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The purpose of this tissue culture study was to determine whether physiological levels of the steroid 17-β-estradiol would stimulate visible changes in morphology or growth of adult human fibroblastic cells derived from the large intestine, a non-target tissue.

Two methods of data collection were used. First, sequential phase-contrast photomicrographs were made of 2 groups of 9 secondary cultures of 79-8924 tissue in plastic flasks at selected times during 3-day intervals. At the end of each experiment, cell monolayers were fixed with 10% neutral buffered formalin. Flasks were rinsed, sawed into 1 X 2½ inch slides and, stained with a modification of Lehman's (1965) polychrome staining procedure to which an initial staining period in 1% Alcian Blue and the final treatment with oil of cedar for clearing and mounting were added for the second type of data collection. This consisted of bright-field microscopic observation of stained slides. Analysis of stained slides included determination of mitotic coefficients, enumeration of lavender- and yellow-staining nuclei among the expected steel blue nuclei, and calculation of nuclear size index values as the product of the greatest length X greatest width of 90 oval nuclei per slide. The t-test was used to compare the difference of pairs of means.

Means of mitotic coefficients for the 2 estradiol treatments were not significantly different from each other even though the amount of estradiol differed by a factor of 10. This does not support the idea proposed by some authors that the effect of this hormone is always a function of concentration. Nuclear-size index values were consistently smaller for estradiol-treated cultures, but this difference was significant only in the second experiment. Number of nuclei and nuclear staining differences based on comparison of numbers within fields containing a dividing cell and numbers in fields not containing dividing cells were not consistently statistically significant.

Discussion of chromosome analysis of the 17½ week old stock cultures, as it describes the stage in culture, is included.

It was concluded that a growth response, as defined by an increase of mitotic coefficient of estradiol-treated tissue cultures, did occur with this non-target tissue, the lamina propria. It is suggested that the response of connective tissue to steroids can alter the dynamic balance of the tissue organization in the intestinal mucosa.

A PILOT STUDY OF THE VISIBLE EFFECTS OF ESTRADIOL ON HUMAN INTESTINAL CELLS IN TISSUE CULTURE

by

Linda Neskaug Curtis

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Arts

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Approved by

Laura G. Anderton
Thesis Adviser

APPROVAL SHEET

This thesis has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

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I am indebted to my husband John for his help and enthusiasm.

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INTRODUCTION

Endocrinologists have often used the term "target tissues" in reference to those tissues that exhibit a pronounced response to a specific hormone. A seemingly clear-cut example is shown in the effects of the follicle stimulating hormone (FSH) which is produced by the pituitary. It is generally considered that the only target tissues of this gonadotrophin are within the ovaries of the female and the testes of the male. The simplicity of this generalization, while often useful, may create the false impression that all hormones can react with only a few very specific tissues. That this is not always the case is shown by the ovarian hormone estrogen. Its recognized target tissues are the pituitary gland and the secondary sex structures. According to Clegg and Clegg (1969),

> Vaginal changes induced by oestrogen include cornification of the epithelium, hyperaemia, hypertrophy and oedema of the vaginal stroma, appearance of glycogen in the epithelium, a fall in pH of vaginal secretions and a change in the electrical potential.

Additional effects on the secondary sex structures are production of rhythmic contractions in uterine smooth muscle and development of mammary glands.

Further research on estrogen has shown that it has widespread effects that are not limited to the reproductive system. In mammals it is known to influence body growth and

weight, to produce changes in the skin and adrenal glands, and to alter water and electrolyte metabolism. Anderton (1970) has stated that larval development of frogs in water containing estradiol results in production of mammalian-like adrenals in these amphibians. Medicinal uses of estrogen in humans such as in birth control pills and estrogen therapy have resulted in recognition of various other "non-target tissues" that respond to estrogen. Among these responses are changes in blood clotting, improvement of acne conditions in adolescents, and alteration of glucose metabolism in the liver. Willmer (1970) has described the reaction of a soil amoeba Naegleria which can be made to alternate between flagellate and amoeboid forms treated with such steroid hormones as progesterone, deoxycorticosterone, testosterone, and estrogen. He suggests that it is of evolutionary significance that

> ... these highly sophisticated hormones, once thought to be peculiar to the physiology of vertebrates, and later of insects too, are evidently substances that can act on the physiological mechanisms of creatures as low in the evolutionary scale as Naegleria probably is. At whatever site in the cell these steroids are ultimately found to act, i.e. at the surface, on mitochondria, or on some part of the nuclear apparatus ..., it is probably very significant that the mechanism, or one very like it, is already present in Naegleria and is, as it were, ready to be exploited in higher forms.

On the basis of this evidence about cells and tissues which were not previously thought capable of response to estrogen stimulation, it appears that future research in this field

should 1) re-evaluate the potential for response of other "non-target tissues" and 2) seek to describe the modes of action of steroid hormones in such ways that each effect on every responsive tissue is explained.

Estradiol is particularly suitable for use in this study since it is a natural estrogen that is readily available in pure form. Its medicinal uses have necessitated the acquisition of information such as the normal amounts of estrogen found in the blood of females. Quite obviously the relevance of the present study depends on use of physiological doses of the drug; toxicity effects must not be confused. As previously noted, responses to estrogen stimulation vary according to type of tissue. Villee (1962) describes the general response of the secondary sex structures as "growth". In this regard estrogen is considered to be a mitogenic agent. Describing its actions on a physicochemical basis, Willmer (1970, 1965, and 1960) designates it as a "surface-active agent". McGuire (1968), Puca (1968), Teng (1968), Toft (1967), and Gorski (1965) have concluded that the initial or primary reaction of estradiol occurs at specific binding sites at the cell surface of reacting tissues and involves formation of a hormone-receptor complex. Following this initial reaction, up to 80% of radioactively labeled estradiol can be located in the nuclear fraction (Puca, 1968). According to Clegg and Clegg (1969), administration of estrogen results in the activation of RNA-DNA

synthesis and increases the rate of RNA and protein synthesis. "This sequence of events is what one could expect if oestrogen triggered off these various changes by a primary effect at the level of the gene, i.e. on RNA synthesis."

The present study is to be an evaluation of the growth response of adult human intestinal cells in tissue culture to 17-β-estradiol. The large intestine is not considered to be a target tissue and no previous reports have been made of any intestinal cellular response to stimulation by steroid hormones. The probable origin of the fibroblastic growth normally obtained with the tissue culture methods to be used is the lamina propria, a connective tissue layer tightly attached to the mucosa. The importance of this study is enhanced by the fact that the cultures to be studied are derived from adult tissue since most work has been done with embryonic tissue and relatively little information is available about cultured adult tissue. Tissue culture provides a simplified system in which variables other than the direct action of the hormone can be controlled (Merchant, Kahn, and Murphy, 1964). However, it must be kept in mind that this simplification of cell environment does not allow conclusions of *in vitro* studies to be directly applied to *in vivo* conditions without substantiating information from *in vivo* studies.

DESIGN OF EXPERIMENTS

Development and Use of a Perfusion Chamber

Rationale for using a perfusion chamber technique has been suggested by many authors.

> Time-lapse cinematography is an excellent method for studying and recording the normal activity of single cells *in vitro* when used in conjunction with a perfusion chamber. In addition, the effect of agents such as drugs, hormones, detergents or carcinogens may be demonstrated in the living cell with this technique. (Merchant, Kahn, and Murphy, 1964)

At the time the experiments were planned, the equipment available included only standard phase-contrast microscopes and photographic equipment that could be used with them for making sequential still photographs. However, neither Leighton tube nor T-flask cultures could be viewed, even under low power, with phase equipment. Dissecting microscopes were not acceptable since they easily heated cultures above tolerable limits and did not offer sufficient magnification. Since at this time our lab did not have an inverted phase-contrast microscope, observation of living cells depended on acquisition of some sort of perfusion chamber. The idea of making observations of single cells or of small numbers of cells suited our purposes since we had limited amounts of tissue. Growth from explants had provided only a few partially covered 11 X 35 mm coverslips in Leighton tubes.

The basic plan for a number of different perfusion

chambers includes a supporting framework of metal or plexiglass for attachment of two coverglasses so that they are no more than 1 or 2 mm apart and an aseptic means of introducing and changing media for prolonged culture. This is accomplished by channels and grooves in plexiglass chambers or by insertion of hypodermic needles through the rubber gasket of the metal plate chambers. Toy and Bardawil (1958) and Christiansen et al. (1953) have developed plexiglass chambers; Rose (1954), Paul (1957), Sharp (1959), and Sykes and Moore (1960) have worked with metal chambers. A commercially available Sykes-Moore perfusion chamber is described by Merchant, Kahn, and Murphy (1964), but it is fairly expensive and is too large in diameter to be used with the Labline "Incu-stage", a highly recommended stage incubator (Christiansen et al., 1953). Construction of our own chambers appeared to be a necessity.

The Curtis modification of the Rose perfusion chamber (1954) consists of metal plates holding together two coverslips sandwiching a silicone rubber gasket. See figure 1 for dimensions and construction details. The desired characteristics that it must provide are as follows: 1) optically flat, parallel surfaces 1 mm to 1.5 mm apart for viewing under high power; 2) change of medium with minimal difficulty using modified 25 gauge hypodermic needles guided by alignment channels provided in the bottom half of the chamber; 3) non-toxic and sterilizable components, Corning

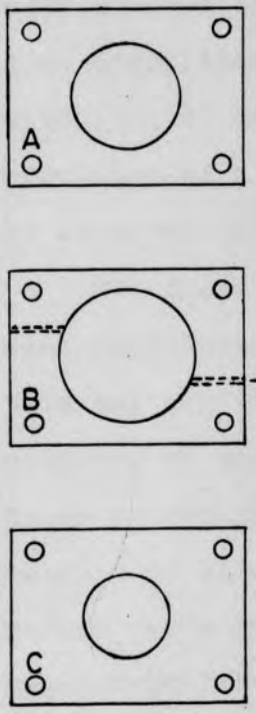
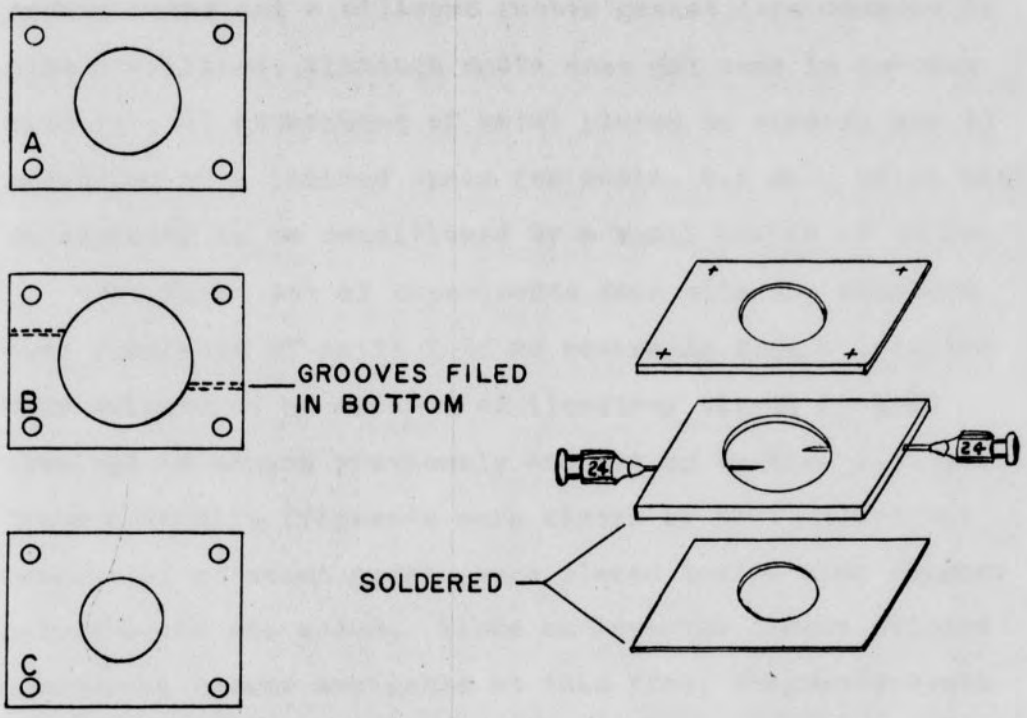
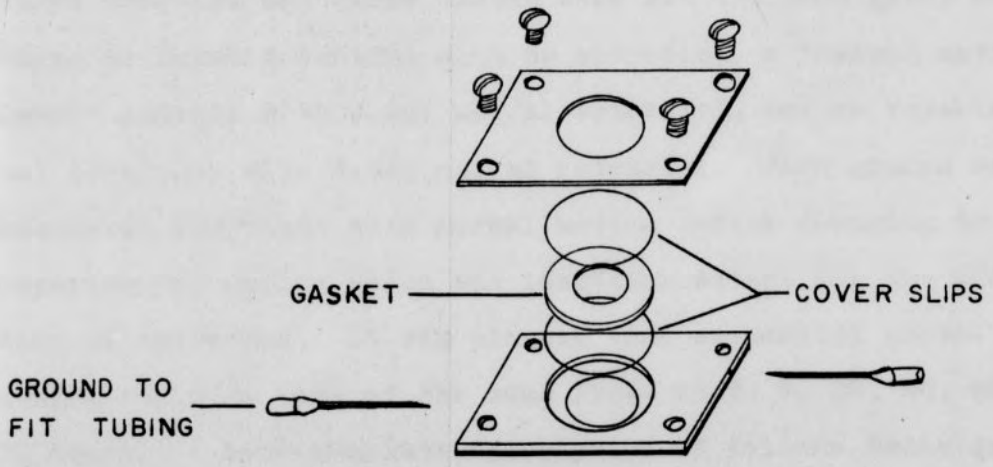


Figure 1. Construction details and dimensions of the modified-Rose perfusion chamber used in the early part of this study. Plates A, B, and C are shown in actual size. Plate A is made from 1/32 inch brass; Plate B is made from 1/16 inch brass; and Plate C is made from 0.010 inch brass shim stock. Plates B and C are soldered together with rosin-core solder while No. 24 hypodermic needles are in place. When the plates have cooled, the stainless steel needles are given a twist to break them loose from the solder and they are removed. The channels, thus formed, provide a steady guide for the smaller No. 25 hypodermic needles used to penetrate the 1/16 inch silicone rubber gasket. Plates A and B,C are clamped together while being drilled and tapped for the four 4-40 X $\frac{1}{4}$ inch round-head brass screws. A model maker's razor saw is used to cut off the excess bolt length while they are in place. The circular coverslips are Corning No. 2, 18 mm. In actual use 3/32 inch I.D. Nalge sterilizable P.V.C. tubing carries medium from a hypodermic syringe to the No. 25 hypodermic needle which has been ground down and polished to fit within the tubing. In a similar manner, medium is carried from the chamber and exits through a piece of sterile, cotton-stoppered P.V.C. tubing.


A
B
GROOVES FILED
IN BOTTOM
C
24
24
SOLDERED


GASKET
COVER SLIPS
GROUND TO
FIT TUBING

coverglasses and a silicone rubber gasket (the chamber is also sterilized, although media does not come in contact with it); 4) attachment of metal plates by screws; and 5) provision of a limited space for media, 0.1 mm^3, which might be expected to be conditioned by a small number of cells.

The first set of experiments done with the chambers used fragments of an 11 X 35 mm coverslip from a Leighton tube culture of an explant of ileostomy tissue 69-2982 obtained 18 months previously and set up by Mrs. J.O. Hall. These coverslip fragments were chosen so that equivalent amounts of attached growth were placed inside each chamber before media was added. Since an inverted tissue culture microscope became available at this time, fragments could also be placed in plastic flasks for treatment with estradiol. Three chambers and three flasks were set up; each group of three included a control with no estradiol, a "normal estrogen level" control with 0.001 mcg/ml estradiol, and an experimental condition with 0.021 mcg/ml estradiol. Both groups were incubated overnight with normal medium before changing to experimental medium which was identical except for the addition of estradiol. It was planned that sequential photographs would be made of the same field at 0, 4, 24, 48, and 72 hours. A more complete description of culture techniques and photographic details is included in the Materials and Methods chapter. As could be expected for a first run, many details remained to be worked out. Coverglass breakage in

two of the three chambers necessitated reassembly which resulted in detachment of cells from the fragments. The one remaining chamber was treated with 0.021 mcg/ml estradiol and its zero hour picture used as a control. In the initial handling of fragments for flasks, the "no-estradiol control was damaged. This was determined later when the zero hour picture for this flask was examined. Growth in the other two flasks appeared normal. Location of the same area for photographs in the single remaining chamber was much easier and, as it turned out, the only area that could be positively identified. Figure 2 illustrates changes that were observed.

A second attempt to run this experiment was successful in terms of chamber assembly with minimum damage to cells. However, the appearance of these cells rapidly deteriorated as characterized by increased granularity of cytoplasm and contraction of processes which were probably due to an insufficient number of cells to properly "condition" the medium. Figure 3 shows these changes. Figure 4 illustrates two other factors that would be important in interpretation of this type of study: 1) dense packing of cultures with growth so heavy that cytoplasmic boundaries are obscured and 2) proximity of remains of the original explant. One of the highlights of our observations was the discovery of large numbers of mitotic cells in the stock culture from which we had obtained coverglass fragments. Figure 5 shows quite a few of these cells with their characteristically rounded-up

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Figure 2. Cell movements and "growth" observed in perfusion chamber studies. This area from a coverglass fragment with cells from the 69-2982 ileostomy stoma culture was photographed at the following times after treatment with 0.021 mcg/ml estradiol.

