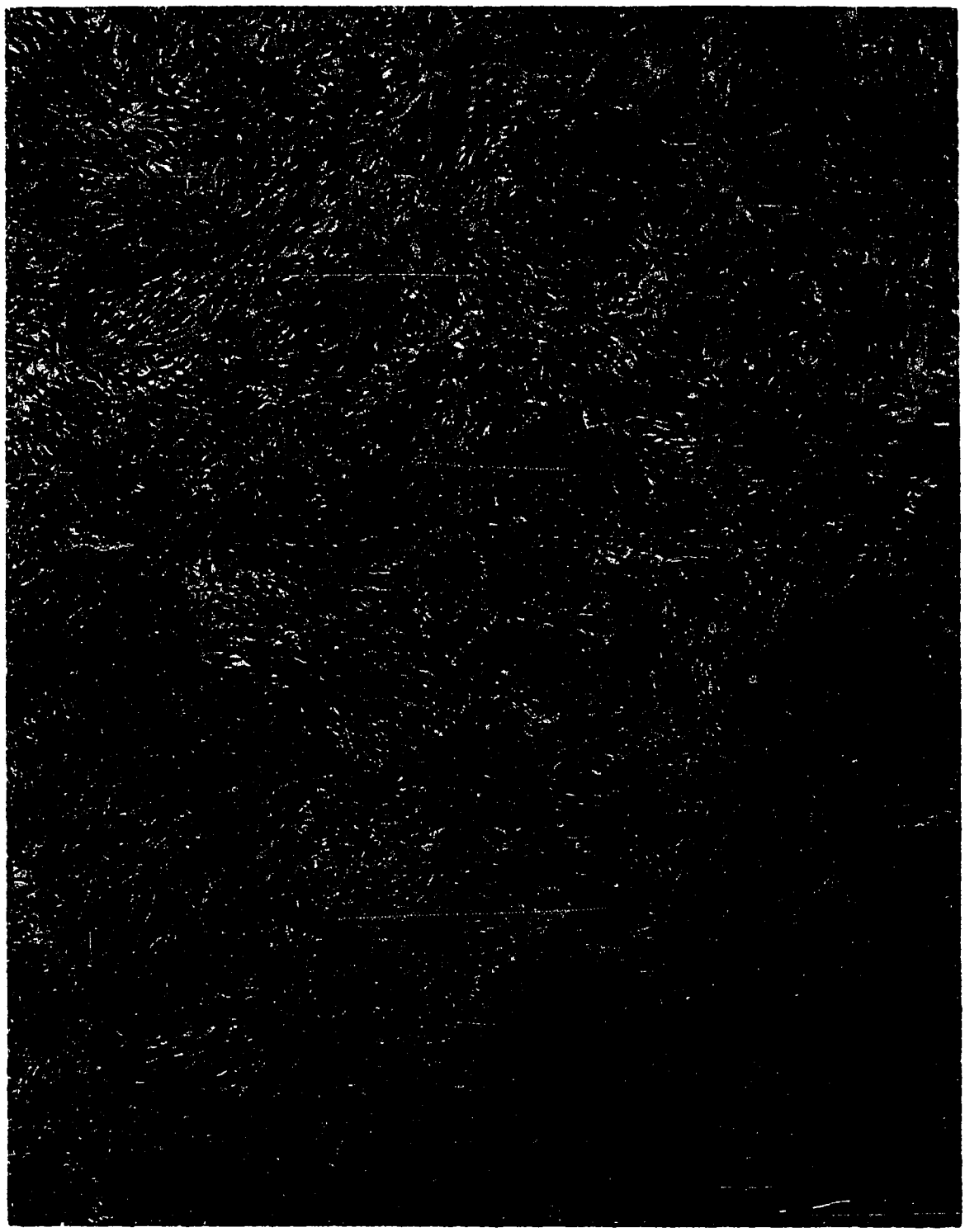


Figure 2. Cells in Phase III fixed with 95% ethanol and stained with 0.5% methylene blue. 55x

Figure 3. Cells in Phase II; not stained. 208x (Phase contrast)

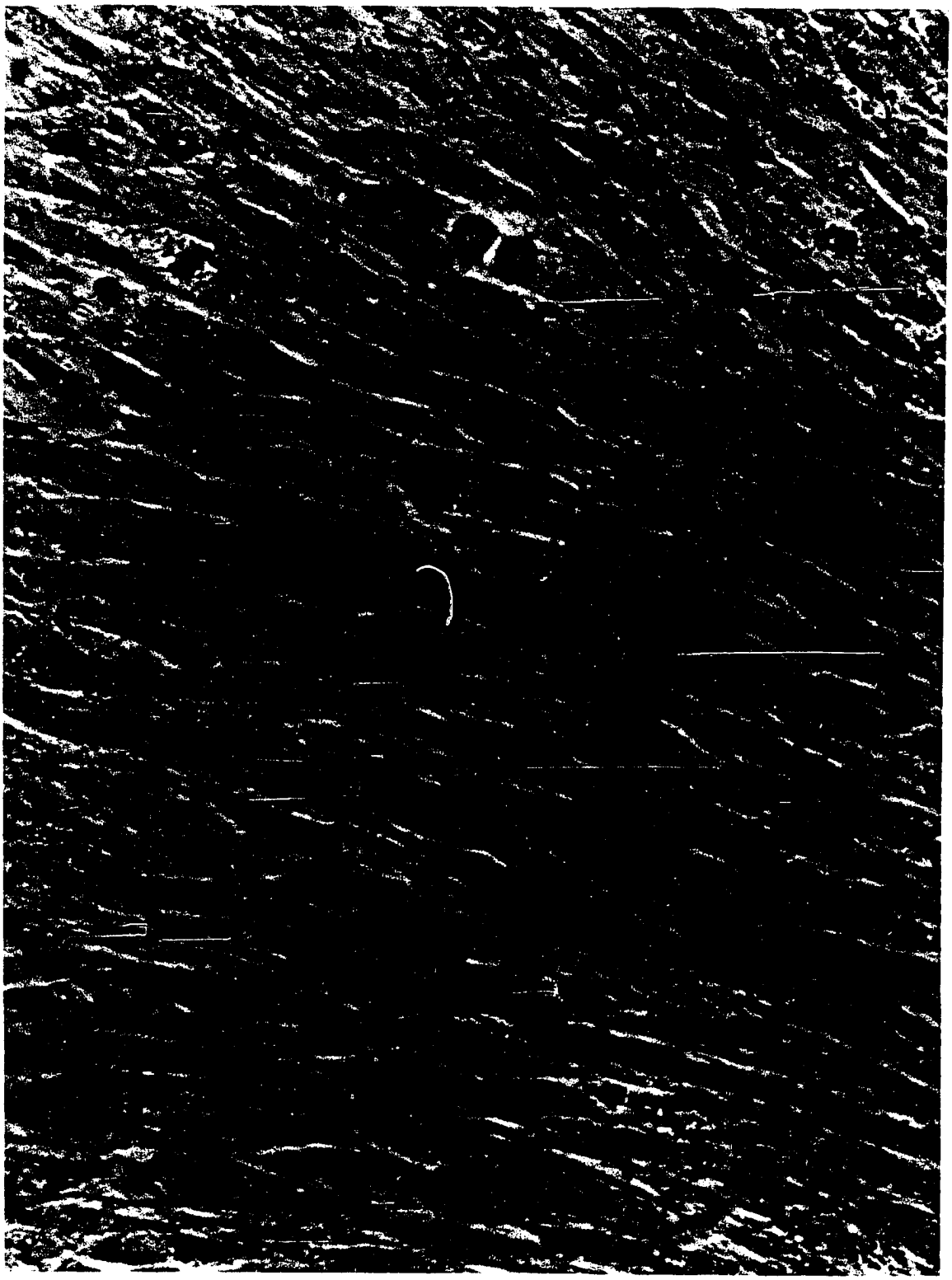
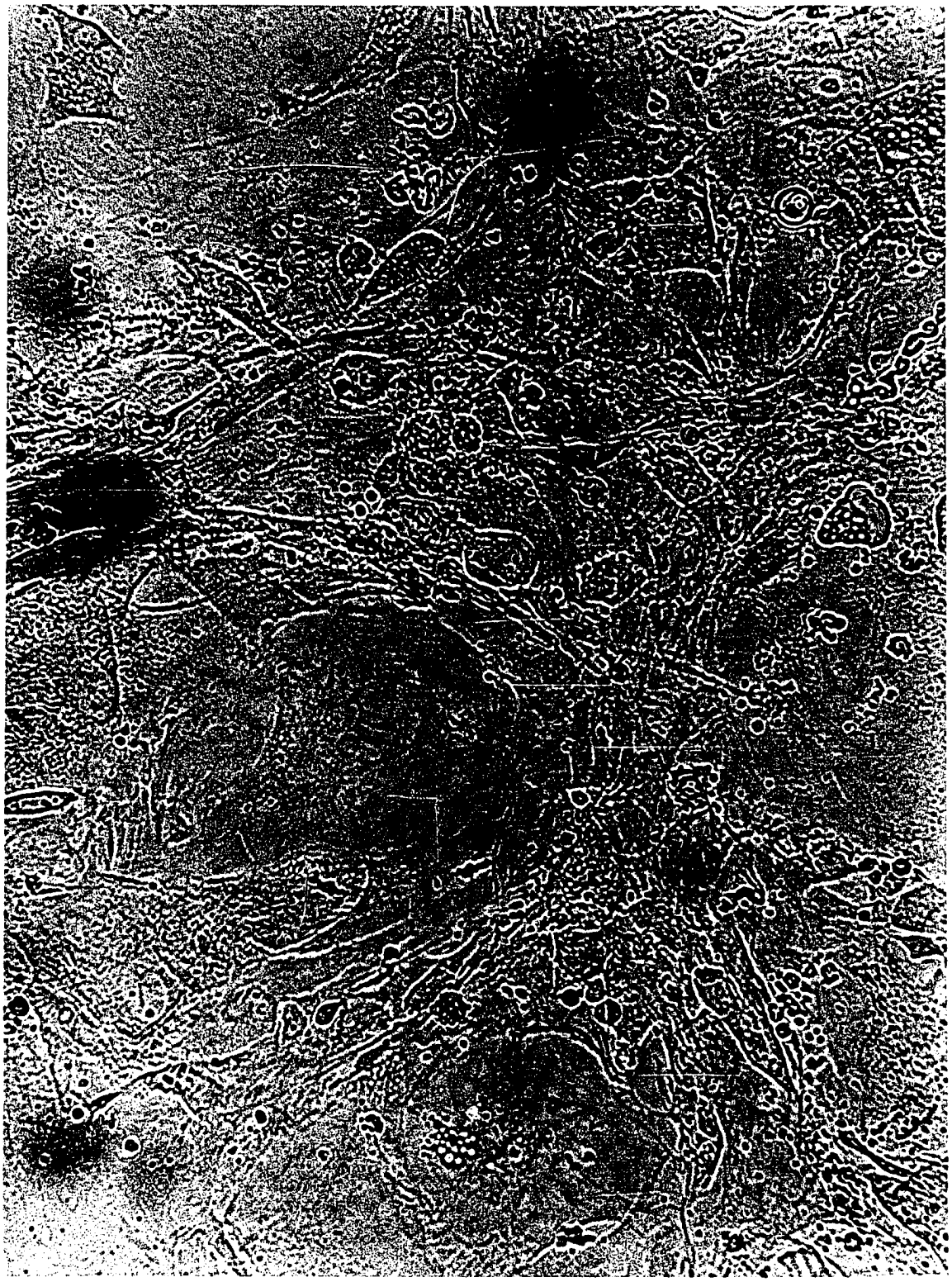