A. 4 hours

B. 24 hours

C. 48 hours

D. 72 hours

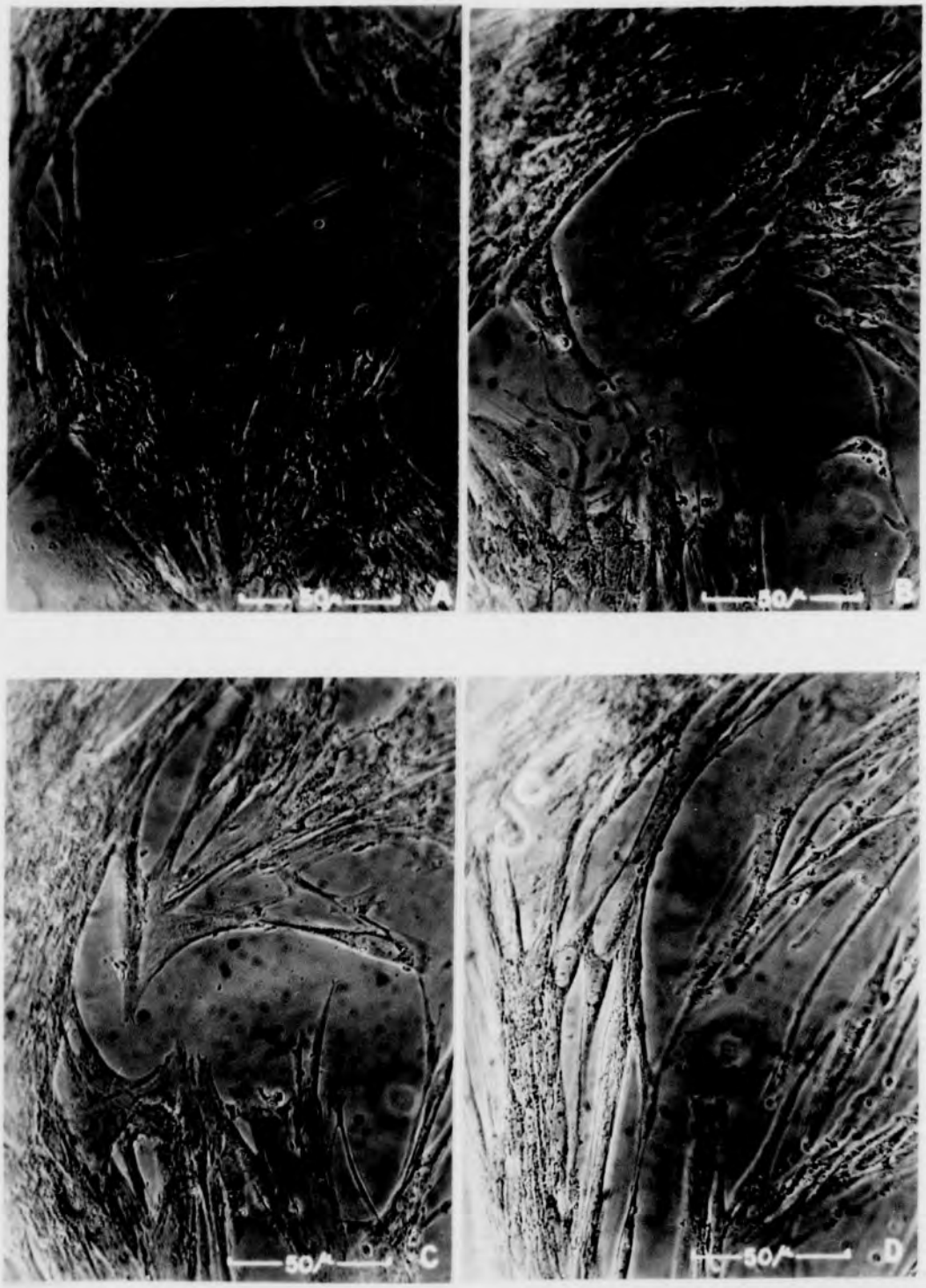

50A
A
50A
B
50A
C
50A
D

Figure 3. Increased granularity of cytoplasm and contraction of processes attributed to an insufficient number of cells to properly condition the medium.

A. Cells photographed immediately after coverslip fragment was placed in the perfusion chamber

B. Another location photographed at the same time as A

C. The same area as photograph A, but showing deterioration after 4 hours.

D. A similar deterioration of cells on a coverglass fragment which occurred in a culture flask

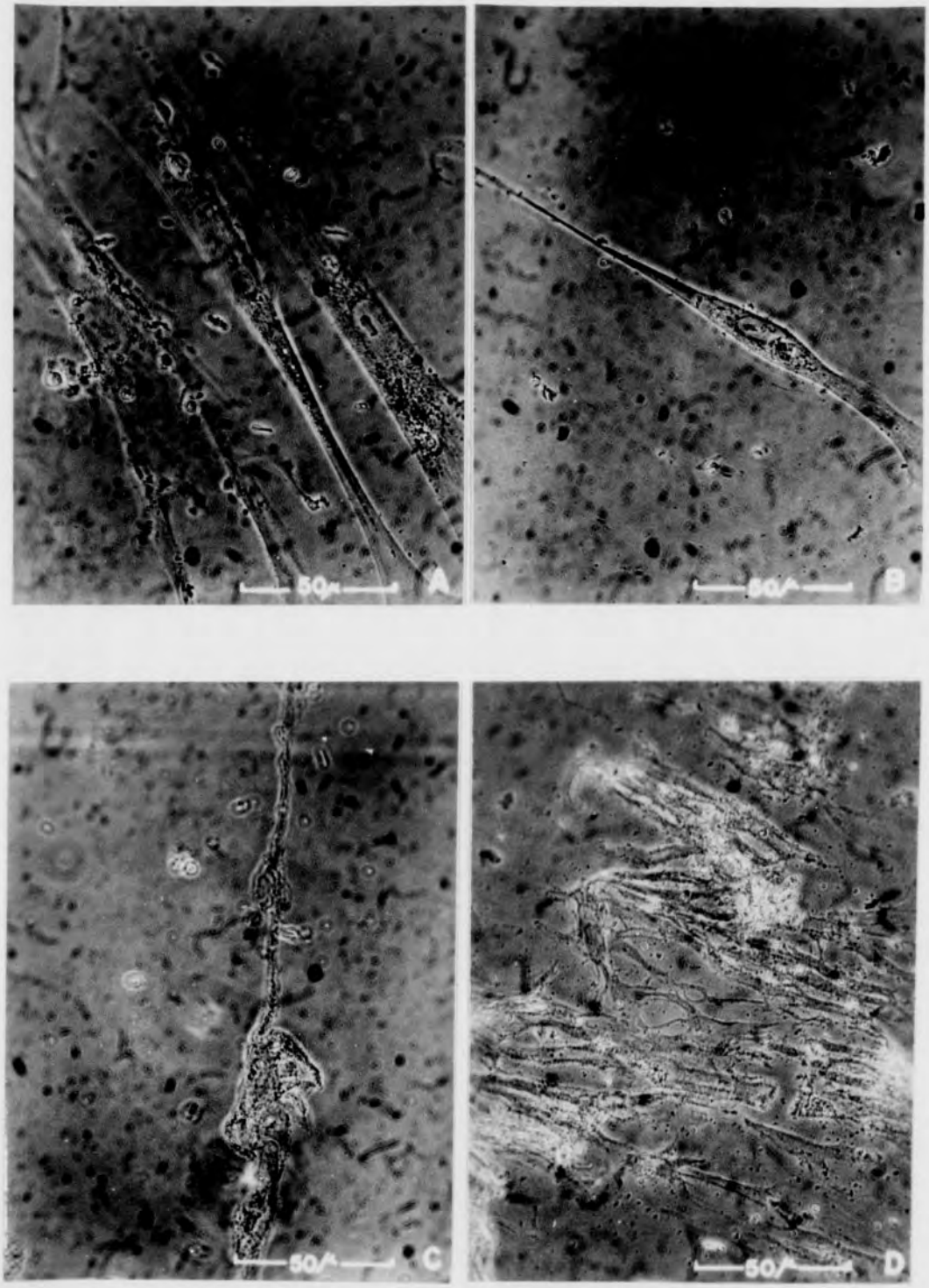

50A
A
50A
B
50A
C
50A
D

Figure 4. Two factors which present interpretation difficulties with coverglass fragments from explant cultures.

A. and B. Inclusion of parts of the original explant in the study areas

C. and D. Areas of densely packed cell growth

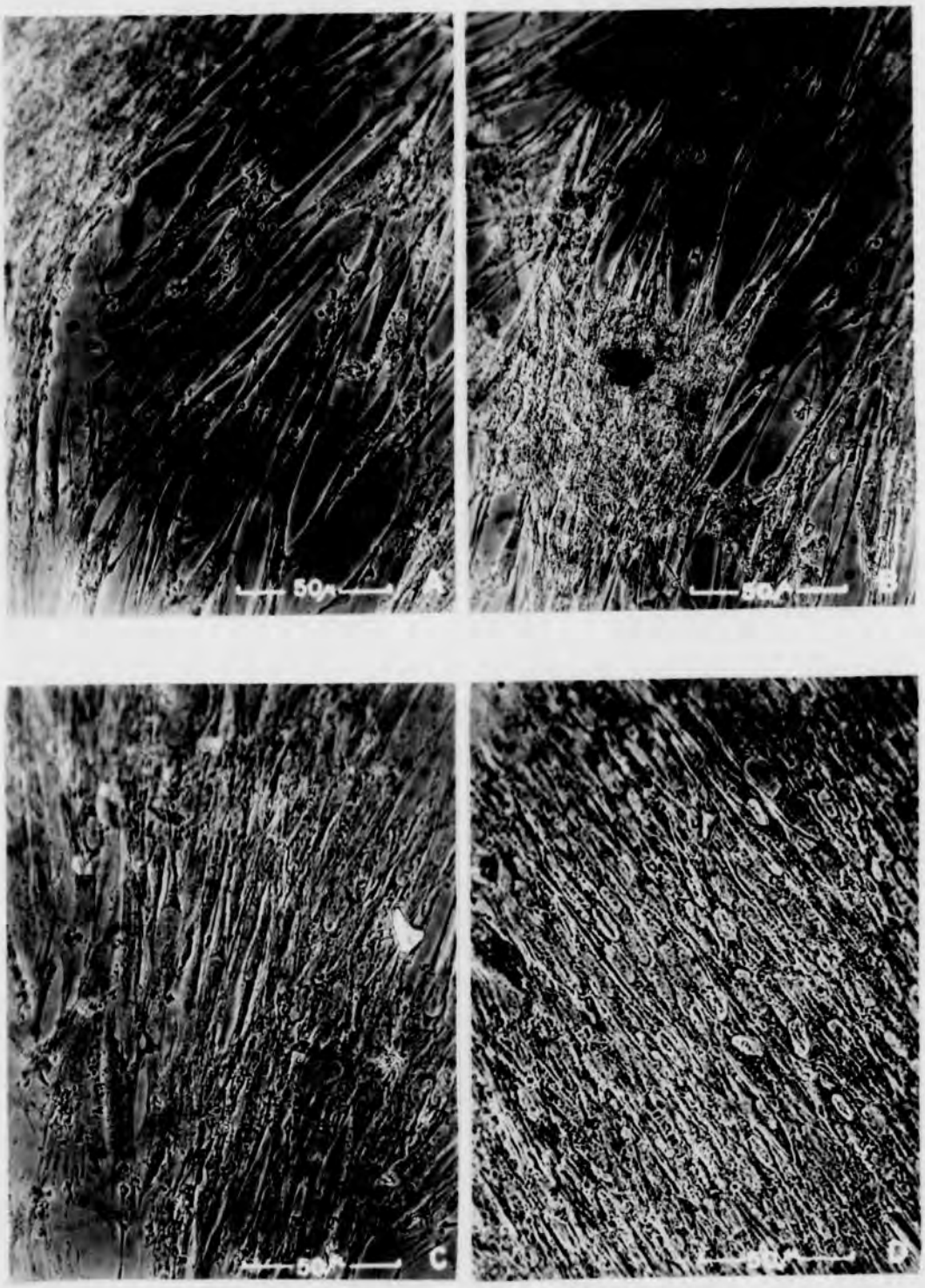

50A
A
B
C
D

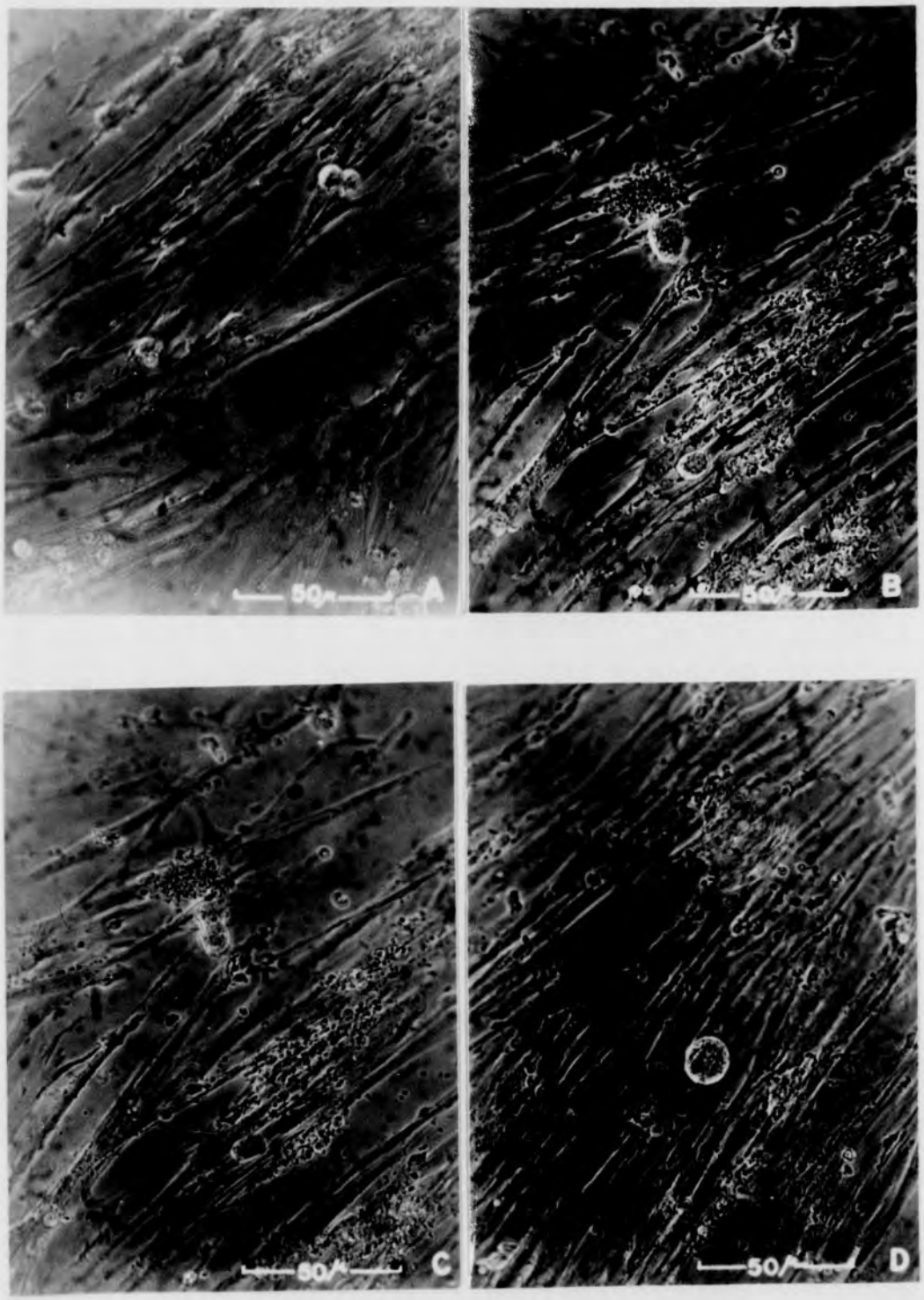
Figure 5. Dividing cells in the 69-2982 ileostomy stoma stock culture.

A. Several dividing cells (identifiable by rounded-up cytoplasm and phase-haloes)

B. Two dividing cells

C. The same area as B. photographed 10 minutes later (complete division required 20 minutes)

D. Suspected polyploid mitotic cell


A
50
B
50
C
50
D
50

cytoplasm and "phase-haloes".

New Materials and Techniques

As mentioned in the previous section, an inverted phase-contrast microscope became available after the construction of the perfusion chambers. This permitted the examination of cultures grown in large flasks that could not be used on a standard microscope. At this point the factor that limited expansion of studies was the small amount of growing tissue.

Two events acted to make a much larger amount of tissue available. First was the acquisition of a colonic biopsy 70-8924 which was set up as will be described in the Materials and Methods section concerned with explant cultures. Growth of fibroblastic cells from this tissue, particularly the part derived from the base of the polyp that was described as "normal" tissue by the pathologist, was unusually good, covering all of the area under the coverglass within a month. The second event that enabled us to increase the growth obtained from this biopsy was the method of subculturing using a combination of trypsin and versene that was shown to us by Mrs. Betty Hammel of Indiana University at Bloomington. In addition to providing large numbers of cells, this method insured a rather uniform dispersion of cells which was desirable since it permitted one to randomly choose approximately comparable areas for quantitative studies.

The most important contribution of this early work was that it made clear the necessity of choosing between a few continuous, phase-contrast observations with perfusion chambers and many phase observations of flask cultures that could be compared with subsequent bright-field observations of fixed and stained preparations of the same material. González-Ramírez's (1963) phase-contrast, high magnification observations of nucleolar function in living tissue culture cells represent a type of information about cells that might best be acquired by perfusion chamber methods. Difficulties with our early attempts suggested that the direction of quantification of differences between estradiol-treated and untreated cultures would be more prudent, especially since the response of a non-target tissue, if there were any, might be expected to be of a lesser order of magnitude and more difficult to recognize. Hanks (1953) makes the following observation about the problem of choosing between qualification and quantification:

> The temptation to compile photographs and an endless volume of notes is almost irresistable. The new investigator must be warned that many capable observers have recorded such material for years, often without being able to employ it as analytical data bearing on any specific problem. This dilemma can be avoided by setting out to learn <u>something in particular</u>, by designing protocals for each essential maneuver, and by constructing tables for the entry of numbers which designate the essential information anticipated. It is of prime importance to devise methods of obtaining <u>actual measurements or of translating observations into numerical data</u>.

MATERIALS AND METHODS

Preparation of Glassware and Media

Cultures were grown in glass and plastic culture flasks, in addition to the perfusion chambers already described. Falcon 30 ml polystyrene flasks were used for most secondary cultures and for all but the perfusion chamber experiments. The plastic flasks exhibited both advantages and disadvantages for tissue culture. They were inexpensive, were nontoxic to cells, and did not require the careful washing and sterilization that the regular glass vessels did. Also, it was discovered that cells grown in media high in fetal bovine serum (20% FBS) adhered so firmly to plastic that they could not be removed even with the trypsin-Versene solution normally used for subculturing. This property was beneficial to our preparation of plastic slides from these flasks. Disadvantages included the fact that plastic flasks could not be flamed without destroying their air-tight quality. Merchant, Kahn, and Murphy (1964) have described a second major disadvantage as "a rather severe limitation on use of common fixatives and staining reagents." Particularly, xylene and xylene-diluted mounting media melted polystyrene and caused it to become opaque.

Primary cultures were set up in Earle's T-15 Pyrex culture flasks having a 15 cm^2 culture area and obtained

from Bellco Glass Co.; these flasks were closed with No. 00 West silicone stoppers. Bellco 11 X 35 mm coverglass inserts were used inside the T-flasks as they were in the 16 X 125 mm Leighton tubes which we also used. Neither the Leighton tubes nor the Earle's T-flasks provided the optical quality desired for phase observations; however, plastic flasks had much flatter bottoms and, therefore better optical quality. The fact that cells often grew on both sides of the coverglass as well as on the bottom of the culture vessel complicated phase observations. On the other hand, staining of the removable coverglass would offer an additional avenue of study. But difficulties with cell detachment from the coverglass during staining appeared to out weigh the benefits.

New glassware was soaked overnight (18 hours) in 1% HCl (Penso and Balducci, 1963); next it was washed in Microbiological Associates' Microsolve washing compound according to directions. All glassware was boiled in ½% Microsolve solution, rinsed 10 times in running tapwater, rinsed 2 times in deionized glass distilled water, dried in an oven, and sterilized in an autoclave at 121° C for 20 minutes. Used glassware was autoclaved if it contained cells; and all glassware was rinsed before waiting to be washed. It was necessary to separate all tissue culture materials from general lab glassware that might have come in contact with toxic chemicals such as formalin and colchicine (Merchant, Kahn, and Murphy, 1964)

The medium used for growth of cell monolayers and tissue explants was Eagle's Minimum Essential Medium (MEM) with Earle's balanced salts solution, which contained the necessary salts, stable amino acids, vitamins, glucose, and phenol red. To this was added fetal bovine serum (FBS) which provided a source of amino acids, glucose, and growth stimulating factor; the antibacterial agents streptomycin, penicillin, and neomycin; the antimycotic agent Fungizone; and glutamine, a less stable amino acid, as follows:

MEM with Earle's BSS	80 ml	
FBS	20 ml	
Pen-Strep	2 ml	of 10,000 units and 10,000 mcg/ml stock
Fungizone	0.1 ml	of 250 mcg/ml stock
Neomycin	1 ml	of 10,000 mcg/ml stock
Glutamine	1 ml	of 100 X stock

Amounts of 2% to 20% fetal bovine serum or other "embryo juice" have often been recommended for tissue culture. Lower concentrations of FBS have been used for maintenance media, and larger amounts were used when active growth was desired. Requirements for FBS are known to vary for different tissues. According to Parker (1962), serum proteins are thought "to provide essential growth factor(s) of small molecular weight, either bound to the serum protein or formed from it on proteolysis."

The primary cultures were set up using FBS obtained from Microbiological Associates and Neomycin supplied by The Moses H. Cone Memorial Hospital. All other ingredients were obtained from Grand Island Biological Company as were all

media components subsequently used. The concentration of each antibiotic was twice the normal amount, as recommended by Mrs. Alice Ashton of the State Public Health Laboratory, since intestinal tissue is not bacteria-free initially and may retain some bacteria in spite of the heavy antibiotic wash used prior to setting up primary cultures. This antibiotic wash consisted of 12.4 ml of Hank's balanced salts with phenol red, 9 ml of Penicillin-Streptomycin with 10,000 units/ml and 10,000 mcg/ml respectively, 1.1 ml Neomycin with 10,000 mcg/ml and sterile 1.4% $NaHCO_3$ to adjust pH.

Control of pH of culture medium was accomplished by using the carbonate-bicarbonate system included in the balanced salts solution, as well as the action of amino acids and serum. Inclusion of phenol red in the medium provided a visual index of pH change since it appears yellow when acid, pH 6.8 and below, and purplish-red when alkaline, pH 7.2 and above. Adjustment of pH could be made with 0.3 N NaOH and 0.3 N HCl or preferably with 1.4% $NaHCO_3$ and gaseous CO_2 (Merchant, Kahn, and Murphy, 1964). Air-tight vessels are essential to pH maintenance.

Preparation of Explant Cultures from Colonic Biopsies

Two small pieces of tissue, approximately 3 mm^3 each, were removed from a 55 year old male, No. 70-8924, on June 25, 1970. One piece, described as normal by the pathologist, had been excised from the base of the stalk of the polyp;

the other was described as an adenomatous polyp and was quite red due to blood infiltration. The tissue was placed on ice while being transported to our lab.

The polyp and normal tissue were aseptically prepared in separate containers by Mrs. Joan O. Hall and myself as follows: 1) tissue was cut with iridectomy scissors and forceps into pieces approximately 1 mm^3 while immersed in the heavy antibiotic wash; 2) these pieces were rinsed a second time in heavy antibiotic wash for 5 minutes; 3) for each specimen, 3 pieces were placed in each of seven Earle's T-15 flasks which contained an 11 x 35 mm coverglass that was flipped over on top of them to keep them in contact with the bottom of the flask (Chu, 1970); 4) each flask was passed briefly through a flame to help attach the tissue; 5) 3 ml of culture medium was carefully added to each flask so as not to dislodge the tissue; and 6) each flask was slowly gassed with 5% CO_2 in air which was passed through a sterile, cotton plugged capillary pipette at approximately 2-3 lbs/in^2 for about 1 minute or until the purplish-red, alkaline color of the indicator had just started changing to bright red. All flasks were incubated at 37° C and examined daily under the tissue culture microscope for migration of cells out from the explant. One flask each of normal and polyp tissue was overrun by bacteria which were shown by Gram staining and growth on EMB plates to be of the kinds normally found in the colon. The remaining 12 flasks showed

cell migration of at least one piece of tissue in each flask within 3 days. Media was changed 3 times a week and further gassing was not required as sufficient acid was produced as a metabolic byproduct.

Preparation of Secondary Cultures or Subculturing

When cell growth covers most of the bottom of the flask, cells can be harvested and transferred to additional containers where they attach to the glass as a monolayer. For our observations the monolayer technique "... has the advantage of being simple and reproducible ...." (Merchant, Kahn, and Murphy, 1964) Besides providing a rather uniform layer, subculturing generates an increase in cell numbers by removing the restriction of limited space.

Subculturing is accomplished by replacing culture medium with an equal volume of a 1: 100 mixture of Difco Bacto-trypsin and GIBCO 1: 5,000 Versene. Cells are incubated at 37° C with this mixture for 5 to 15 minutes or until gentle shaking produces a cloudy suspension of detached cells. This suspension is diluted with a 3 X volume of culture medium and divided among the original and additional containers.

This technique was recommended by Mrs. Betty Hammel (1970), Culture Preparator of the Microbiology Department of Indiana University at Bloomington. It is a modification of the method of Madin and Darby (1958) for using a combination

of trypsin and Versene. Our attempts to use either of these agents separately have been unsuccessful. One feature of this method, which is unlike that of Madin and Darby, is that no attempt is made to separate the enzyme-chelating agent mixture from the cell suspension by centrifugation. According to Mrs. Hammel the serum component of the culture medium abolishes the effects of the trypsin-Versene combination. Cells can be subcultured from plastic flasks if they have been grown in 2 to 5% serum media but not 20% serum medium. It appears that the amount of serum determines how tightly the cells attach to the surface. Cultures growing on glass do not adhere as tightly, but they do adhere tightly enough with 20% serum that trypsin or Versene alone cannot loosen them, thus causing our initial difficulties.

Choice of Estradiol Concentration

Schering Corporation's Progynon aqueous estradiol suspension for injection was chosen since it provided a sterile suspension of crystalline estradiol in isotonic saline. One 10 cc multiple-dose vial of Progynon provided 0.25 mg/ml estradiol, an amount sufficiently large for dilution. Choice of a widely-available, prepared suspension inhances the "repeatability" of this study.

It was desired that the concentration of estradiol to be used in this study represent the average levels found in

adult females during the normal monthly cycle, during pregnancy, or during the administration of estrogen for such purposes as birth control and

> treatment of menopausal disorders, hypogenitalism, sexual infantism, amenorrhea and oligomenorrhea associated with hypogonadism, postpartum breast engorgement, inoperable breast carcinoma in post-menopausal women ..., senile vaginitis and pruritus or kaurosis vulvae. (Schering Product Information, 1968)

Estimation of average physiological concentrations of estradiol is based on determination of blood levels of this steroid. According to Svendson (1964), the normal adult female range is 0.1 to 1.0 mcg/liter or 0.0001 to 0.001 mcg/ml; in pregnancy estradiol is estimated at 15.0 to 30.0 mcg/liter or 0.015 to 0.030 mcg/ml. Birth control pills of the 2 mg size add a maximum of 0.1 mg of the estrogenic component to the average 5,000 ml of blood in the body; and assuming that all estrogen is absorbed and equally distributed, this represents 20 mcg/liter or 0.02 mcg/ml. British medical research groups have encouraged women to switch to the 1 mg pill size which has an actual estrogen content of 0.05 mg or 0.01 mcg/ml. The range of doses suggested by Schering for intra muscular injections 2 to 3 times per week for estrogen therapy is 0.5 mg/5 liters to 1.5 mg/ 5 liters or 0.1 mcg/ml to 0.3 mcg/ml. An intermediate value of 0.2 mcg/ml was selected. The three conditions used in these experiments were 1) no estradiol, control; 2) 0.02 mcg/ml estradiol, normal female taking the 2 mg pill or pregnant female; and

3) 0.2 mcg/ml estradiol, estrogen therapy. In the perfusion chamber experiments, but omitted later, another concentration 0.001 mcg/ml was used to represent a normal female in mid-cycle; this was to provide a comparison with a normal estrogen level.

Other authors (Ozello, 1964; Rao, 1969; and Goldberg, 1966) have studied the effects on tissue of estradiol concentrations up to 21 mcg/ml and described these as physiological concentrations with minimal toxicity. These amounts are well above the 0.2 and 0.02 mcg/ml concentrations chosen for the present study.

Phase-Contrast Observations

Since the experimental plan required sequential photographs of estrogen-treated cultures at 2, 4, 8, 24, 48, and 72 hours, the factor of time needed to locate and photograph the same position in a flask, approximately 3 minutes each, limited the number of flasks that could be studied in this manner. Three groups of 3 flasks or a total of 9 appeared to be as many as could be handled. A period of slightly less than 30 minutes in which the cells were not incubated at 37° C, but at room temperature which was between 25° and 30° C, occurred at each photographic interval as well as the approximately 10 to 15 minutes required to change media each day following photographs.

Cells for the first experiment, October 2-5, 1970, were subcultured September 28, 1970 as follows. Stock cultures

which were 13 weeks old and had been subcultured 6 times, of 70-8924 normal that appeared healthy and relatively free of cellular and extracellular granules were chosen. Fifteen plastic Falcon flasks were filled 3 at a time from a 10.0 ml Plasti-Pak syringe with 3.0 ml each of pooled cell suspension in serum medium (20% FBS in MEM). After all flasks were filled, gassing of these for 45 seconds each at 2 lbs/ minute was begun. At this point a viable cell count by the Trypan Blue method (Merchant, Kahn, and Murphy, 1964) was made of the cell suspension, showing 3.6×10^4 cells/ml. Haemocytometer counts are subject to several types of error that make them unreliable estimates. Time is a critical factor in these processes since cells begin to attach to available surfaces within 30 minutes of addition of serum-containing medium and return to normal morphology in 24 hours. It was hoped that use of pooled cell suspension and rapid filling of flasks, 3 at a time, would insure uniformity. The first syringe-full of cell suspension was used to fill flasks designated 1-0, 1-0.02, and 1-0.2 as shown below. Estradiol designation was chosen randomly.

Estradiol designation	E-0 (Control)	E-0.02 mcg/ml (Pill or pregnancy)	E-0.2 mcg/ml (Estrogen therapy)
1st 3 flasks filled	1-0	1-0.02	1-0.2
2nd 3 flasks filled	2-0	2-0.02	2-0.2
3rd 3 flasks filled	3-0	3-0.02	3-0.2

A period of 4 days elapsed before estradiol was added; the first 3 days were required for the cells to increase in number to a point where the observation fields would contain enough cells to be worthwhile. One day was used in working out a way to mark locations to be photographed. This was done by finding a suitable location at 100 X magnification with the tissue culture microscope and placing a grided "C-THRU" ruler with a 1.5 mm diameter hole under the flask until the field and hole corresponded; then, while the flask and ruler were held in place, a dot of India ink was made at one side of the hole with a Kohinoor No. 1 Rapidograph pen. Accuracy in finding the same area for later photographs was improved by making additional marks around the area. In the first experiment, October 2-5, all positions were located at the far end of the flask away from the neck where disturbance by media changing might occur. Also, it was realized that cell growth was heavier at the far end of every flask due to the fact that the tray upon which they were placed for incubation was warped so that after subculturing more cells attached to the "deeper" end of the flask. This peculiarity invalidated our estimation of the cells per unit area from the haemocytometer viable cell counts. It also required that cell locations be placed the same distance from the end of the flask for comparable areas of cell density.

On October 16, 1970 subcultures for the second experiment were made from the 70-8924 normal stock cultures which

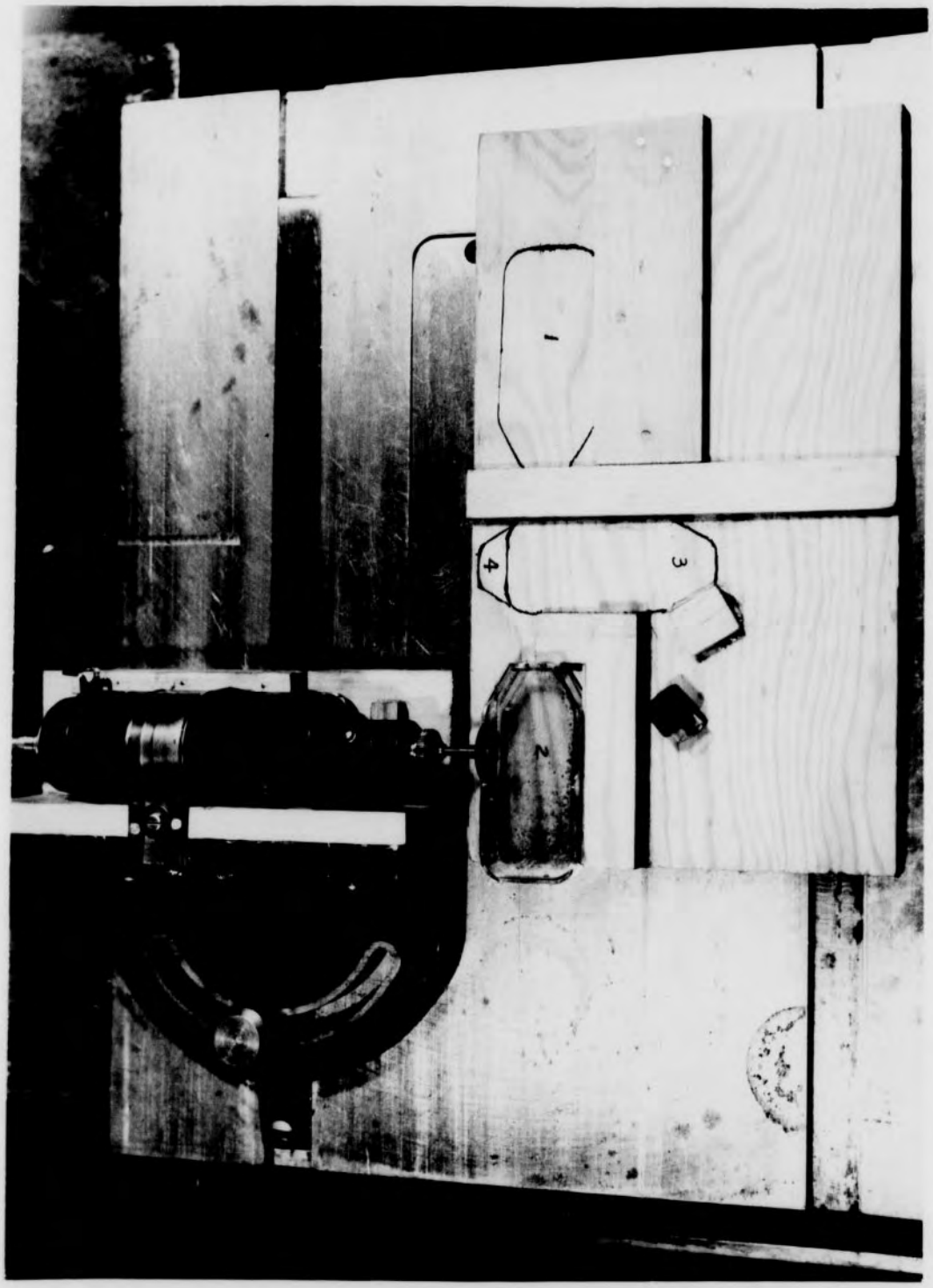
were 16 weeks old at this time and had been subcultured 9 times. Preparation of these subcultures was identical with that for the first experiment except that 5 days, due to poor growth, instead of 4 elapsed between subculturing and addition of estradiol.

Fixation, Preparation of Plastic Slides, and Polychrome Staining

After 3 days each experiment was terminated by replacing culture medium with 10% neutral buffered formalin. According to the method of fixation of Merchant, Kahn, and Murphy (1964), culture medium was poured off and two rinses of warm balanced salts solution were made before adding 3.0 ml of 10% neutral buffered formalin (Carolina Biological Co.) to each flask. Flasks were rinsed 12 hours in running tap water before sawing.