Figure 4. Cells in Phase III fixed with 95% ethanol and stained with 0.5% methylene blue. 365x

Figure 5. Cells in Phase III; not stained. 1080x (Phase contrast)

Figure 6. Cells in Phase III; not stained. Cells had been in culture for 75 days. 140x



Initially, cultures were maintained for two months after the last subcultivation to determine if cells would resume active proliferation and transform to a permanent cell line. However, cells did not regain their capacity to divide, although 60-65% of the culture remained viable (as determined by trypan blue exclusion). Haff and Swim (1956) maintained chick embryo fibroblasts for a period of nine months after the last subcultivation and noted that although cells remained viable, they did not regain their capacity to divide.

Several investigators have noted changes in karyology prior to entry into Phase III (Sax and Passano, 1961; Saksela and Moorhead, 1963; Yoshida and Makeno, 1963), or during Phase III (Moser, 1960; Katz et al., 1969). Therefore, an attempt was made to investigate any possible changes in the number of chromosomes in Phase III as contrasted to Phase II. Although good chromosomal preparations were obtained (Figure 7), reliability of any change in number would be questioned because dipoidy in chicken tissues (Gallus domesticus) has not been definitely established due to the presence of microchromosomes. Current estimates for diploidy range from 78 (Ohno, 1961) to 100-200 (Schneider et al., 1965).

The most significant difference between cells in Phase II, compared to Phase III cells, is in rate of cellular division. Therefore, growth curves were conducted from the second passage to passage 14 to establish when cells entered Phase III. Results of this study are shown in Figures 8 and 9.

Figure 7. Chromosome spread of cells in Phase II. 1080x (Phase contrast)

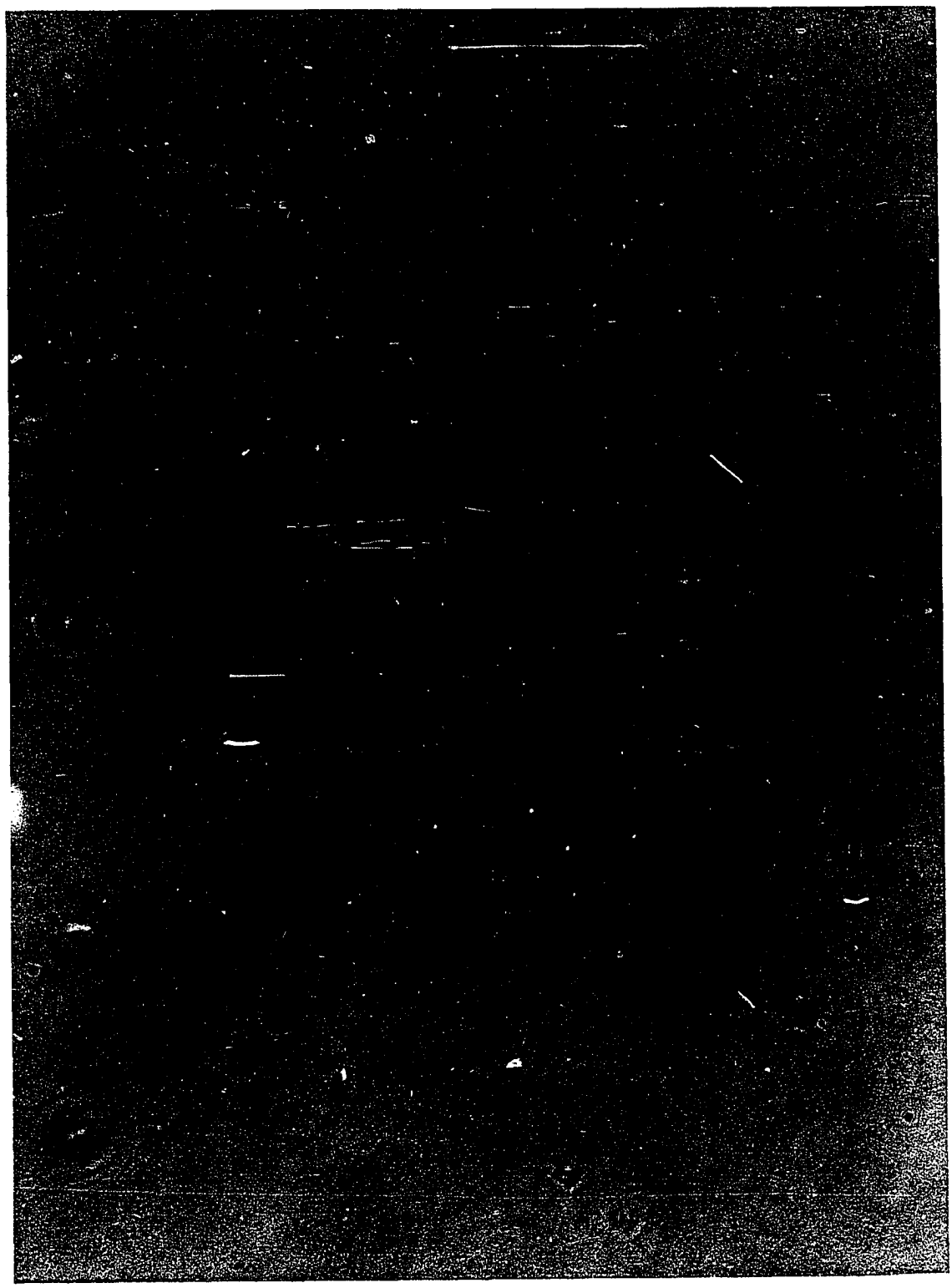


Figure 8. Cell population 65 to 72 hours after initial constant inoculum of 27.9 to 31.0 x 10^6 cells/ bottle

All cell counts from passage 11 to 14 were made at 72 hours post inoculation.

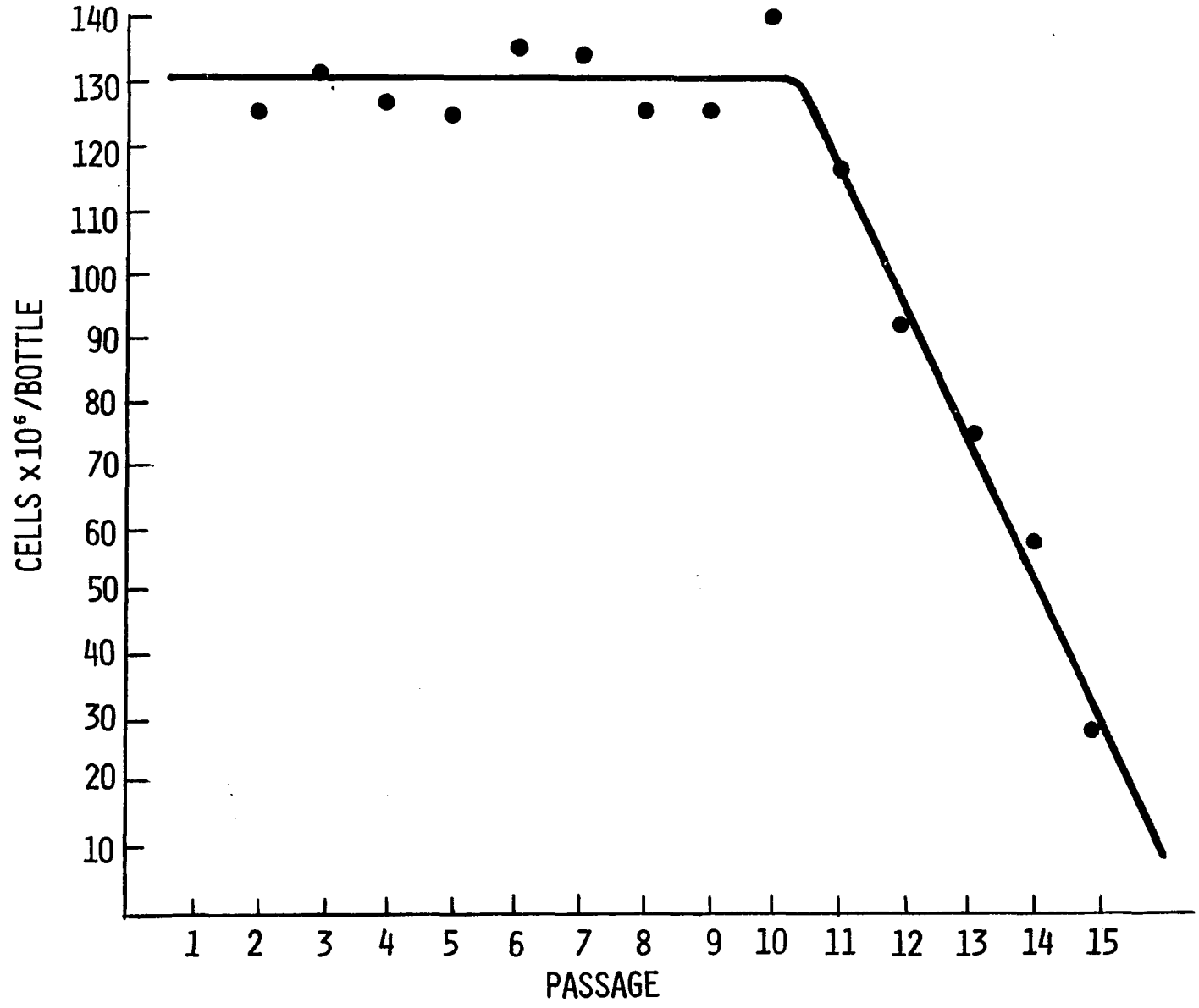
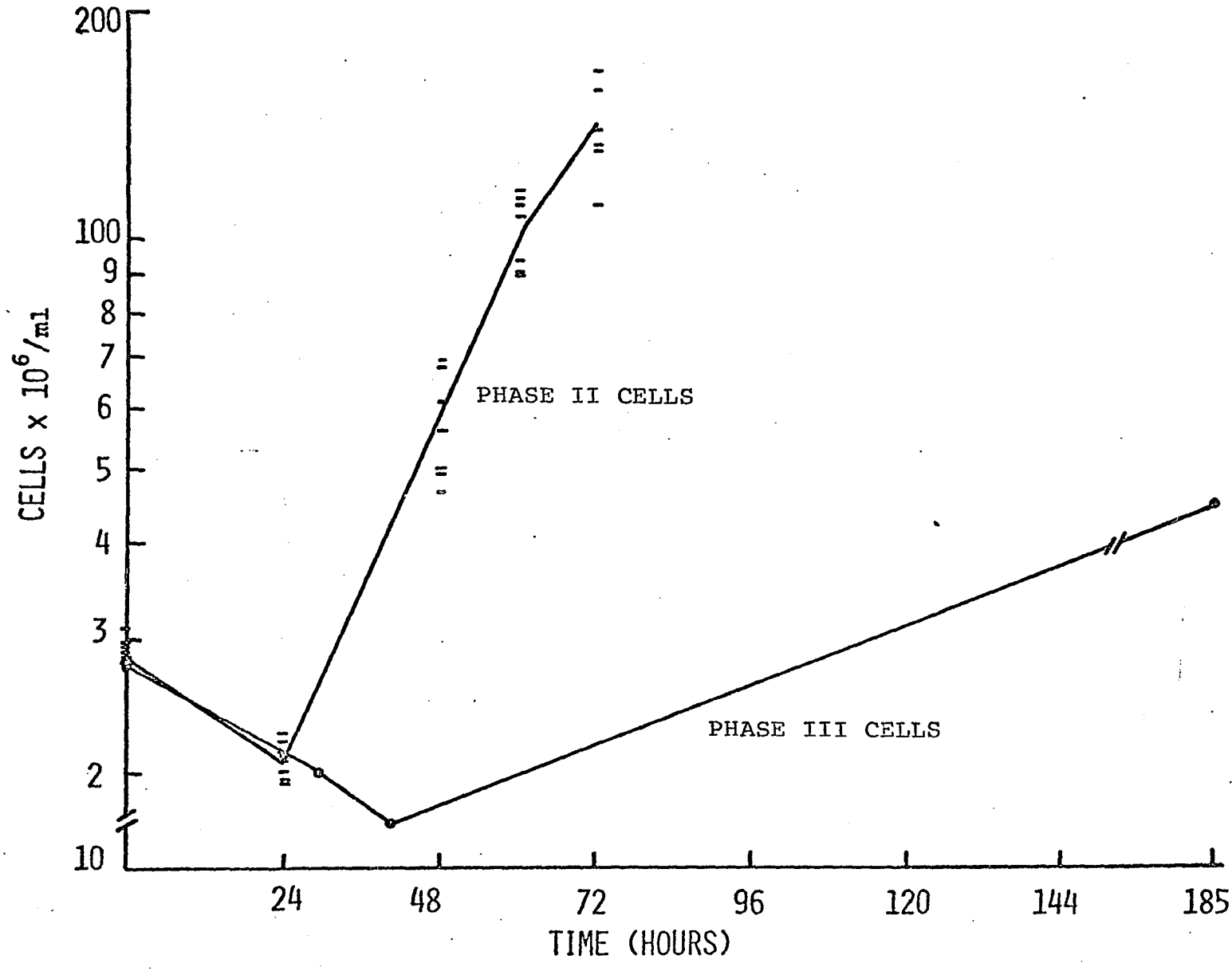