Conversion of 15 ml Falcon plastic flasks into plastic slides of approximately 1 X 2½ inch size for staining and histological study was accomplished by sawing the slide-size sections from the bottoms of the flasks while the flasks were held in a special jig set up for this operation as can be seen in Figure 6. The jig was mounted on a table saw in order to utilize the guide to which a Dremel Moto-tool was rigidly affixed. The high speed of the Moto-tool, 27,000 RPM, and the very thin (.005 inch), fine-toothed blade, Dremel No. A-6, allowed precision cutting of the styrene plastic with little wastage of material. After 4 sides of the slide were sawed, it remained in place in the

Figure 6. Jig and Moto-tool arrangement for making microscope slides from plastic flasks.


1
2
3
4

flask due to a slight re-weld from saw blade heat, and gentle thumb pressure was used to free the slide which was immediately immersed in water to prevent drying of the tissue. While the slide was submerged, a No. 2 X-Acto knife was used to scrape away the flashing that accumulated on the edges of the slide during sawing.

Finished slides were temporarily stored in Coplin jars filled with water for return to the lab where they were stained. All slides were stained at the same time to avoid variation that might occur with aging of the stains. The staining method chosen was a modification of Lehman's (1965) polychrome staining procedure that had been used previously in Dr. Anderton's lab and might be used later for comparison of tissue sections with tissue culture monolayers. Choice of this procedure is warranted since it "... will demonstrate in single sections the distribution and relative amounts of nucleic acids and mucopolysaccharides and will distinguish acid or neutral proteins from basic and -SH rich proteins." (Lehman, 1965) Directions were followed in detail except for the addition of 1) an initial 30 minute staining period in 1% Alcian Blue in 3% acetic acid and the subsequent 2 minute rinse in running tap water and 2 minute rinse in distilled water and 2) clearing and mounting with oil of cedar instead of xylene and xylene-diluted mounting medium which would cause the polystyrene slides to become opaque.

Lehman (1965) describes the colors of different

cellular components as follows:

> Celestine blue stains nuclei steel blue and cytoplasmic RNA lavender. Napthol yellow S stains histones, hemoglobin, keratin, and other basic proteins or proteins rich in -SH groups yellow. Aniline blue stains all mucopolysaccharides various shades of clear blue. Chromotrope 2R stains acid and neutral proteins scarlet. When two of the above molecular components are concentrated in the same cellular region, the multiple dye binding will result in complementary colors. For example, mitotic chromosomes appear green by virtue of celestine blue staining of nucleic acids and napthol yellow S staining of chromosomal histones.

Alcian blue stain is specific for neutral and sulfated mucopolysaccharides.

Analysis of Stained Slides

Three types of assessment of differences between estrogen-treated and control cultures were made with bright-field observations: 1) mitotic coefficient, 2) histochemical index by staining differences, and 3) nuclear size index.

Measurement of increase of cell numbers was made by determining the mitotic coefficient of "number of mitoses per 100 cells" (Merchant, Kahn, and Murphy, 1964) of polychrome stained slides. Where mitotic coefficient is low, total cells counted must be increased for greater accuracy. For the first experiment a minimum total of 2,000 cells were counted by scanning the slide with 312 X magnification and noting the number of mitoses per field and total number of cells per field before moving over exactly one field to repeat this operation. Counts were made at the same distance

from the end of the slide using the coordinate system on the mechanical stage of the microscope. Very low cell counts in the second experiment required that additional rows of fields be counted.

At the same time that mitotic coefficients were determined, a count of unusually colored nuclei was made. According to Lehman most nuclei would stain steel blue; however, some nuclei appeared distinctly lavender, green, or yellow indicating a difference in relative chemical composition.

A Bausch and Lomb ocular micrometer was used to measure maximum length and width of the predominantly oval nuclei of 30 fibroblastic cells per slide. An index value was obtained by multiplication of length times width measurement for each nucleus to include both parameters.

Chromosome Analysis of the Stock Culture

Following the second estradiol experiment, chromosome studies were made to assess the state of the stock culture of 70-8924. Merchant, Kahn, and Murphy (1964) suggested that cultures to be studied should be recently subcultured and therefore in "early or mid log phase of growth" and that the cultures should be incubated 8 hours with colchicine before harvesting. Harvesting and preparation of slides from the cell suspension, which was made by treating the flask with trypsin-Versene mixture used in subculturing, was done

according to Gibco directions (1970) for peripheral blood cultures. The procedure of Moorhead and Nowell (1964) was followed for Feulgen staining of chromosomes.

Determination of modal number of chromosomes was based on a minimum of 30 nuclear spreads in which chromosomes could be counted. Counts were made using 1,000 X oil immersion magnification. Four of these spreads were photographed on Kodak Plus-X-Pan Professional film using the Zeiss microscope, a wide band interference filter (Carl Zeiss Inc.), and roll film back attachment. Chromosomes from enlargements printed on Kodabromide F-4, 8 X 10 inch photographic paper were cut out, matched in pairs for diploid chromosome complement, and arranged according to the Chicago Conference (1966) combining the Denver and London systems of classification.

Other Photographic Equipment and Procedures

Phase-contrast photomicrographs were made with an Olympus tissue culture microscope with 10 X and 20 X phase objectives and a trinocular head on which a 4 X 5 inch film back was mounted by means of a Zeiss vibration-free camera support. Plus X Pan Professional film was used. Magnification of 100 X was used since this had been satisfactory for similar photographs made in June 1970 with both plastic flasks and chambers. Unfortunately, when the equipment was moved during the summer the 5 X ocular was removed from the photographic tube and replaced with a 10 X ocular. Since

the 5 X ocular was not found until after both experiments had been terminated, it was necessary to substitute the 10 X ocular and the 10 X objective combination for the 5 X ocular and 20 X objective combination used in June. The rule that significant resolution is gained by power of the objective and not the ocular was clearly demonstrated. Due to the decrease in quality, substitution was made of a 7 X ocular for the 10 X, for a total of 70 X instead of 100 X, in hopes that some sharpness could be gained with lower magnification.

An advantage of the 4 X 5 inch film size is a sufficiently large image not to require further magnification so that it can be contact printed. Negative size and print size remain the same so that objects photographed with the same microscopic magnification are shown as the same relative size in the print. Prints were made on Kodak Azo F-5 contact print paper.

Color photographs of locations on stained slides were made with a Miranda Sensorex 35 mm camera attached to the Zeiss microscope. Agfachrome transparencies were made using an Ednalite 82 A blue filter and the blue Olympus microscope filter above the field diaphragm; this diaphragm was set at 16.5 and the transformer was set at the red line position. Color prints of the slides were obtained from Eastman Kodak in Atlanta.

Other black and white photographs were made with the same equipment and film type used for chromosome pictures

except that appropriate magnification and exposures were used.

RESULTS

The sequential photomicrographs in Figures 7 and 8 show at least one kind of change that could be observed in the control and 0.02 mcg/ml estradiol-treated cultures, respectively. They form a descriptive record of an increase in cell division in some of the estradiol-treated cultures. Table 1 includes the number of mitotic figures observed in the photographic record of all flask locations of the first and second experiemnts.

TABLE 1. Mitotic Cells Recorded in Sequential Photomicrographs

Group 1

Flask No.	0 Estradiol	0.02 mcg/ml Estradiol	0.2 mcg/ml Estradiol
1	0	8	0
2	1	1	1
3	2	3	2
Total	3	12	3

Group 2

Flask No.	0 Estradiol	0.02 mcg/ml Estradiol	0.2 mcg/ml Estradiol
1	4	8	2
2	0	1	5
3	4	6	3
Total	8	15	10

Figure 7. Sequential photographs of the same area in flask 3 of Group 1, or the first experiment, which received no estradiol treatment (the control). The following designations of time for each photograph represent the amount of time that had elapsed since changing to experimental medium.

A. 0 hours (immediately before medium change)

B. 2 hours

C. 4 hours

D. 8 hours

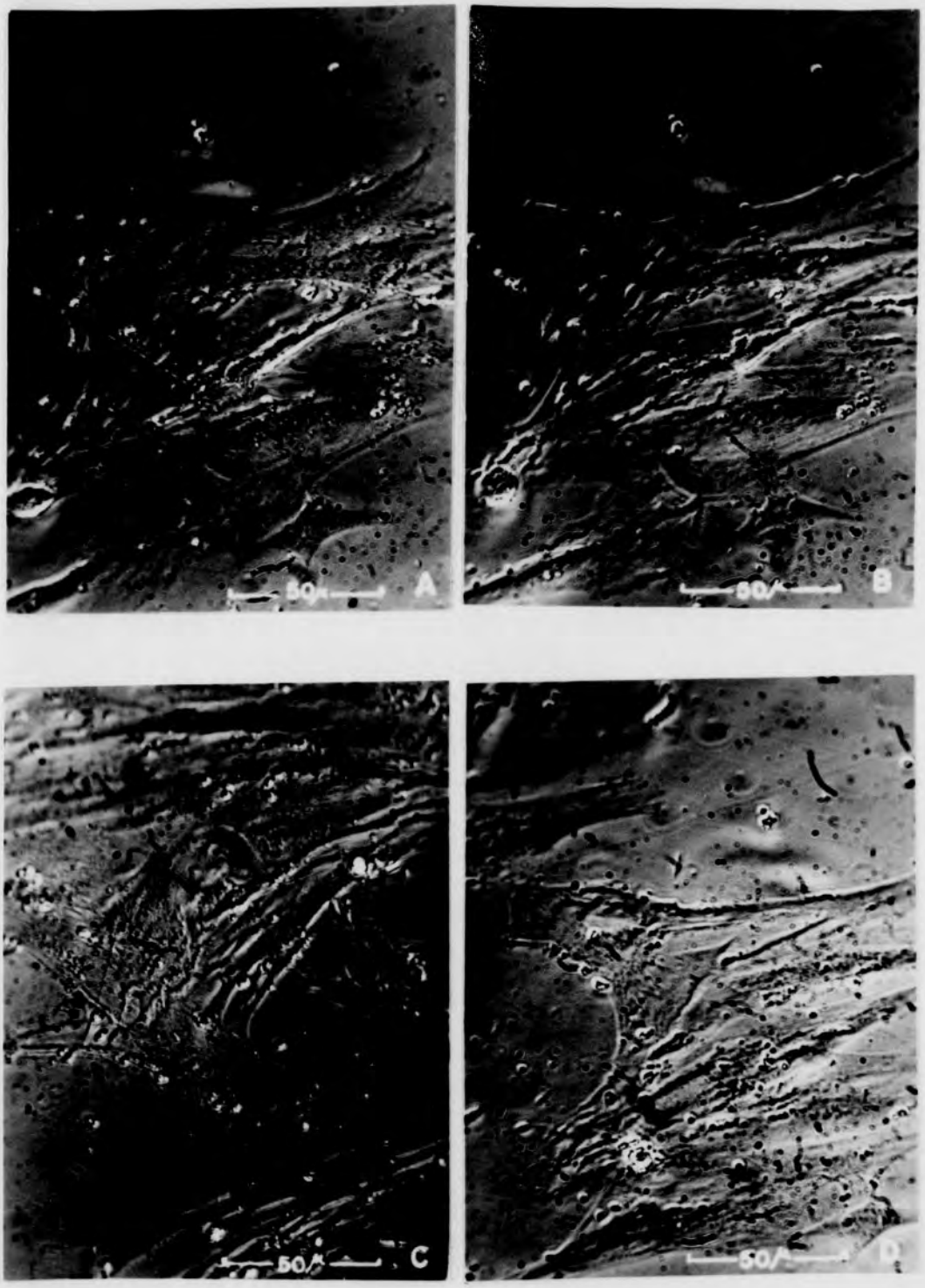

50Å
A
50Å
B
50Å
C
50Å
D

Figure 7. (Continued)

E. 24 hours

F. 48 hours

G. 72 hours


50μ
G

Figure 8. Sequential photographs of the same area in flask 1 of Group 1 which received treatment with media containing 0.02 mcg/ml estradiol. The following designations in time refer to the amount of time following addition of estradiol-containing medium.

A. 0 hours (immediately before medium change)

B. 2 hours

C. 4 hours

D. 8 hours

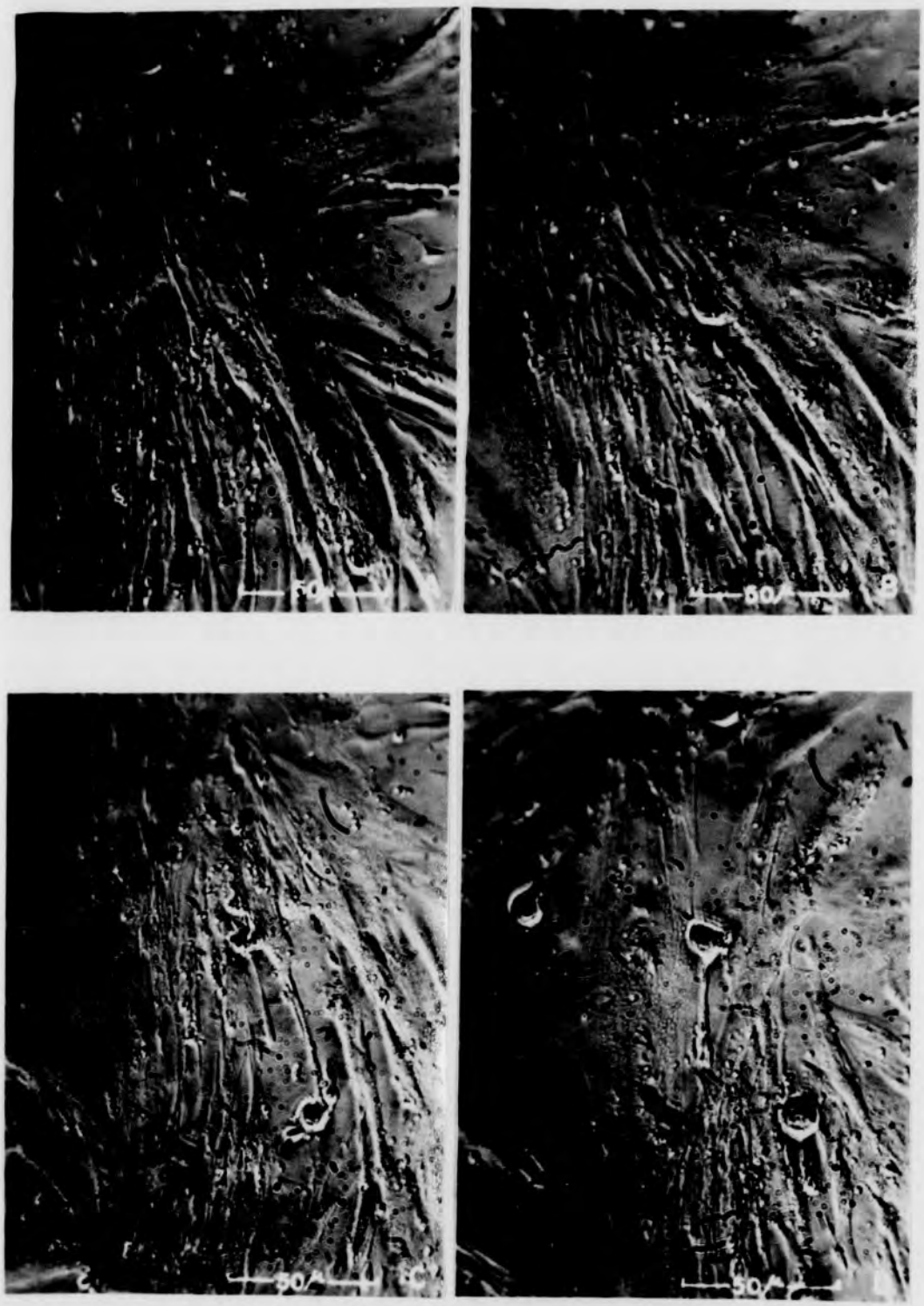

50Å
50Å
50Å
50Å

Figure 8. (Continued)

E. 24 hours

F. 48 hours

G. 72 hours


50


50


50

The observation of a possible mitotic increase as shown by the phase-contrast photomicrographs led to the determination of mitotic coefficients, or number of dividing cells per 100, from the polychrome-stained slides that had been made from the plastic flasks that contained the cell monolayers. When mitotic coefficients are low, larger cell counts must be made. For the first experiment, or Group 1, the total cell count per slide was 2,000 and the mitotic coefficients represent the number of dividing cells/ 500 X 100%. For the second experiment, Group 2, the total cell count was 1,000 and the mitotic coefficient represents the number of dividing cells/ 250 X 100%. Mitotic coefficient data is listed in Table 2. Comparisons of the different estradiol treatments were made using the t-test to compare the difference of two means. These calculations were made using the Olivetti Underwood Programma 101 available in the Biology Department. Table 3 lists the t-test information obtained from the mitotic coefficient data.

Nuclear size index values were obtained as the product of the greatest length multiplied by the greatest width of 30 oval fibroblastic nuclei per polychrome-stained plastic slide. It was hoped that this data might provide information about changes in nuclear size as related to cell division or polyploidy. Table 4 includes t-test information on estradiol-treated and control cultures. Figures 9 and 10 represent distribution of these index values.

TABLE 2. Mitotic Coefficient Data.

Group 1

Slide	0 Estradiol	0.02 mcg/ml Estradiol	0.2 mcg/ml Estradiol
1	0.60%	2.70%	1.36%
	1.11%	1.14%	1.66%
	1.14%	2.35%	2.78%
	1.15%	1.52%	0.79%
2	0.59%	1.36%	1.14%
	0.39%	2.60%	1.36%
	1.33%	1.75%	2.38%
	1.58%	1.56%	0.41%
3	0.80%	1.62%	1.49%
	1.38%	3.09%	1.79%
	0.82%	1.61%	2.29%
	0.92%	0.96%	0.81%

Group 2

Slide	0 Estradiol	0.02 mcg/ml Estradiol	0.2 mcg/ml Estradiol
1	1.20%	1.20%	1.60%
	1.20%	1.60%	0.79%
	1.60%	0.80%	1.20%
	0%	1.74%	0%
2	0.40%	1.60%	0.80%
	0%	2.80%	3.20%
	1.20%	2.00%	1.20%
	1.11%	1.20%	1.23%
3	1.20%	0.40%	2.41%
	1.60%	1.20%	1.82%
	0.80%	1.20%	0.80%
	0%	0.68%	1.46%

TABLE 3. t-Test Information on Mitotic Coefficients.

Group 1

Amount Estradiol	Mean % of 9 Calculations	S.D.	S.E.
0	0.98	0.375	0.100
0.02 mcg/ml	1.86	0.668	0.192
0.2 mcg/ml	1.51	0.697	0.200

t-Test Comparisons	t-Value	Level of Significance
0 vs. 0.02 mcg/ml Estradiol	4.032	t. 995 %
0 vs. 0.2 mcg/ml Estradiol	2.340	t. 975 %
0.02 vs. 0.2 mcg/ml Estradiol	1.259	t. 80 %

Group 2

Amount Estradiol	Mean % of 9 Calculations	S.D.	S.E.
0	0.86	0.606	0.173
0.02 mcg/ml	1.37	0.641	0.184
0.2 mcg/ml	1.38	0.832	0.238

t-Test Comparisons	t-Value	Level of Significance
0 vs. 0.02 mcg/ml Estradiol	2.019	t. 95 %
0 vs. 0.2 mcg/ml Estradiol	1.755	t.95 %
0.02 vs. 0.2 mcg/ml Estradiol	0.023	below t. 60 %

TABLE 4. t-Test Information on Nuclear Size Index Values

Group 1

Amount Estradiol	Mean of 90 Measurements	S.D.	S.E.
0	226	87.3	9.2
0.02 mcg/ml	216	81.8	8.6
0.2 mcg/ml	210	72.0	7.6

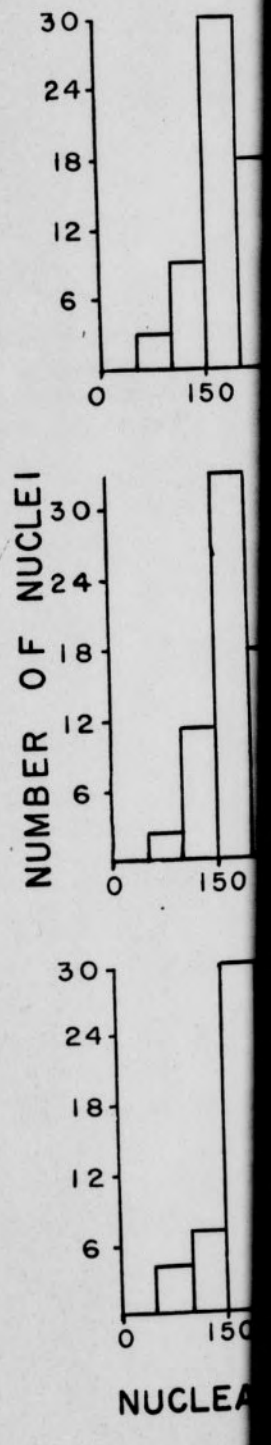
t-Test Comparisons	t-Value	Level of Significance
0 vs. 0.02 mcg/ml Estradiol	0.748	t. 70 %
0 vs. 0.2 mcg/ml Estradiol	1.334	t. 90 %
0.02 vs. 0.2 mcg/ml Estradiol	0.563	t. 60 %

Group 2

Amount Estradiol	Mean of 90 Measurements	S.D.	S.E.
0	307	150.7	15.9
0.02 mcg/ml	270	88.2	9.3
0.2 mcg/ml	263	148.4	15.6

t-Test Comparisons	t-Value	Level of Significance
0 vs. 0.02 mcg/ml Estradiol	2.053	t. 975 %
0 vs. 0.2 mcg/ml Estradiol	1.992	t. 975 %
0.02 vs. 0.2 mcg/ml Estradiol	0.363	t. 60 %

Figure 9. Graphs comparing distribution of nuclear size index values for the first experiment, Group 1. Index values are the product of the greatest lenght in microns X the greatest width in microns of nuclei. Each graph represents 90 nuclei.



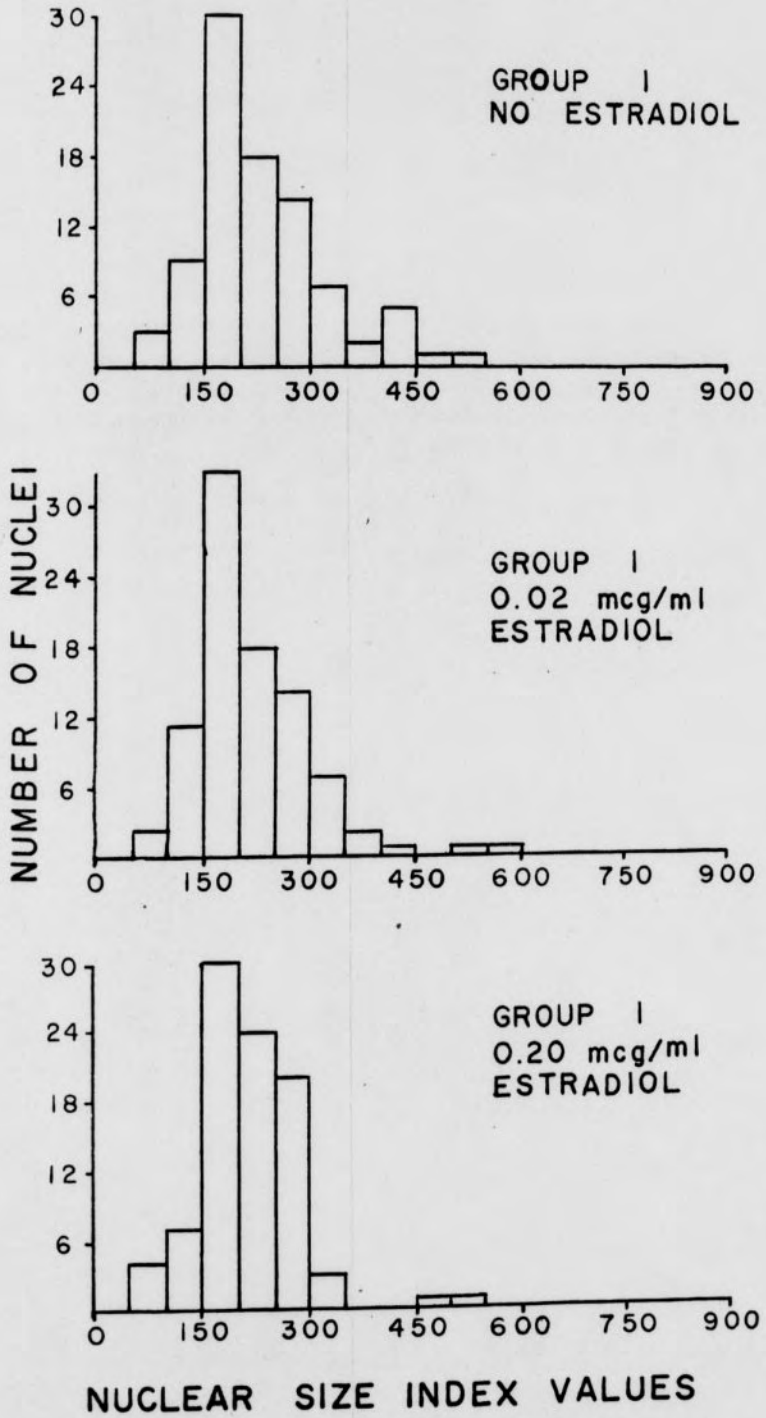
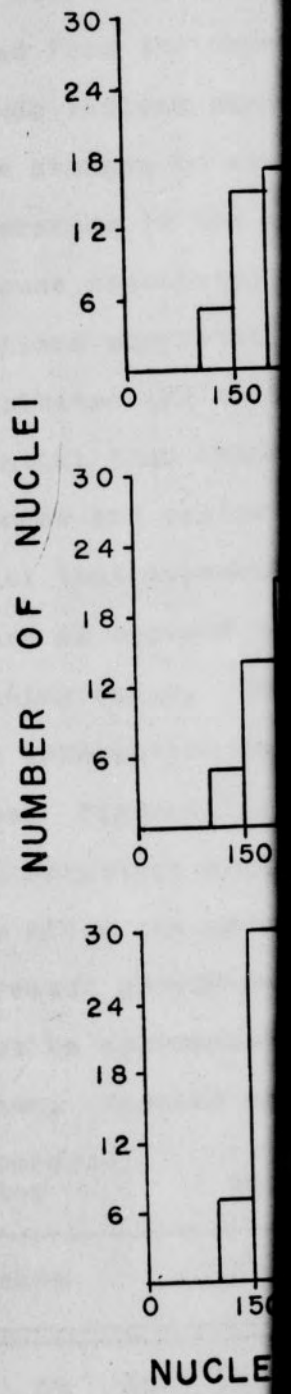
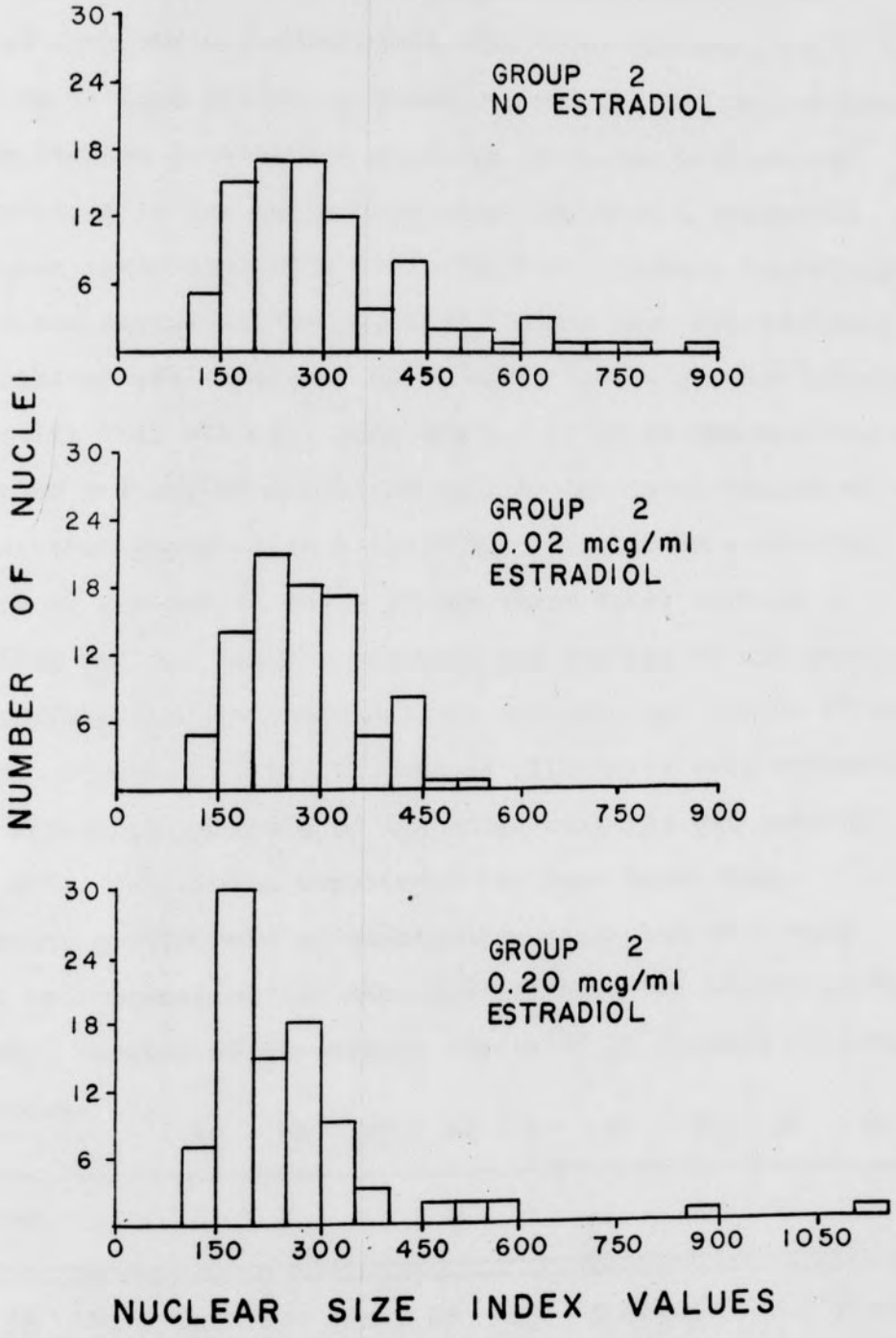

GROUP I
NO ESTRADIOL
GROUP I
0.02 mcg/ml
ESTRADIOL
GROUP I
0.20 mcg/ml
ESTRADIOL
NUMBER OF NUCLEI
30
24
18
12
6
0
150
300
450
600
750
900
NUCLEAR SIZE INDEX VALUES

Figure 10. Graphs comparing the distribution of nuclear size index values for the second experiment, Group 2. Index values are the product of the greatest length in microns X the greatest width in microns of nuclei. Each graph represents 90 nuclei.




GROUP 2
NO ESTRADIOL
GROUP 2
0.02 mcg/ml
ESTRADIOL
GROUP 2
0.20 mcg/ml
ESTRADIOL
NUMBER OF NUCLEI
NUCLEAR SIZE INDEX VALUES
0
150
300
450
600
750
900
1050
6
12
18
24
30

Nuclear staining in the polychrome-stained slides varied from the expected steel blue color (Lehman, 1965) to include various shades of lavender, yellow, and yellow-green. These changes in staining possibly reflected biochemical differences in the cells that might indicate a synthetic response associated with cell division. Cursory inspection of slides suggested that mitotic figures were non-randomly distributed and that some areas might have a greater mitotic potential than others. Data was collected on the numbers of lavender and yellow nuclei as well as the total number of nuclei that appeared in a field which contained a mitotic figure as opposed to those fields where there were no dividing cells. Table 5 includes the average values and t-test information on these nuclear staining and number differences. Figures 11, 12, 13, and 14 illustrate some variations.

Karyotype analysis of the stock cultures was made 10 days after the second experiment had been terminated. Decreased growth rate of subcultures suggested that they might be approaching the degenerative phase of serial cultivation. Results of chromosome counts of 32 spreads follow.

Chromosome Number	< 40	42	43	44	45	46	48	50	54
Spreads	5	2	1	1	1	1	1	1	1

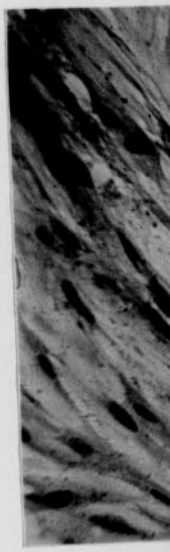
55	58	62	64	65	66	67	85	% Modal	% Non-Modal
1	2	1	3	1	8	1	1	25	75

TABLE 5. Nuclear Staining and Number Differences in "Mitotic Fields" as compared with "Non-Mitotic Fields"

Category	Staining or Number Difference	0 Estradiol	0.02 mcg/ml Estradiol	0.2 mcg/ml Estradiol
Group 1				
Average Number of Nuclei in "Mitotic Fields"	Total No.	76.1	63.0	57.9
	Lavender	12.2	9.5	5.9
	Yellow	4.3	1.8	2.1
Average Number of Nuclei in "Non-Mitotic Fields"	Total No.	61.8	44.6	37.9
	Lavender	12.4	8.4	3.6
	Yellow	4.6	2.0	1.7
Level of Significance	Total No.	t. 80 %	t. 90 %	t. 995 %
	Lavender	less than t. 60 %	t. 60 %	t. 90 %
	Yellow	less than t. 60 %	t. 60 %	t. 60 %
Group 2				
Average Number of Nuclei in "Mitotic Fields"	Total No.	28.6	24.2	42.3
	Lavender	8.7	5.8	10.0
	Yellow	1.2	0.6	0.7
Average Number of Nuclei in "Non-Mitotic Fields"	Total No.	12.0	11.2	10.0
	Lavender	4.1	3.0	2.9
	Yellow	0.5	0.1	0.4
Level of Significance	Total No.	t. 975 %	t. 99 %	t. 90 %
	Lavender	t. 975 %	t. 995 %	t. 90 %
	Yellow	t. 80 %	t. 975 %	t. 80 %

TABLE 5. Nuclear Staining and Number Differences in "Mitotic Fields" as compared with "Non-Mitotic Fields"

Category	Staining or Number Difference	0 Estradiol	0.02 mcg/ml Estradiol	0.2 mcg/ml Estradiol
Group 1				
Average Number of Nuclei in "Mitotic Fields"	Total No.	76.1	63.0	57.9
	Lavender	12.2	9.5	5.9
	Yellow	4.3	1.8	2.1
Average Number of Nuclei in "Non-Mitotic Fields"	Total No.	61.8	44.6	37.9
	Lavender	12.4	8.4	3.6
	Yellow	4.6	2.0	1.7
Level of Significance	Total No.	t. 80 %	t. 90 %	t. 995 %
	Lavender	less than t. 60 %	t. 60 %	t. 90 %
	Yellow	less than t. 60 %	t. 60 %	t. 60 %
Group 2				
Average Number of Nuclei in "Mitotic Fields"	Total No.	28.6	24.2	42.3
	Lavender	8.7	5.8	10.0
	Yellow	1.2	0.6	0.7
Average Number of Nuclei in "Non-Mitotic Fields"	Total No.	12.0	11.2	10.0
	Lavender	4.1	3.0	2.9
	Yellow	0.5	0.1	0.4
Level of Significance	Total No.	t. 975 %	t. 99 %	t. 90 %
	Lavender	t. 975 %	t. 995 %	t. 90 %
	Yellow	t. 80 %	t. 975 %	t. 80 %



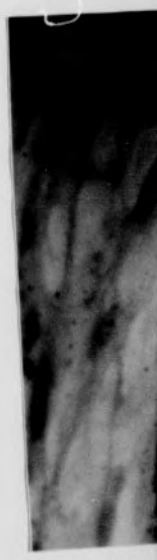


Figure 11. Color photographs of nuclear staining of polychrome stained slides illustrating the presence of lavender- and yellow-staining nuclei in addition to the expected steel blue color.