CELLS x10⁶/BOTTLE
140
130
120
110
100
90
80
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
PASSAGE

Figure 9. Comparison of the mean growth curve of cells in Phase II as compared to a growth curve for cells in passage 14 (Phase III)


200
100
9
8
7
6
5
4
3
2
10
CELLS x 10^6/ml
PHASE II CELLS
PHASE III CELLS
24
48
72
96
120
144
185
TIME (HOURS)

In Figure 8 the cell population at 65 to 72 hours incubation time, at 38 C with an initial constant inoculum of 27.9 to 31.0 x 10^6 cells/bottle, remains relative constant through passage 10. Subsequent passages indicate, however, a gradual decrease in cell population maxima 72 hours after inoculation and incubation. A decrease in the rate of cellular division throughout the experimental period is illustrated in Figure 9, which compares the mean growth curve of cells in Phase II with a growth curve during passage 14 (Phase III). The above results are similar to findings by other workers. Hayflick (1965) working with WI-38 (human lung) cells demonstrated consistancy in cell population maxima during Phase II and a gradual decrease in cell population maxima during Phase III. Macieira-Coelho et al. (1966) showed that the overall growth rate of WI-38 cells during Phase III was very reduced when compared to Phase II growth curves.

Cells maintained for extended periods after inoculation during Phase III never yielded cell populations approaching those in Phase II. Thus, under the conditions employed in this study, Phase II was characterized by healthy appearing cells growing in vitro for 30 days or 9 passages. Phase III was characterized by increasing intracellular granulation and extracellular debris during an additional 20-25 days, or 4-5 passages. The entire life span of chick embryo fibroblasts was usually confined to 14-15 passages in a period of approximately 51 days (Table 1). These findings are supported by Hay and

Table 1. Total time *in vitro* as related to passage number

Date culture initiated	Time (days) *in vitro*	Total passages
3-16-69	50	17
5-22-69	46	13
7-3-69	55	14
7-15-69	54	16
11-18-69	51	15
1-8-70	55	15
3-11-70	49	14
Average	51	15

Strehler (1967) who found that the life span of chick embryo fibroblasts was limited to 13-26 passages in less than two months. The slight discrepancy in this study as compared to Hay and Strehlers' (1967) work is probably due to differences in growth medium employed, as the latter authors supplemented their medium with chick embryo extract.

Tests for Presence of Mycoplasma in Tissue Cell Cultures

To check the possibility that contamination of cell cultures by mycoplasma may have occurred during the cultivation of cells, cultures were tested for the presence of these organisms as described in Materials and Methods. Contamination

of cell cultures by mycoplasma(s) was never detected. Although the presence of mycoplasmas in cell cultures is not always easily detected, the presence of mycoplasma(s) influencing the current studies may be argued against by consideration of Table 1. Table 1 compares the life span of seven different cell cultures originating from different 10 day old chick embryos at different times. As is seen, the average life span of chick embryo fibroblasts *in vitro* is 15 passages in an average time of 51 days. If mycoplasma(s) was influencing any of the above cultures, it is unlikely that such consistancy would be noted in culturing time as related to passage number *in vitro*. The ability of mycoplasma test plates to support growth of mycoplasma was verified by the presence of a positive control for each test. Although the presence of a positive control does not confirm the ability of all mycoplasmas to grow on the test plate, it does confirm the ability of the test plate to support growth of some mycoplasmas.

Histone Properties and Values

At various intervals during the growth of cells in Phase II and Phase III, cells were harvested, nuclei were isolated and histone and DNA were isolated as previously described. Because proteolytic enzymes for nuclear proteins are active during the isolation of nuclei, it was essential that nuclei, and subsequently extracted histone, be isolated as rapidly as possible to prevent loss of test material. Nuclei were

obtained in approximately 5 1/2 hours (Figure 10) and histone was isolated 2-2 1/2 hours later. Panyim et al. (1968) have shown that proteolysis of histones is at a minimum if nuclei are isolated within 8-12 hours at 0 C; thus, degradation of histone in this study was kept at a low level.

Histone obtained from isolated nuclei was characterized by its solubility in dilute acid (0.5 N H_2SO_4) and base (1N NaOH), and its insolubility in strong acid (20% trichloroacetic acid). In addition to these characteristics, a spectrophotometric scan is presented in Figure 11. From 29 histone samples, the characteristics listed in Table 2 were obtained.

The characteristics obtained are in close agreement with criteria established by Bonner et al. (1968a) and Bonner et al. (1968b) for chromatin. Chromatin is characterized by its low absorbancy at 280 nm and 260 nm and its appreciable absorbancy at 220-230 nm. All preparations used in this study met the above standards. Histone isolated from nuclei may be effectively compared to histone from chromatin since it has been demonstrated that both are indistinguishable qualitatively and quantitatively (Sadgopal and Bonner, 1969).

Because the amount of histone isolated varied from experiment to experiment, it was necessary to relate the amount of histone obtained to a reliable index. Content of nuclear DNA was chosen to serve as this index and total histone was evaluated against DNA on a microgram to microgram relationship.

Figure 10. Isolated nuclei from chick embryo fibroblasts cultivated *in vitro*. 1080x (Phase contrast)