A. The top photograph which includes several mitotic figures was made at 125 X microscopic and 175 X actual magnification.

B. The lower photograph which includes several yellow nuclei was made at 200 X microscopic and 280 X actual magnification.

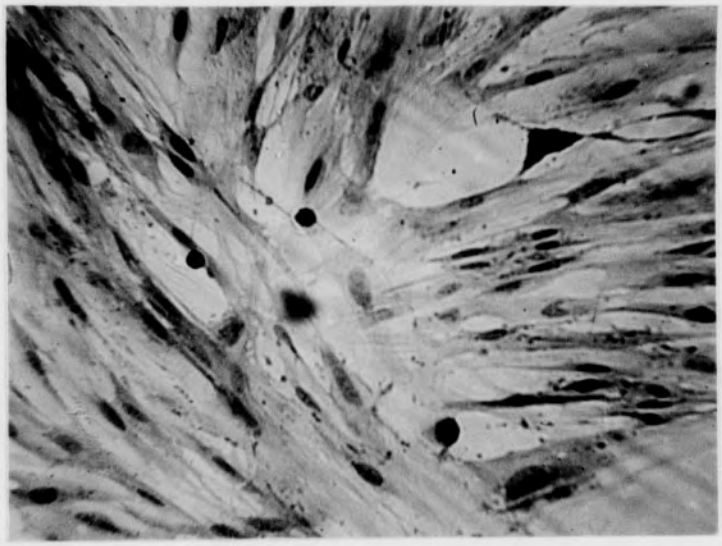


Figure 12. Color photographs of yellow- and green-staining nuclei.

A. The top photograph shows a yellow-green nucleus at the upper left (200 X microscopic and 280 X actual magnification).

B. The lower photograph shows two green mitotic cells at 500 X microscopic and 710 X actual magnification.

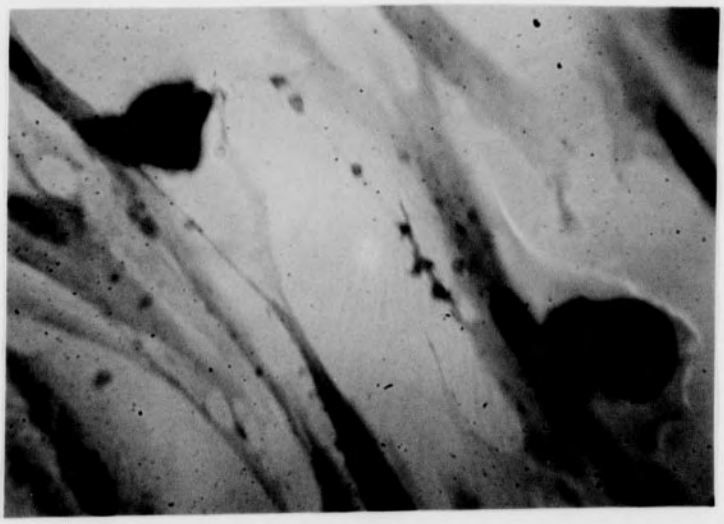
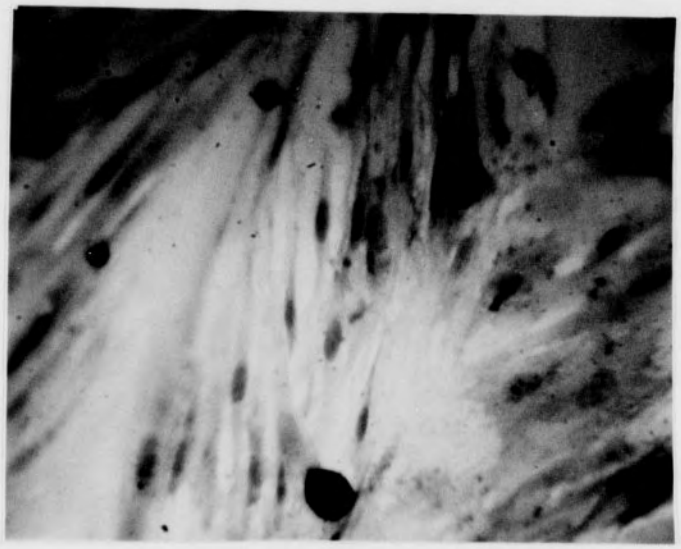


Figure 13. Color photographs of size differences in mitotic cells.

A. The top photograph includes 4 mitotic figures. The largest is 24μ across at the widest part; and the other three, from left to right, are 10μ, 12μ, and 14μ across (200 X microscopic and 280 X actual magnification).

B. The lower photograph contains a large metaphase which measured 40μ in length (200 X microscopic and 280 X actual magnification).



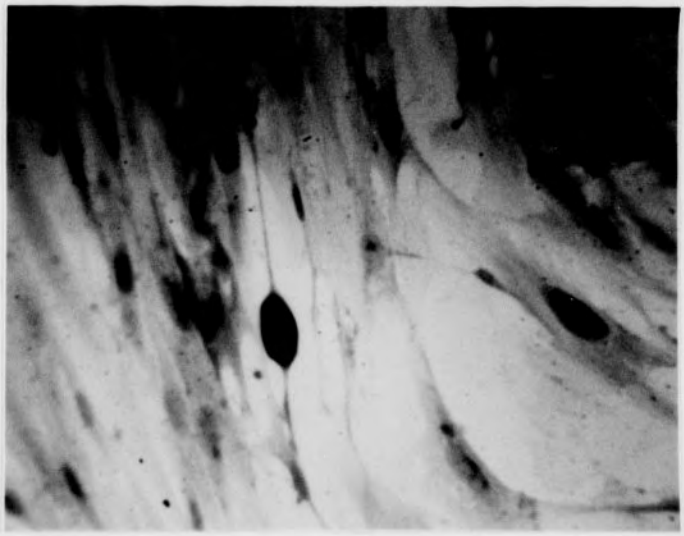
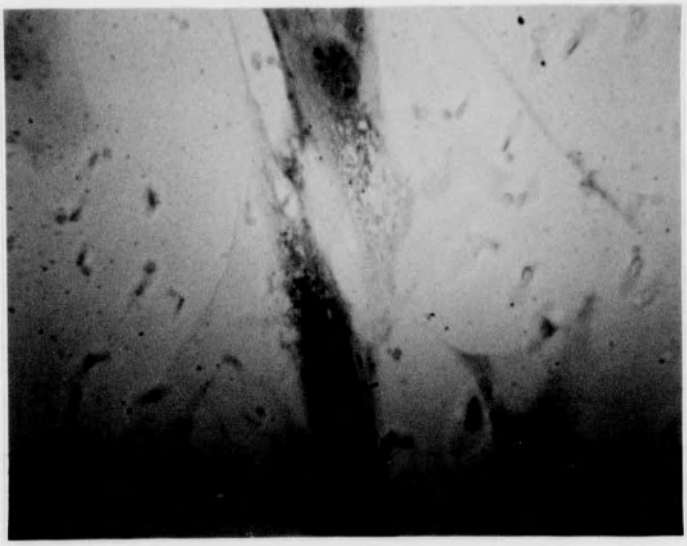


Figure 14. Variations in cell morphology.

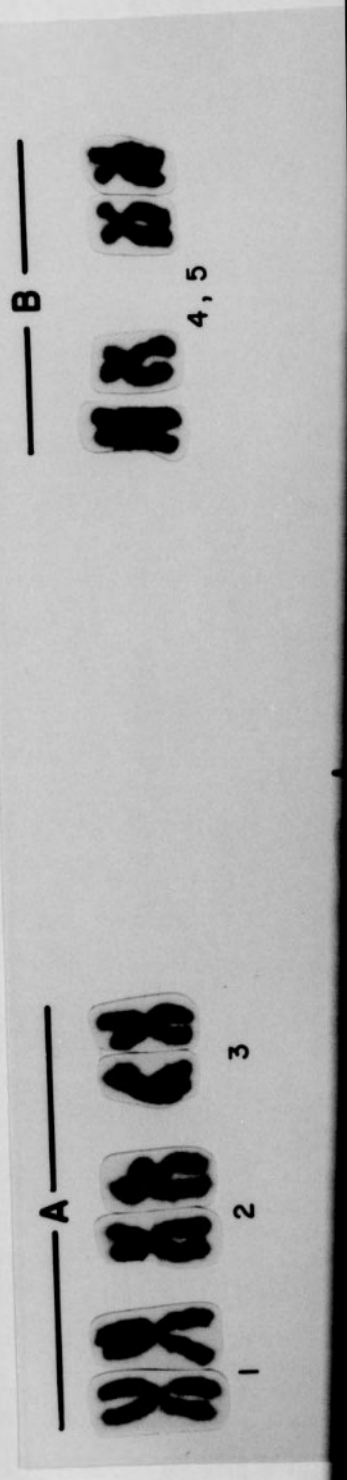
A. The top photograph shows cells with granular or vacuolar cytoplasm (200 X microscopic and 280 X actual magnification).

B. Lower photograph includes several lavender nuclei or nuclear fragments at the center (200 X microscopic and 280 X actual magnification).



Of the spreads counted 25% (8) had the modal number of 66 chromosomes; and 44% (14) had counts between 60 and 69. Spreads with fewer than 30 chromosomes were not included in the 32 spreads upon which percentages were based. Hypodiploid counts, which constituted 32% (10), often result as a technical artifact but are thought to have some significance in aneuploid degeneration (Saksela and Moorhead, 1963). Four representative karyograms and corresponding photographs of metaphase chromosome spreads are shown in Figures 15 through 22. Presence of markers such as dicentrics and fragments is noted on the karyograms. Figure 23 is a photomicrograph of a polyploid cell which was estimated to have 6 times the diploid number of 46 chromosomes. An abnormal mitotic figure is shown in Figure 24.

Figure 15. Karyogram of stock culture 70-8924 after 17½ weeks in culture showing the normal human male chromosome compliment of 46 chromosomes.



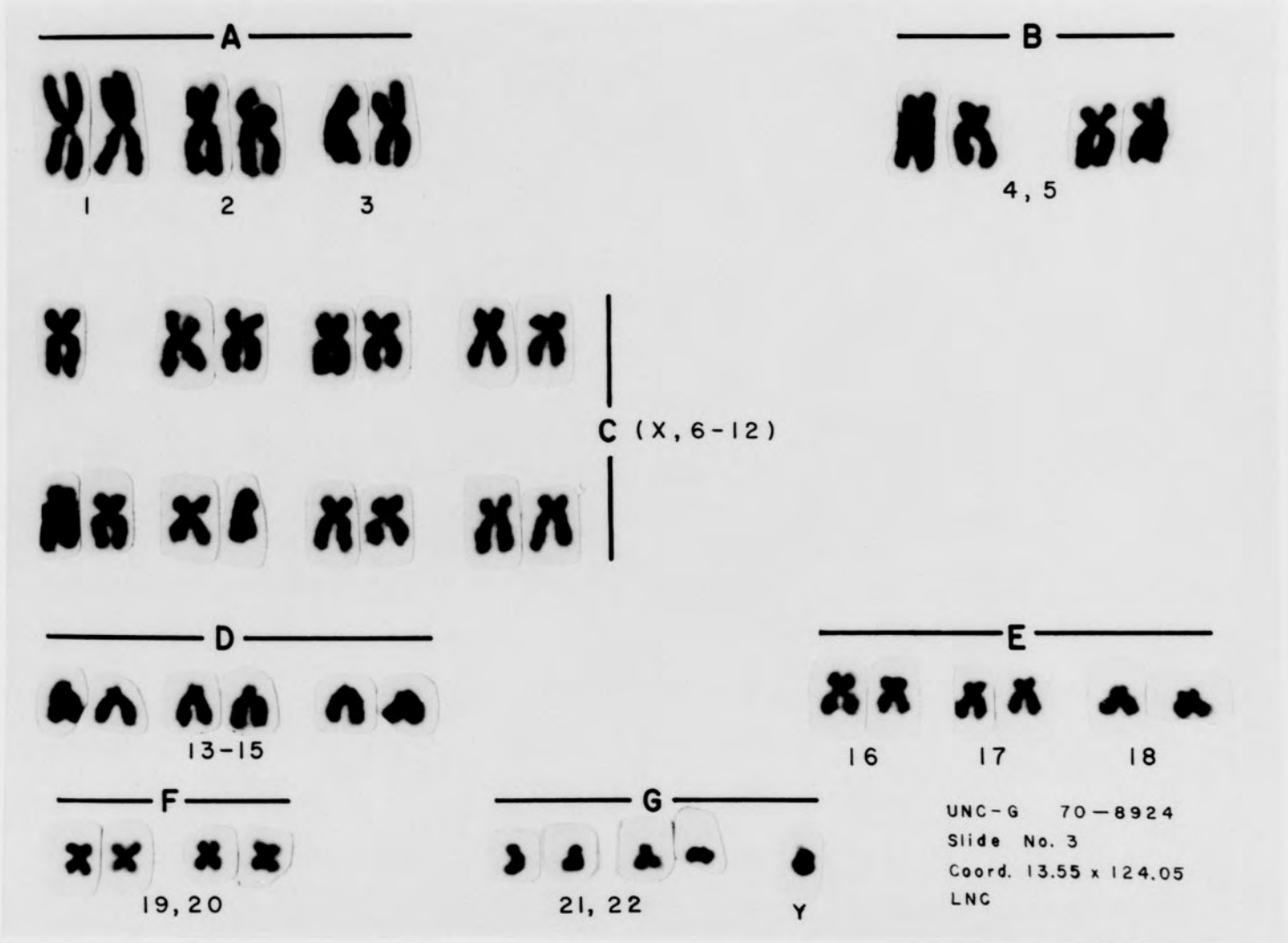

A
1
2
3
B
4, 5
C (X, 6-12)
D
13-15
E
16
17
18
F
19, 20
G
21, 22
Y
UNC-G 70-8924
Slide No. 3
Coord. 13.55 x 124.05
LNC

Figure 16. Metaphase chromosome spread for Fig. 15.

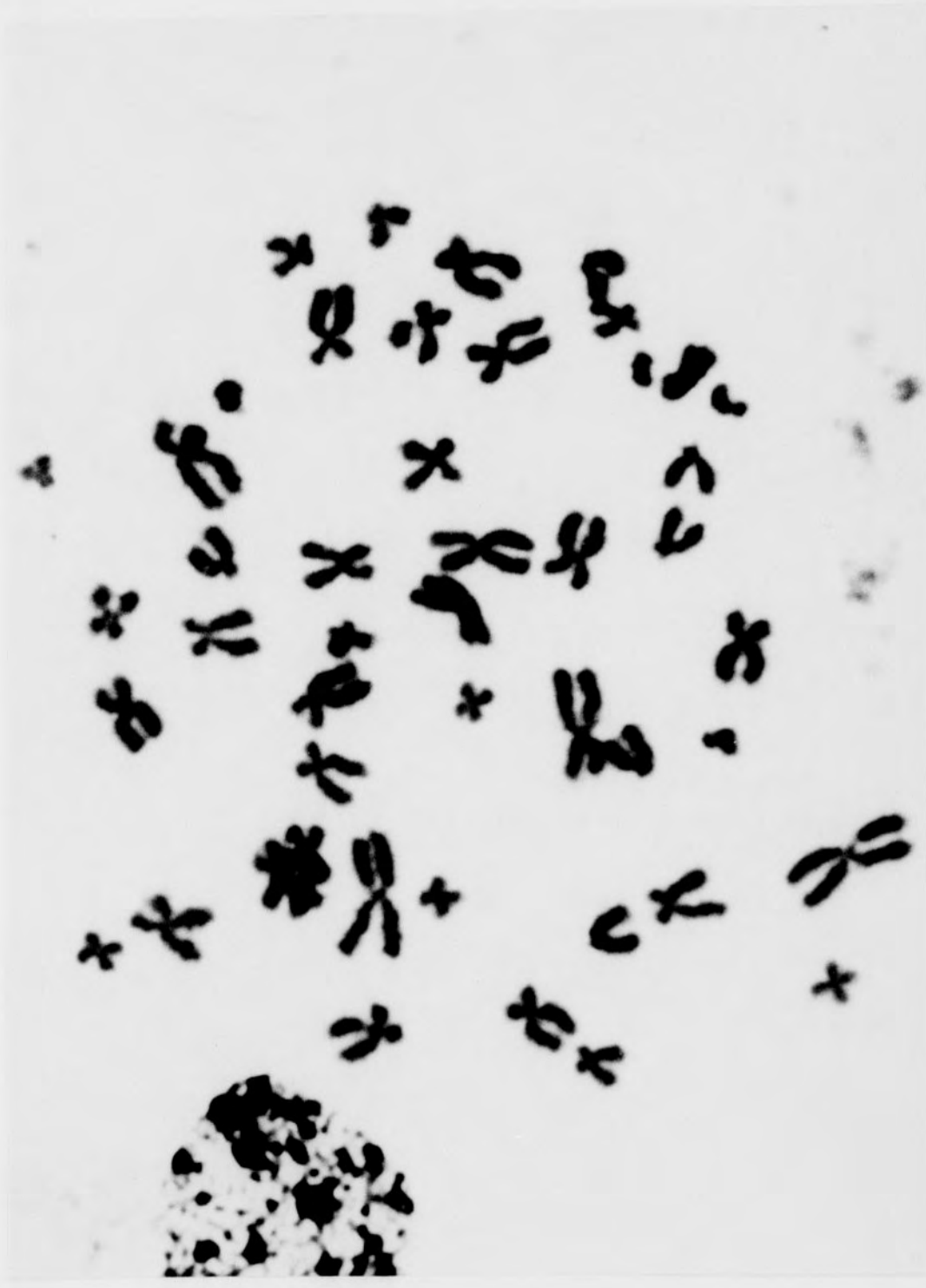
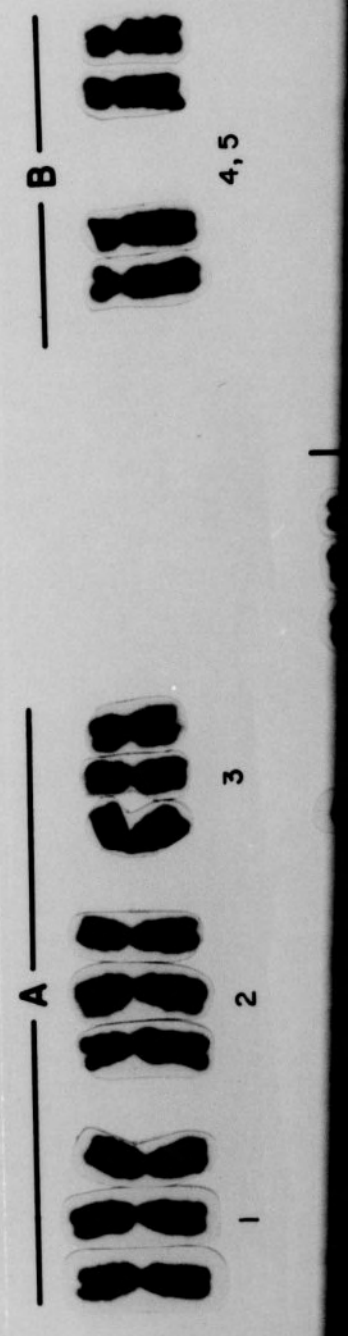


Figure 17. Karyogram of stock culture 70-8924 showing 58 chromosomes.



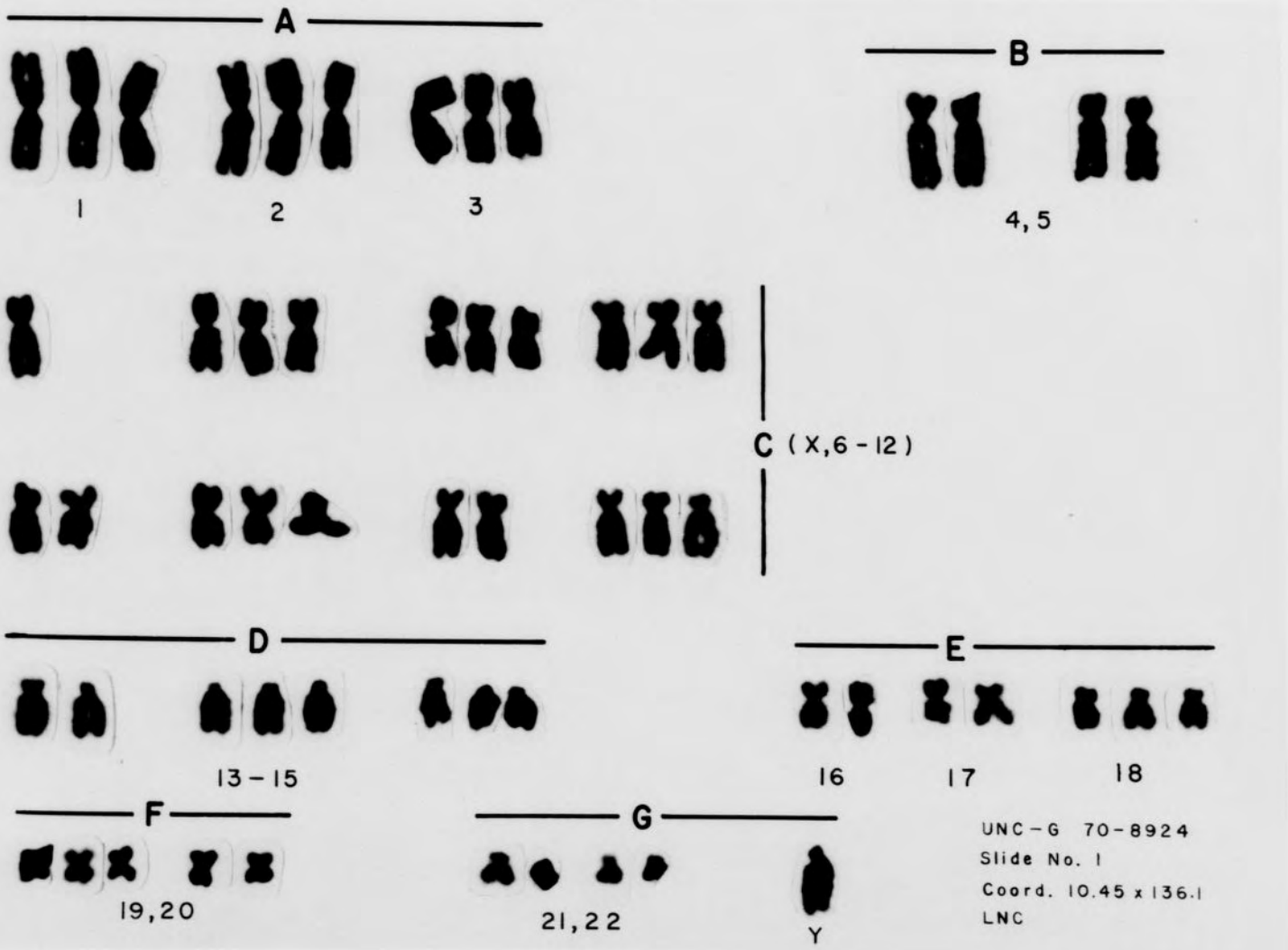

A
1
2
3
B
4,5
C (X,6-12)
D
13-15
E
16
17
18
F
19,20
G
21,22
Y
UNC-G 70-8924
Slide No. 1
Coord. 10.45 x 136.1
LNC

Figure 18. Metaphase chromosome spread for Fig. 17.

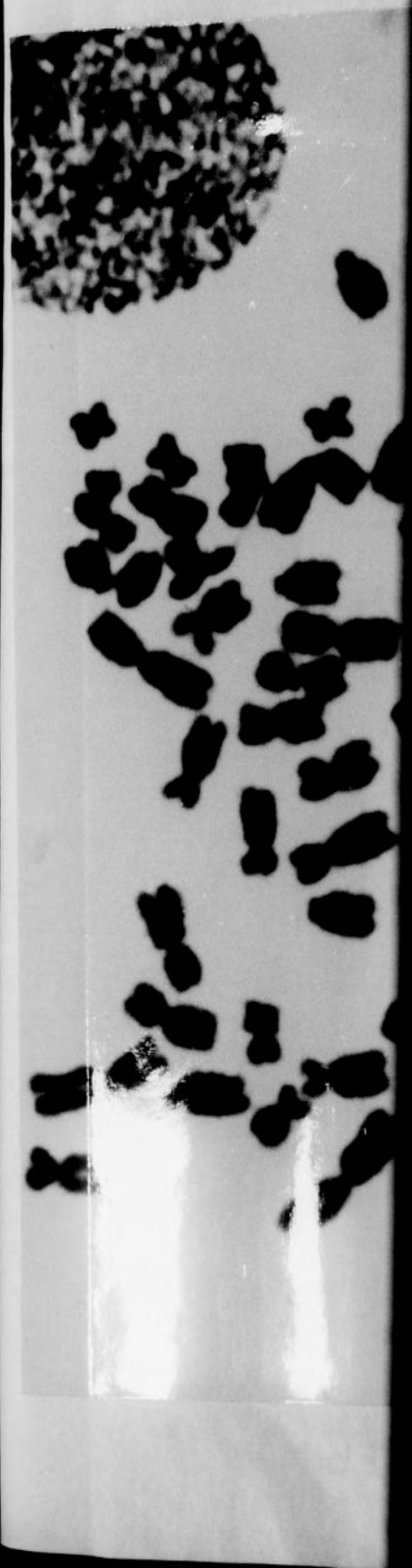


Figure 19. Karyogram of stock culture 70-8924 showing 65 chromosomes and 1 acentric.

A. Marker 1 is a dicentric

B. Marker 2 is an acentric fragment

C. Markers 3 and 4 are centric fragments

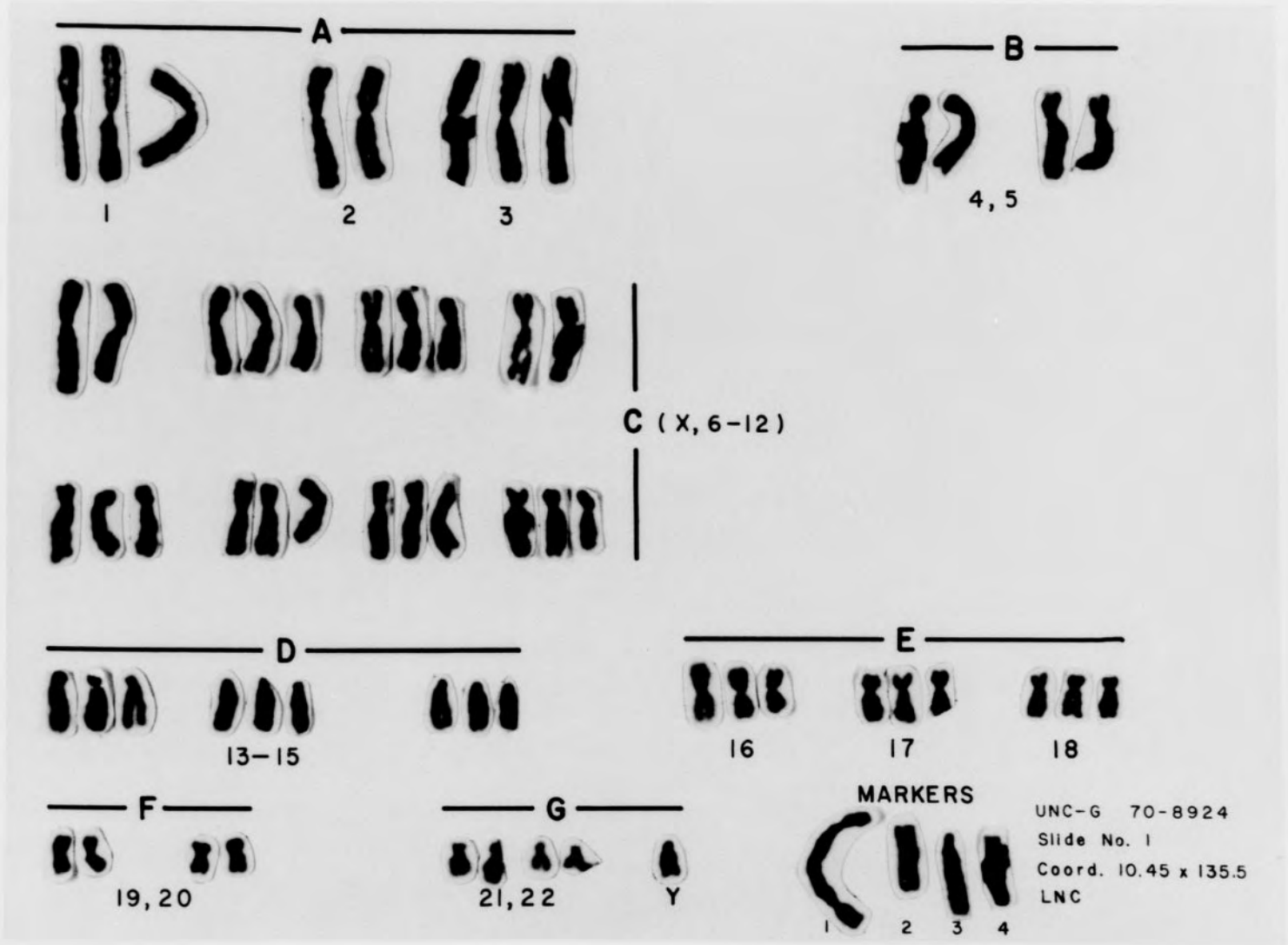

A
1
2
3
B
4, 5
C (X, 6-12)
D
13-15
E
16
17
18
F
19, 20
G
21, 22
Y
MARKERS
1
2
3
4
UNC-G 70-8924
Slide No. 1
Coord. 10.45 x 135.5
LNC

Figure 20. Metaphase chromosome spread for Fig. 19.

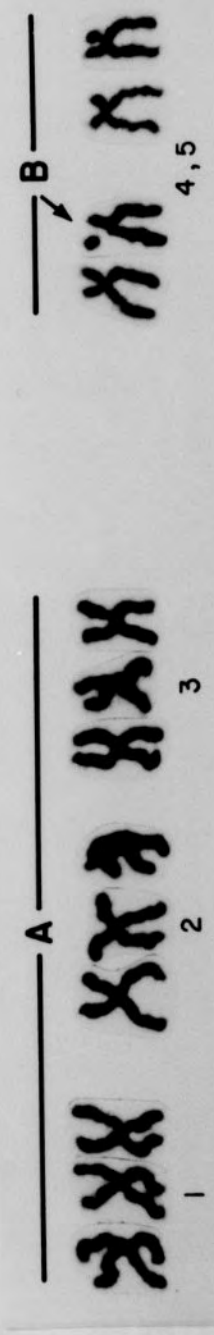


Figure 21. Karyogram of stock culture 70-8924 showing 67 chromosomes.

A. Marker 1 is an apparently normal B group or X chromosome obscured in the photograph by some trash on the slide.

B. Marker 2 is a dicentric

C. The arrow pointing toward a B group chromosome indicates a gap

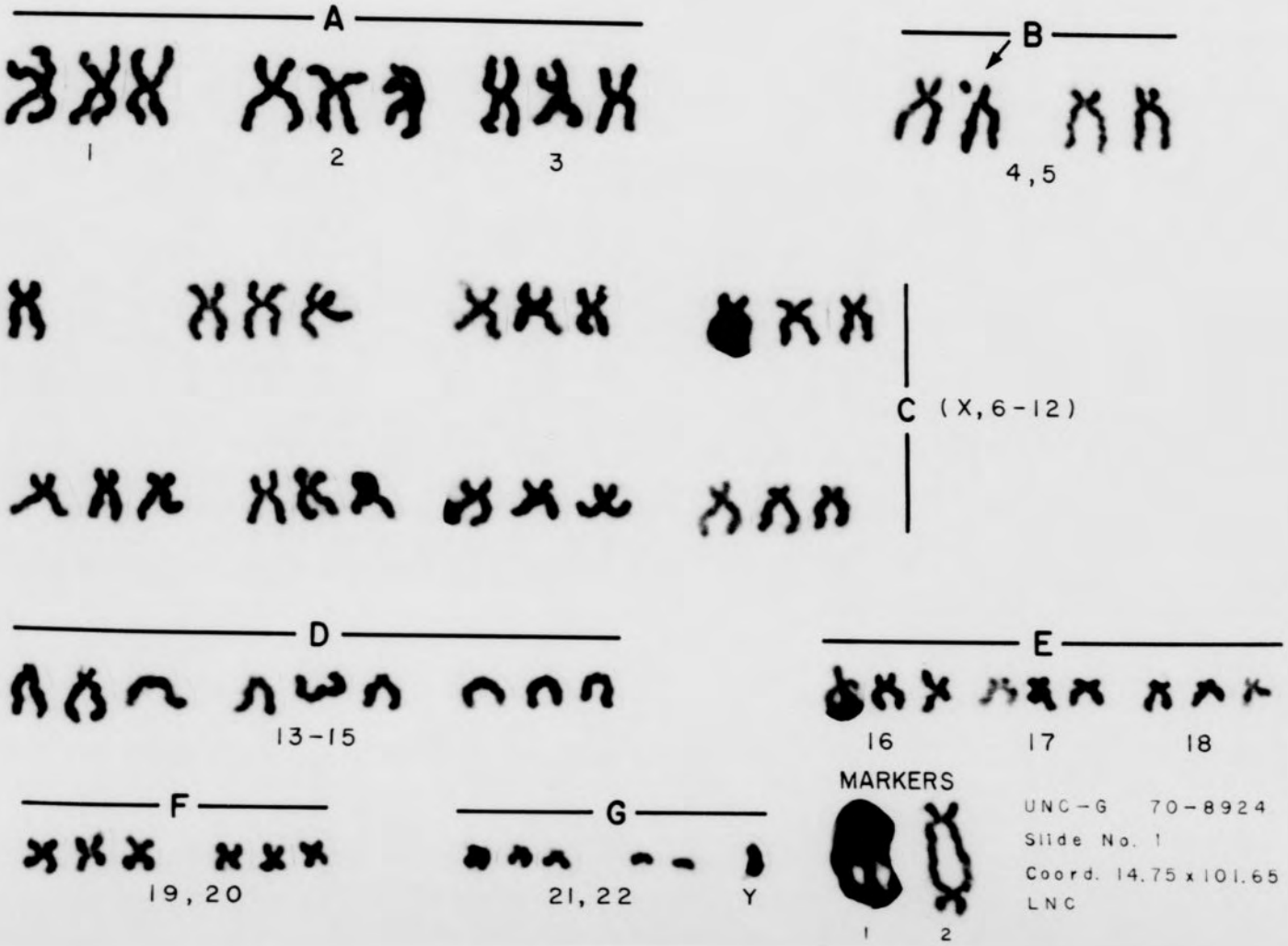
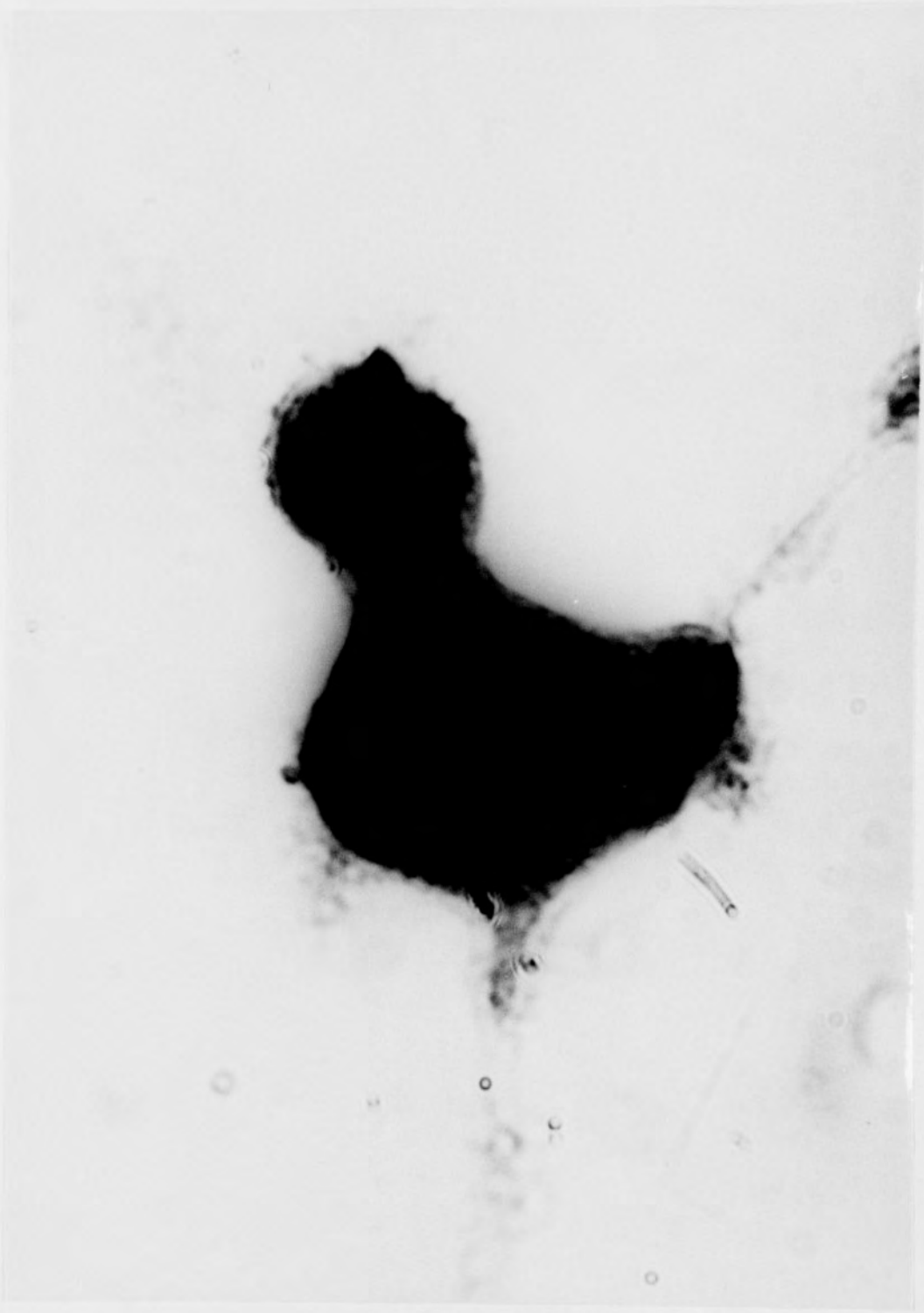