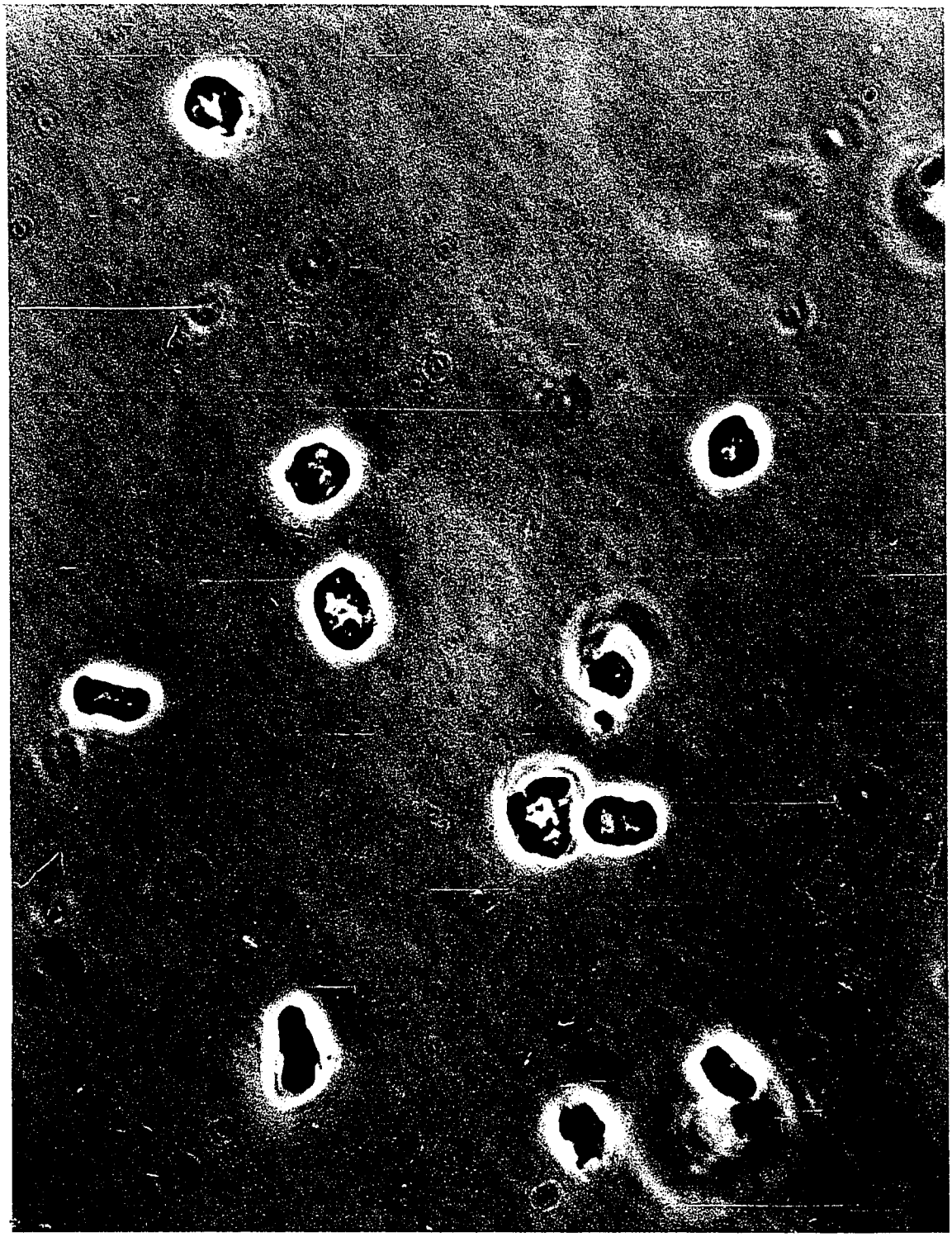


Figure 11. Spectrophotometric scan of a typical histone preparation isolated from chick embryo fibroblasts *in vitro*

_____ Calf thymus histone obtained from Sigma Chemical Co., St. Louis, Mo.
____.____.____. Chick histone extracted from cells cultivated *in vitro*.

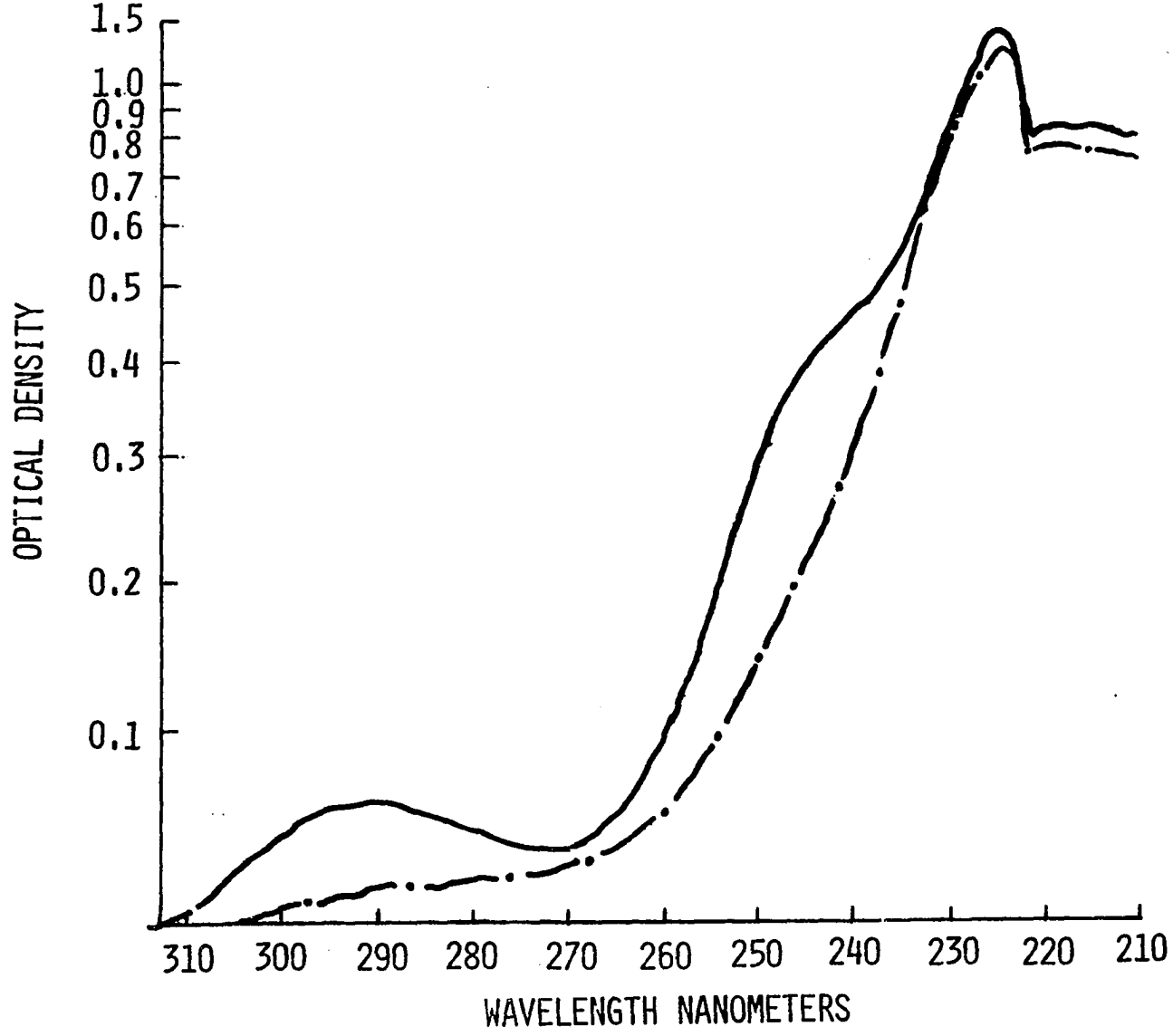

OPTICAL DENSITY
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
310
300
290
280
270
260
250
240
230
220
210
WAVELENGTH NANOMETERS

Table 2. Spectrophotometric characteristics of isolated chick histone

Number of histone samples	Optical density at 280 nm	260 nm	222-225 nm
27/29	<0.05	<0.08	>0.9

Values obtained from histone extractions during Phase II and Phase III are presented in Figures 12 and 13.

Figure 12 illustrates histone/DNA ratios vs. cell population obtained during growth curves for cells harvested in Phase II. Initially, the histone/DNA ratio rose to a maximum value of 2.095 when cells entered mid-lag phase. At mid-log phase, the histone/DNA ratio approached a minimum value of 0.94, after which a steady increase occurred. It is of interest to note that the histone/DNA ratio obtained for cells in stationary phase (1.43-1.63) is similar to that obtained if histone and DNA were isolated a few hours after being subcultivated (1.39-1.59). Thus, values obtained at each cell population tended to confirm each other.

As stated in the literature review, no reports have been found concerning histone/DNA values for diploid tissues in vitro. Thus, the above results cannot be compared directly to results obtained by other investigators. There have been, however, many investigations concerned with establishing histone/DNA values in various organs and cell lines capable of

Figure 12. Histone/DNA ratios vs. cell population for cells in Phase II

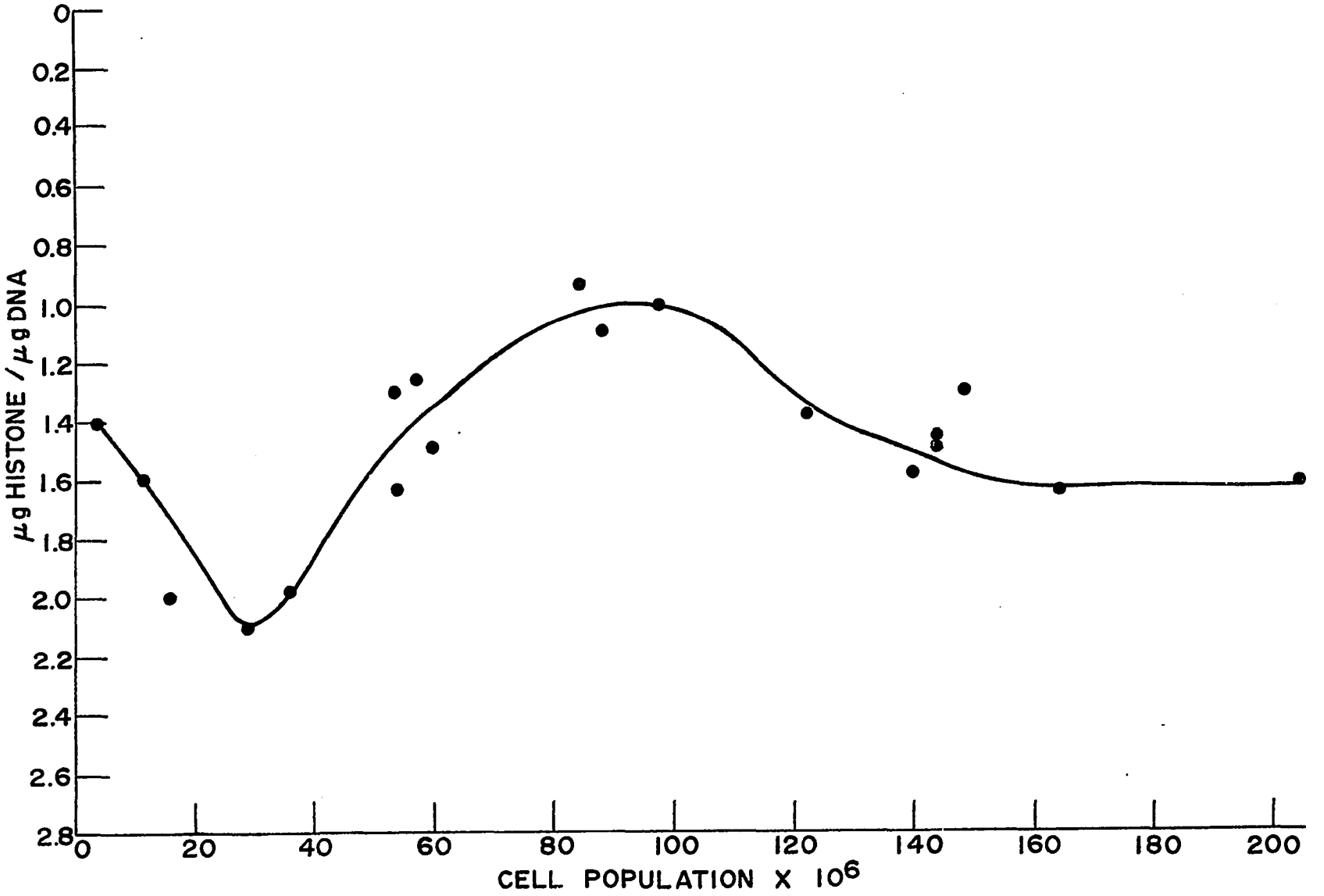
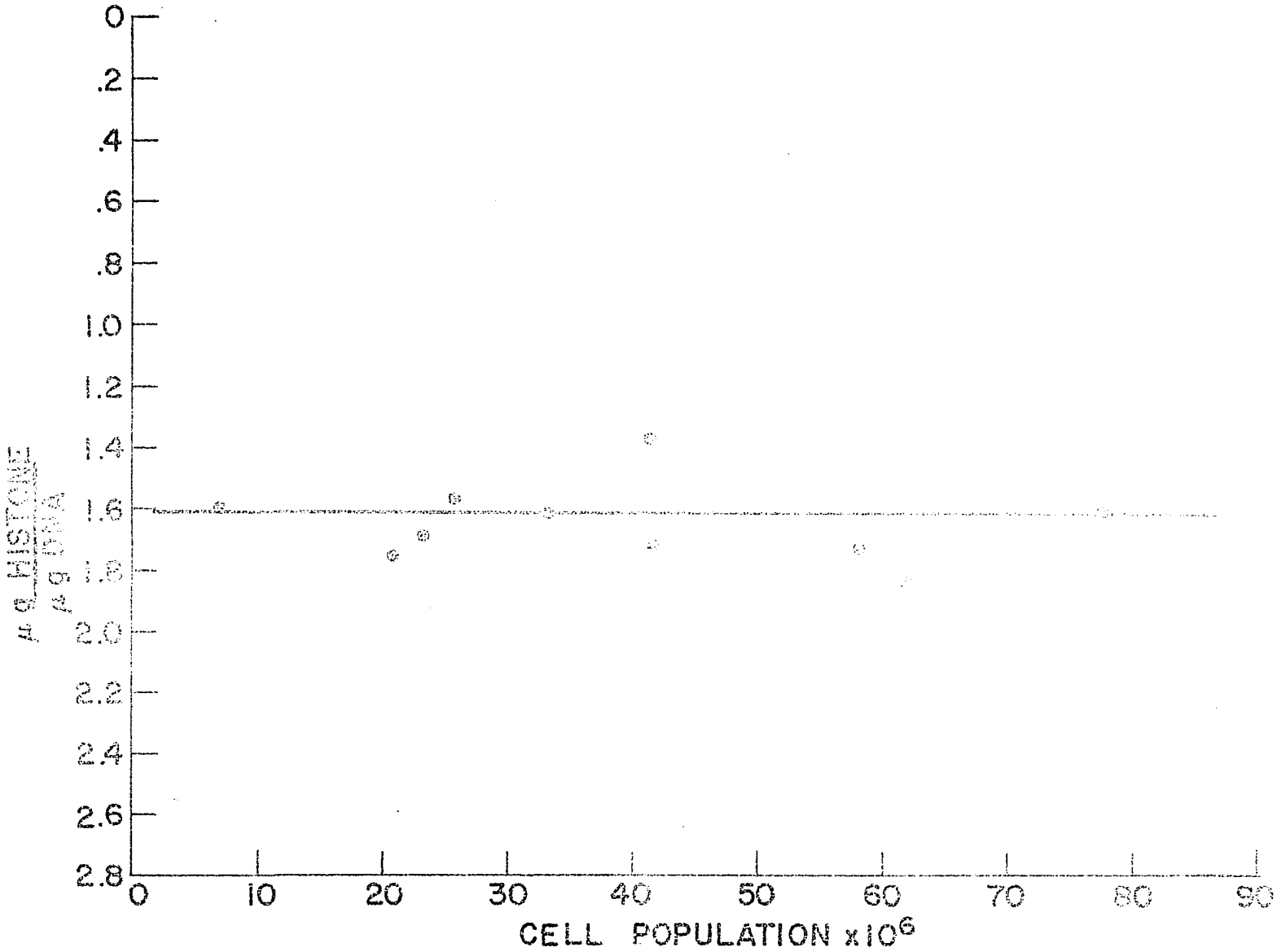

0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
μg HISTONE / μg DNA
0
20
40
60
80
100
120
140
160
180
200
CELL POPULATION X 10⁶

Figure 13. Histone/DNA ratios vs. cell population for cells in Phase III


0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
μ g HISTONE / μ g DNA
0
10
20
30
40
50
60
70
80
90
CELL POPULATION x10⁶

indefinite cultivation *in vitro*. Unfortunately little work establishing the change in this ratio during the growth period of cell cultures has been conducted. The data obtained is largely derived from organs or cultures in mid-log phase of the growth cycle. A summary of histone/DNA values taken from the literature is presented in Table 3. From Table 3 it can be seen that wide variations in nuclear histone/DNA ratios are cited even for similar cellular materials. Undoubtedly, this variation is the resultant of the techniques employed in obtaining the final products, the physiological state of the biological material when extraction was initiated, and differences inherent in the starting material. However, several conclusions may be derived from the data in Table 3. For actively dividing tissues, a histone/DNA value of approximately 1.0 (range 0.83-1.12) is obtained. For cultures in stationary phase (only *Tetrahymena pyriformis* may be evaluated), a significantly higher value is noted (1.9-2.0). Interpretation beyond this point of the data presented in Table 3 is difficult; however, if data from Hancock (1969), Bonner *et al.* (1968b) and Stone (1969) are combined, a cyclic pattern resembling the results presented in Figure 12 is obtained.