A
1
2
3
B
4,5
C (X, 6-12)
D
13-15
E
16
17
18
F
19, 20
G
21, 22
Y
MARKERS
1
2
UNC-G 70-8924
Slide No. 1
Coord. 14.75 x 101.65
LNC

Figure 22. Metaphase chromosome spread for Fig. 21.

Figure 23. A polyploid cell with an estimated 6 X 46 chromosomes.

Figure 24. An abnormal mitosis.



DISCUSSION

Aneuploidy in the Stock Cultures

The first serious problem that must be considered in evaluating the results of these estradiol experiments is that of the karyotype analysis. The chromosome counts revealed distinct aneuploidy with a modal chromosome number of 66 instead of the normal 46. The chromosome pattern of the hyperdiploid spreads, as determined by arranging chromosomes according to size, centromere location, and relative length of long and short arms, appeared to be hypotriploid. A spread with 66 chromosomes, then, seemed to fit the pattern of 69 - 3. Appearance of aneuploidy in excess of 30% and of dicentric chromosomes is characteristic of the degenerative phase of serial subcultivation equivalent to the 46th passage or 23rd week in culture with twice weekly subculturing (Saksela and Moorhead, 1963). The two estradiol studies had been made on cultures of 70-8924 at 13 and 16 weeks. This was short of the 20 weeks described by Saksela and Moorhead (1963) and Tjio and Puck (1958) as having 1-2% or no aneuploidy respectively, and it had been expected that cultures would be relatively normal. Increased granularity of cytoplasm and decreased growth of the subcultures made at 16 weeks were hints of degeneration.

Can the cells used in these experiments be considered

representative of normal human fibroblastic cells *in vitro*? Unfortunately there is not a clear answer as to when the increase in aneuploidy began. On the basis of the time required for subcultures to form confluent monolayers, it could possibly be inferred that the 13 week old cultures were more nearly normal than at 16 weeks.

The mean of the nuclear size index values for the control in the first experiment was 226; and for the second experiment it was 307. Figures 9 and 10 depict the distribution of index values and show a decrease of values between 100 and 200 and an increase in values above 300 for the second experiment.

Response to Estradiol

Data on the numbers of mitotic cells seen in the sequential phase-contrast photographs, Table 1, and mitotic coefficients determined from stained slides, Tables 2 and 3, indicate that cultures exposed to medium containing 0.02 mcg/ml estradiol tend to show a greater rate of mitosis than those with medium to which no estradiol or 0.2 mcg/ml estradiol, an amount 10 times greater than 0.02 mcg/ml, had been added. Comparisons of mitotic coefficients testing the difference of means using the t-test indicate that control values are significantly less than either the 0.02 or 0.2 mcg/ml estradiol groups. Mitotic coefficients for the 0.02 and 0.2 mcg/ml estradiol groups are not significantly

different from each other.

Nuclear size index values were consistently smaller for estradiol-treated cultures than for controls in both the first and second experiments. But this difference was significant only in the second experiment. As with the mitotic coefficients, values for the two estradiol treatments were not significantly different for either experiment.

Nuclear staining and number differences of fields with dividing cells as compared to fields without dividing cells, are shown in Table 5 of the Results section. These differences were significant in only 1 of 9 cases in the first experiment while there were 5 of 9 cases that were significant for the second experiment. Inconsistency of results for the staining differences does not appear to justify the intended use of such differences as a histochemical index related to mitotic potential on the basis of presence or absence of dividing cells as was done for Table 5.

According to Goldberg and Atchley (1966) who were studying the effect of estradiol on the melting point of placental DNA, "... the effect of the estradiol was a function of the hormone concentration" within the range of concentration 1.5 to 17.3 mcg/ml. This supposition is not borne out by the data on mitotic coefficients in the present study. Kuchler (1962) found that 9.5 mcg/ml estradiol inhibited both synthesis and growth of L strain mouse fibroblasts. Ozello (1964) used smaller amounts, 0.1 and 0.4

mcg/ml estradiol to depress growth of human neonatal fibroblasts and stimulate acid mucopolysaccharide production. However, Lasnitzki (1965) in reviewing tissue culture studies on the effects of estradiol on growth described the results of these studies as "inconsistent and often contradictory". She stated that estrogen dilutions of 10^{-7} produced growth stimulation of fibroblasts while those of 10^{-5} caused growth inhibition. This same sort of system in which a certain tissue is stimulated to grow by a low concentration and inhibited by larger amounts is exemplified by the effects of the steroid hydrocortisone on lymphocytes. At higher levels hydrocortisone is very toxic to lymphocytes. Whitfield (1968) explains the action of cortisone on the basis of a "cell damage" or "release" mechanism. Sublethal damage results in the conversion of relatively densely packed lymphocyte heterochromatin, which is inactive in RNA synthesis, into loosely packed lymphocyte euchromatin which is active in RNA synthesis. Balinsky (1965) described the similar but nonspecific role of various chemical agents in embryonic induction as "sublethal damage (cytolysis)" which, as he suggested, "'unmasks' some substance present in the ectodermal cells themselves, and that this substance, once released or activated, changes the presumptive epidermal cells into neural cells."

Willmer (1954) proposed that

> a high division rate may sometimes be connected with a high rate of disintegration, a process which might well be autocatalytic since the processes of cell breakdown are known to provide conditions suitable for building up new cells. Extracts of autolysed tissues ... are said to be strong growth-promoters.

This basic concept has been expanded by Bullough (1965) who agreed with the concept "that the inducing factors may act as triggers only and that the main part of the differentiation mechanism evidently lies inside the reacting cells." He provides a broad theory of mitotic homeostasis based on the presence of protein substances within cell surfaces, called "chalones", which inhibit mitosis. According to Bullough's theory cell damage or stimulation by mitogenic hormones such as estrogen reduce the effective level of this substance which had suppressed synthesis of substances necessary for mitosis.

The Possible Role of Fibroblasts in the Formation of Polyps

There exists the strong possibility that a causal relationship exists between changes in estrogen or other steroid levels during puberty and climacteric and the initial development of polyps and the occurrence of malignancy, respectively, within the familial polyposis family studied in our lab. In this family the very early polyps can be recognized by a proliferation of the lamina propria, which is predominantly connective tissue and is of mesodermal origin, underlying mucosa which has normal-appearing

glands.

The adenomatous polyp 70-8924 with the "normal" tissue of the base providing the cultures for these experiments was not derived from a family member but is similar in that it also shows hyperplasia of the connective tissue. This is illustrated by Figures 25 and 26. According to the pathologist's report (McLendon, 1970) the mucosa of the base and stalk were free of abnormal glands, But "The glands forming the polyp show nuclear hyperchromatism and slight pleomorphism with decreased mucous secretions." These changes, as can be seen in Figure 27, did not include the disorganization characteristic of malignancy.

In early embryonic development of the intestine, the mesoderm, which forms the connective tissue of the submucosa and lamina propria as well as muscle and blood vessels, induces the formation of the mucosa from endoderm. The proliferative rate of the connective tissue, then, might constitute for the mucosa one of the "extrinsic control factors" described by Grobstein (1970) as important to cytodifferentiation.

Figure 25. A cross section of polyp 70-8924 stained with Harris hematoxylin and eosin. CT indicates connective tissue extending into the polyp. Actual magnification is 12.6 X.

with
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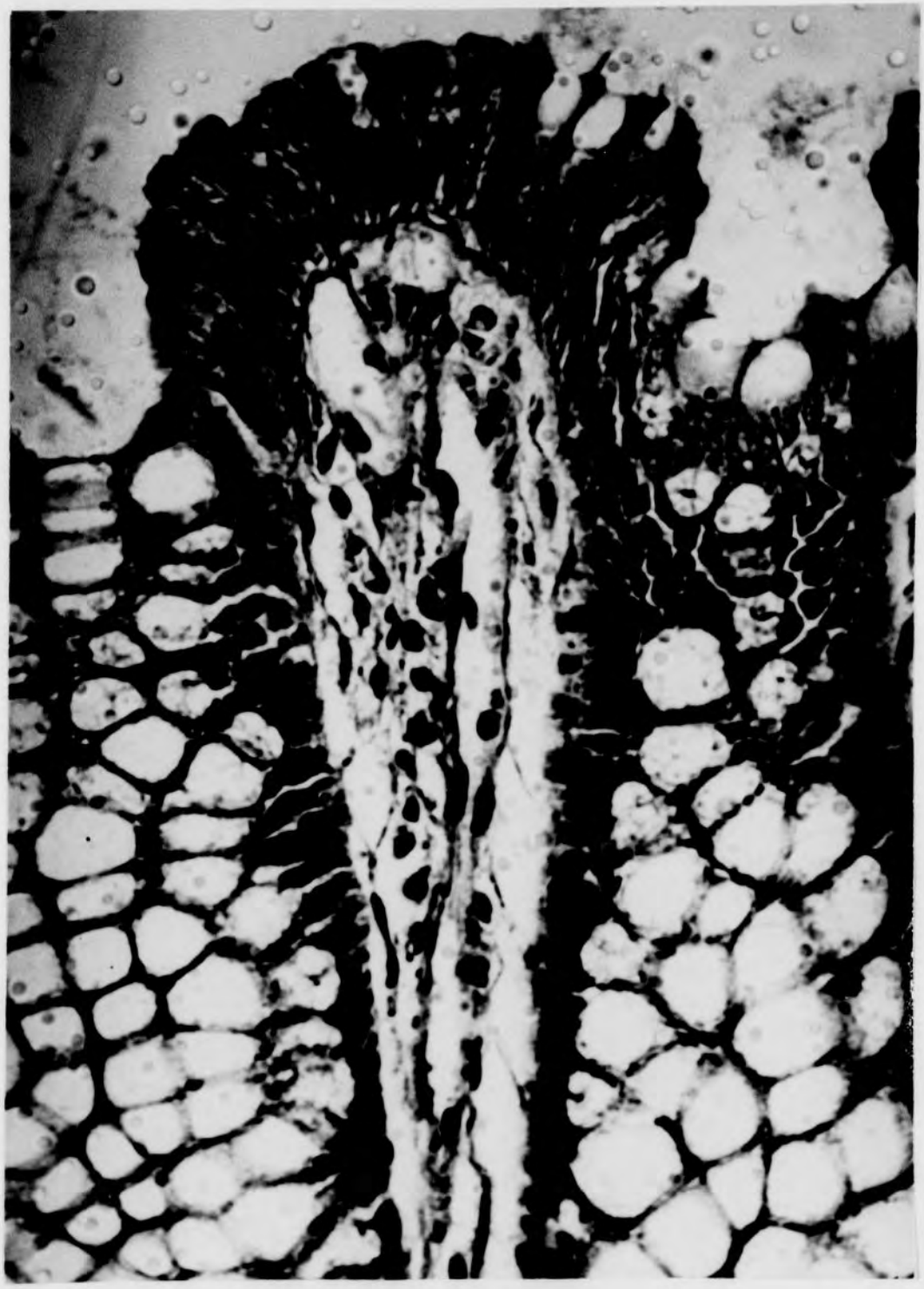
cation

Figure 26. A photograph of connective tissue at lower right underlying the mucosa in the polyp shown in Fig. 25. Microscopic magnification is 200 X.

Figure 27. A photograph of the mucosa of the polyp shown in Fig. 25 including parts of 2 crypts separated by connective tissue of the lamina propria. Microscopic magnification is 200 X.



SUMMARY

1. A tissue culture study was made to determine whether physiological levels of estradiol stimulate visible changes in morphology or growth of adult human fibroblastic cells derived from the large intestine, a non-target tissue.

2. A section is included on the construction and use of a modified-Rose perfusion chamber used in the early part of this study.

3. Concentrations of 17-β estradiol used in this study are based on concentrations found in the blood of adult females. The 0.02 mcg/ml estradiol treatment represents the 0.02 mcg/ml added by the pill or pregnancy (0.015-0.03 mcg/ml range); and the 0.20 mcg/ml treatment represents the amount added by estrogen therapy (0.1-0.3 mcg/ml range).

4. Sequential phase-contrast photomicrographs were made of locations within each of 18 flasks. Each experiment included 9 flasks of cultures: 3 flasks with no estradiol comprised the control and 3 each were treated with 0.02 and 0.20 mcg/ml respectively.

5. At the end of each experiment, cell monolayers were fixed with 10% neutral buffered formalin. After rinsing, plastic flasks were sawed into 1 X 2½ inch slides that were stained by a modification of Lehman's (1965) polychrome staining procedure to which an initial staining period in

1% Alcian Blue was added. Slides were cleared and mounted with oil of cedar.

6. Analysis of stained slides included determination of mitotic coefficients, enumeration of lavender- and yellow-colored nuclei among the expected steel blue nuclei, and calculation of nuclear size index values as the product of greatest length X greatest width of 90 oval nuclei per slide. The t-test was used to compare the difference of pairs of means. Means of mitotic coefficients for control flasks were found to be significantly less than for either estradiol treatment. Means for the two estradiol treatments were not significantly different from each other even though the amount of estradiol differed by a factor of 10. This does not support the idea that the effect of this hormone is always a function of concentration. Amounts of 0.1 to 20 mcg/ml have been reported to inhibit fibroblast growth; it has been thought, however, that smaller amounts might stimulate growth. Nuclear size index values were consistently smaller for estradiol-treated cultures, but this difference was significant only in the second experiment. Number of nuclei and nuclear staining differences based on comparison of numbers within fields containing a dividing cell and numbers in fields not containing dividing cells were not consistently statistically significant.

7. Chromosome analysis of the stock cultures was made $17\frac{1}{2}$ weeks after cultures had been set up. Chromosome counts of

1% Alcian Blue was added. Slides were cleared and mounted with oil of cedar.

6. Analysis of stained slides included determination of mitotic coefficients, enumeration of lavender- and yellow-colored nuclei among the expected steel blue nuclei, and calculation of nuclear size index values as the product of greatest length X greatest width of 90 oval nuclei per slide. The t-test was used to compare the difference of pairs of means. Means of mitotic coefficients for control flasks were found to be significantly less than for either estradiol treatment. Means for the two estradiol treatments were not significantly different from each other even though the amount of estradiol differed by a factor of 10. This does not support the idea that the effect of this hormone is always a function of concentration. Amounts of 0.1 to 20 mcg/ml have been reported to inhibit fibroblast growth; it has been thought, however, that smaller amounts might stimulate growth. Nuclear size index values were consistently smaller for estradiol-treated cultures, but this difference was significant only in the second experiment. Number of nuclei and nuclear staining differences based on comparison of numbers within fields containing a dividing cell and numbers in fields not containing dividing cells were not consistently statistically significant.

7. Chromosome analysis of the stock cultures was made 17½ weeks after cultures had been set up. Chromosome counts of

32 spreads revealed aneuploidy with a modal chromosome number of 66. According to Saksela and Moorhead (1963) the amount of aneuploidy to be expected at 20 weeks was less than 1-2%. It could not be predicted when the increase of aneuploid cells began, but on the basis of nuclear size index values it was suggested that an increase could be seen between the first and second experiments.

8. The possibility is suggested that there might be a causal relationship between alteration of dynamic balance by changes in steroid levels at puberty and climacteric and initial development of polyps and occurrence of malignancy, respectively, in the polyposis family being studied by our lab. This is based on the inductive potential of connective tissue underlying the mucosa as in embryonic development.

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