The cyclic pattern obtained from comparisons of the histone/DNA ratio of different cell populations is probably the result of changes in the amounts of DNA/cell and of histone/cell, rather than being totally dependent on only one variable in the system. Although there is much evidence supporting

Table 3. Histone/DNA values taken from the literature

Histone origin	Histone/DNA	Reference
Whole chick embryo	1.17-1.23	Huang and Huang (1969)
Chick erythrocytes and organs	0.76-0.98	Dingman and Sporn (1964)
Rat liver	1.8	Dounce and Umana (1962)
Pea seedlings	1.3	Bonner and Huang (1963)
Physarum polycephalum	1.05	Mohberg and Rusch (1969)
Mouse mastocytes (mid-log phase)	1.12	Hancock (1969)
HeLa (1/3 confluency)	1.20-1.30	*Ibid.*
HeLa (log phase)	1.02	Bonner *et al.* (1968b)
Rat ascites tumor	1.16	*Ibid.*
Sea urchin	0.86	*Ibid.*
Tetrahymena pyriformis (early log phase)	1.1	Stone (1959)
Tetrahymena pyriformis (log phase)	0.9- 1.02	*Ibid.*
Tetrahymena pyriformis (late log phase)	1.63	*Ibid.*
Tetrahymena pyriformis (Stationary phase)	2.0	*Ibid.*
Tetrahymena pyriformis (log phase)	1.0	Lee and Scherbaum (1966)
Tetrahymena pyriformis (stationary phase)	1.9	*Ibid.*
Hypotetraploid lymphoma[a]	0.98	Kit and Gross (1959)
Diploid lymphoma[a]	0.83	*Ibid.*

[a]Cells grown intraperitoneally in mice.

concurrent synthesis of DNA and of histone (Niehaus and Barum, 1965; Spalding et al., 1966), there are increasing amounts of data indicating that the synthesis of histone also occurs prior to DNA synthesis (Evans et al., 1962; Butler and Cohn, 1963; Umaña et al., 1964; Sadgopal and Bonner, 1969; Gurley and Hardin, 1970). The significant change in histone/DNA ratio occuring in lag phase is probably the result of a decrease in the rate of DNA synthesis and the associated deposition of a nonchromatin pool of histone. Indeed, Gurley and Hardin (1970) maintain that the deposition of histone in a nonchromatin pool cannot be detected unless the culture is slowed down in respect to DNA synthesis, or is completely stopped from growing. Umaña et al. (1964) have demonstrated that nuclei isolated from tissues in which a great number of cells are in interphase have a histone/DNA ratio close to 2 or somewhat higher.

The steady decline in histone/DNA values noted as cells enter log phase is probably a reflection of increasing amounts of DNA per cell since a greater proportion of cells are entering mitosis. Umaña et al. (1964) confirms this explanation by demonstrating that nuclei isolated from tissues in which a large number of cells are dividing have a histone/DNA ratio close to 1.0.

Figure 13 illustrate histone/DNA values obtained during cultivation of cells during Phase III. The graph obtained indicates a physiological condition resembling stationary phase

of Phase II cells. Since only a small proportion of cells in Phase III are dividing, as is the prevalent condition in stationary phase, the histone/DNA values obtained would be expected to maintain a value comparable to that found in stationary phase.

One of the major questions posed at the beginning of this thesis was, "Do histones, as a class of proteins, differ quantitatively in Phase II compared to Phase III?" Consideration of Figures 12 and 13 indicates that cells in Phase III do not contain significantly more histone per microgram of DNA than do cells in Phase II. However, instead of exhibiting a cyclic variation in histone/DNA ratio, cells in Phase III maintain a histone/DNA ratio throughout their growth period resembling stationary phase in Phase II cells. This difference is, however, probably due to differences in rates of DNA synthesis.

Previous studies concerned with possible changes in amounts of histone in aging tissues have been confined to in vivo investigations. Dingman and Sporn (1964) did not find any significant differences in the histone/DNA ratio for chromatin extracted from chick embryo tissue as compared to adult tissue. Kischer and Hnilica (1967) failed to find any qualitative changes in over-all composition of histone extracted from adult and embryonic chicken brain, liver and dorsal skin with feather organ. Beeson and Triplett (1967) have supported the above

reported finding by demonstrating that histone from the spleen and liver of embryonic and adult chickens did not differ either qualitatively or quantitatively. Thus, this study, involving aging of chick embryo fibroblasts *in vitro*, appears to support earlier findings concerned with possible changes of histone with age *in vivo*; i.e., there is no detectable change in the histone/DNA ratio as cells age.

The original objective after obtaining purified histone preparations and quantitizing each sample, was to subject each sample to polyacrylamide gel electrophoresis and to quantitize each fraction via densitometry. It was found, however, that fractions comprising calf thymus histone (dissolved in 1 N NaOH) underwent considerable smearing when subjected to polyacrylamide gel electrophoresis. Application of chick histone samples in 1 N NaOH to polyacrylamide gel electrophoresis did not yield any results because of the failure of samples to enter the gels. Attempts to reprecipitate chick histone via strong acids or strong bases failed.

Studies *in vivo* concerned with possible changes in histone fractions during aging have yielded negative results. Beeson and Triplett (1967) using polyacrylamide gel electrophoresis, followed by densitometry analysis, could not reveal any quantitative changes in histone fractions from embryonic chicken organs compared to adult organs. Kischer and Hnilica (1967) also could not demonstrate any fractional changes in histone content from chick organs during embryonic development.

Coupling data that quantitatively histones do not change during aging *in vitro* or during embryogenesis, with data that most cells of an adult organism possess the same amounts of the same histones (Dingman and Sporn, 1964; Beeson and Triplett, 1967; Kischer and Hnilica, 1967), the question whether histones are the natural repressors of genetic activity in the cell may be considered. Increasing numbers of investigators are considering that chemical modification of histones may be an important factor in regulation of genetic activity and possibly of aging rather than changes in quantity of histone. Indeed, modifications such as acetylation (Allfrey *et al.*, 1964; Pogo *et al.*, 1966), methylation (Allfrey *et al.*, 1964; Tidwell *et al.*, 1968; Kaye and Sheratzky, 1969) or phosphorylation (Benjamin and Goodman, 1969; Murthy *et al.*, 1970) are currently being considered in the role of regulation of genetic activity and the role this phenomenon plays in aging, if any.

SUMMARY

The most significant difference between cells in Phase II, compared to Phase III cells, was the rate of cellular division. Cell population 65 to 72 hours after a constant inoculum remained relatively constant through passage 10. Subsequent passages indicated, however, a gradual decrease in cell population maxima. A decrease in the rate of cellular division throughout the growth period was also illustrated.

Under conditions employed in this study, Phase II was characterized by healthy appearing cells growing in vitro for 30 days or 9 passages. Phase III was characterized by increasing intracellular granulation and extracellular debris during an additional 20-25 days, or 4-5 passages. The entire life span of chick embryo fibroblasts was confined to 14-15 passages in a period of 51 days. Tests for the influence of mycoplasmas during this study were negative.

At various intervals during the growth of cells in Phase II and Phase III, cells were harvested, nuclei isolated and histone and DNA isolated. For cells in Phase II, the histone/DNA ratio rose to a maximum value of 2.095 when cells entered mid-lag phase. At mid-log phase, the histone/DNA ratio approached a minimum value of 0.94, after which a steady increase occurred. The histone/DNA ratio obtained for cells in stationary phase (1.43-1.63) was similar to that obtained if histone and DNA were isolated a few hours after being sub-

cultivated (1.39-1.59).

Histone/DNA values throughout the growth cycle for cells in Phase III were similar to values obtained for Phase II cells in stationary phase. This was interpretated to mean that cells throughout Phase III are in a physiological condition resembling stationary phase of Phase II cells.

Further consideration of histone/DNA values for cells in Phase II as compared to Phase III cells indicated that cells in Phase III do not contain significantly more histone/DNA than do cells in Phase II.

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APPENDIX

Figure 14. Standard curve for protein determination (via Lowry reaction)


75
70
60
50
40
30
20
10
0
μg PROTEIN
0.1
0.2
0.25
OPTICAL DENSITY

Figure 15. Standard curve for DNA determination (via Ceriotti reaction)

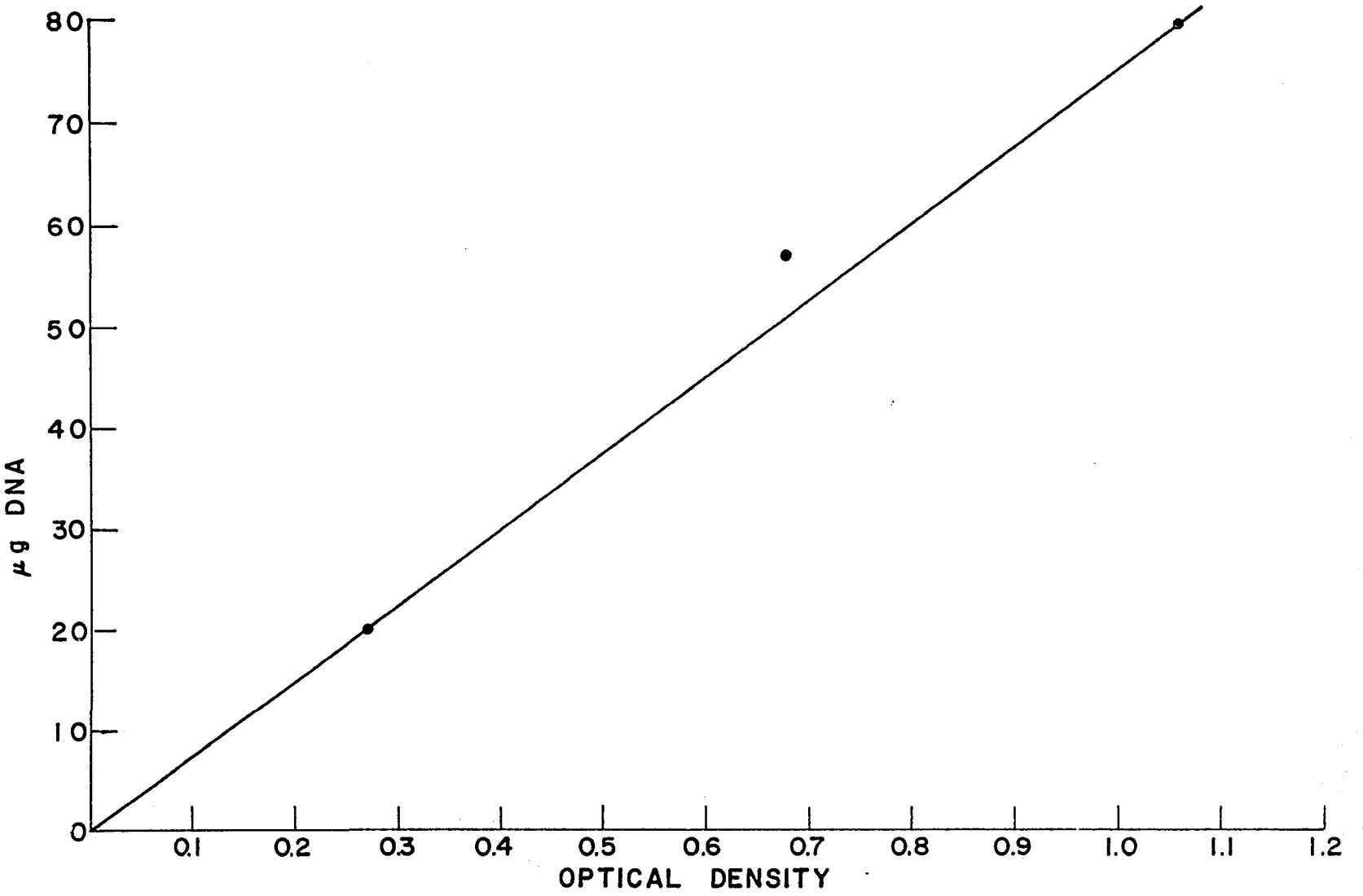
80
70
60
50
40
30
20
10
0
μg DNA
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
OPTICAL DENSITY

Table 4. A summary of histone and DNA extraction for Phase II cells

Passage number	Cell population $\times 10^6$	µg DNA / 0.5 ml		µg Histone / 0.5 ml		µg Histone / µg DNA
6	3.9	±0.25	7.0	±0.5	9.75	1.39
10	11.7	±0.06	12.06	±0.75	19.25	1.596
10	15.4	±0	4.50	±0.37	9.0	2.0
4	28.9	±0.25	18.50	±1.25	38.75	2.095
6	36.0	±0.81	18.30	±0.67	36.17	1.98
10	53.0	±0.66	19.78	±0.38	25.62	1.30
2	53.4	±0.32	12.18	±0.67	19.92	1.64
2	57.0	±0.13	23.87	±1.5	30.0	1.26
3	59.8	±0.06	12.93	±0.37	23.87	1.49

Table 4 (Continued)

Passage number	Cell population x 10^6	µg DNA / 0.5 ml	µg Histone / 0.5 ml	µg Histone / µg DNA
2	84.0	±1.0 50.0	±1.06 53.18	0.94
2	88.3	±0.25 14.0	±0.5 15.5	1.11
2	97.9	±0.06 6.43	±0 6.50	1.01
2	124.4	±0.12 28.67	±0.50 39.50	1.38
10	13.97	±0 30.0	±1.75 47.25	1.58
5	143.3	±0 9.67	±0.12 14.37	1.49
9	144.0	±0.25 89.0	±2.0 129.25	1.45
5	148.0	±0 64	±0 83.5	1.31
10	164.8	±0.25 40.67	±0 66.50	1.63
2	204	±1.75 78.25	±2.0 126.0	1.61

Table 5. A summary of histone and DNA extractions for Phase III cells

Passage number	Cell population x 10^6	µg DNA / 0.5 ml		µg Histone / 0.5 ml		µg Histone / µg DNA
11	6.7	±0.15	4.52	±0	7.25	1.60
13	20.8	±0	5.0	±0	8.75	1.75
12	23.3	±0.31	5.56	±0.67	9.42	1.69
13	25.7	±0.12	5.74	±0	9.0	1.57
12	33.3	±0.19	4.19	±0.75	6.75	1.61
12	41.5	±0.08	6.04	±0.80	8.30	1.37
12	41.8	±0.25	4.0	±0.12	6.87	1.72
11	58.2	±0	3.75	±0.55	7.45	1.73
11	61.9	±0.5	4.87	±0	9.0	1.84
12	77.4	±0.12	5.87	±0.67	9.42	1.61