SOME EFFECTS OF ELEVATED ENVIRONMENTAL POTASSIUM LEVELS ON THE GROWTH AND PROLIFERATION OF A MAMMALIAN CELL LINE IN CULTURE

by

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* * *

To my wife and son,

Margie and Karl,

Who Patiently

Waited.

* *
*

ABSTRACT

When E4 cells, a cell type selected for its high cloning efficiency from cloned baby hamster kidney cells (BHK-21), was exposed to high potassium medium (10mM Na^+ and 114mM K^+), proliferation, DNA synthesis, fine structure, and morphology were affected. Prolonged exposure to a high potassium environment resulted in cell death. Restoration of normal environmental Na^+-K^+ conditions (118mM and 5.6mM respectively) after 24 hours in high potassium medium induced a moderate, but variable, proliferative synchrony.

The morphological response of E4 to high potassium was marked by cytoplasmic vacuolization, nucleolar condensation, an apparent increase in cell size, and a noticeable increase in cytoplasmic projections. The most striking effects on fine structure were a decrease in cytoplasmic density, the appearance of microtubular and microfibrillar material, the aggregation of ribosomes into rosettes, and an increase in the amount of cytoplasmic membrane systems in the cells.

The proliferative synchrony induced in E4 populations after release from high potassium blockade was moderate, and variable, (35 - 65%). The quality and quantity of the synchrony was enhanced when a metaphase arresting agent, colcemid, was used to segregate the largest population of synchronously proliferating cells from the rest. This method reliably produced a large number of highly synchronous cells suitable for the study of G1 or S phase cell cycle events.

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I INTRODUCTION

THESIS

Statement of Thesis

In mammalian cells, as well as other cell types, substances are unequally distributed across the intracellular and extracellular boundary. Thus, concentration and charge gradients are established across the plasma membrane.

The unequal distribution of potassium ions across the plasma membrane is in part reflected by a trans (plasma) membrane potential, the magnitude of which is thought to depend partly on the relative concentrations of that ion inside and outside of the cells (Troshin 1966, Schoffeniels 1967). The characteristic membrane potential in non-proliferating cells is kept relatively constant by maintaining a relatively constant transmembrane ion distribution. However, intracellular potassium levels are known to fluctuate during the cell

cycle in proliferating cells (Jung and Rothstein 1967) and the membrane potential must fluctuate accordingly.

Cellular transmembrane potential is a consequence of electro-osmotic balance in all cells, however a functional role for transmembrane potential beyond electro-osmotic balance is only understood for cells of neuro-musoular tissues. The depolarization and repolarization of the plasma membrane with consequent changes in the transmembrane potential is at the basis of the function of these cells. Cone (1970), suggested that transmembrane potentials may also serve to regulate the events of cell division. While Cone's arguments are theoretical, there is some evidence that transmembrane potentials may play a significant role in the regulation of this process (Orr et al. 1972, MacDonald et al. 1972). The alteration of extracellular potassium concentration and/or shifts in transmembrane potential are known to alter significantly the growth rate of mammalian cells in culture (Kuchler 1967), but the processes underlying these potassium-induced phenomena are not defined.

The working hypothesis for this thesis suggests in fact a role for transmembrane potential in the regulation of cell division. It is the intent of this thesis to identify the influence of altered extracellular potassium ion concentration on the proliferation, cell cycle, and structure of mammalian cells in culture.

LITERATURE REVIEW

Ions, Transmembrane Potential, and Cell Division

Heilbrunn quotes a paper by Bethe (Bethe 1930, cited by Heilbrunn 1952) in which the following lines appear: "Just as in sea water, no matter whence it comes or how concentrated it may be, the ratios of Na to Ca, K and Mg are always the same. So too in the body fluids (juices) of animal tissues the same approximate ratio between Na, K, and Ca is found. Only with Mg are considerable variations in ratio to be found...". Besides this, it can also be stated that the intracellular Na to K ratio is commonly much less than one, while the extracellular or environmental Na to K ratio is always much greater than one.

The universality of these characteristics intracellular and extracellular ionic environments suggests that such ionic conditions are necessary for life. Since these specific intracellular and extracellular ionic environments also define the electro-osmotic conditions for viability, alterations in these conditions may affect

viability of a cell or an organism.

Some ions are known to play a role in cell division. Calcium is recognized as a principal ionic regulator of cell division *in vivo* and *in vitro* (Whitfield et al. 1973). Iron has also been implicated as a specific regulator in the events of mitosis (Robbins and Pederson 1971). It is likely then, that other ionic species or ion related phenomena are also involved in the regulation of cell division since changes in fluxes for one ionic species are almost certain to affect fluxes of other ionic species.

The early experiments of J. Loeb with the marine fish *Fundulus heteroclitus* (Loeb and Wasteneys 1911), showed that these fish were rapidly killed in pure potassium solutions, even when the ion's concentration was equivalent to that found in sea water. The ability of the fish to survive was enhanced when Na was added to the environment. Fish survived indefinitely when the ratio of Na to K in the solution was 17:1. This observation may serve only to prove that marine fish

have evolved to survive best in the marine environment, but this observation also implies that high extracellular or extra-organismic Na and K can affect viability.

Jung and Rothstein (1967) observed fluctuations in the intracellular sodium and potassium contents of synchronously proliferating L5178Y cells (Mouse lymphoblasts). In an exponentially proliferating cell population, the cell number, cell volume, and intracellular Na-K content increased exponentially. In a synchronous population, the cell number increased in a stepwise manner, cell volume increased exponentially but the sodium and potassium content of the cells fluctuated throughout the cell cycle. The potassium content of these mouse lymphoblasts decreased sharply below the control level just prior to, and including, the early part of S phase (i.e. a discrete period of time in the cell division cycle during which DNA is synthesized). The decrease in potassium content was equal to approximately 20% of the total cellular potassium, but there was a definite trend in the Na fluctuations. The sodium content fell at mitosis and began to

rise again when the potassium content began to fall, but fell again when the potassium content began to rise. The Na content returned to control levels in time for the next mitosis. One might assume that these fluctuations only reflected osmotic balance adjustments. However if this were true, the sodium and potassium contents of synchronously proliferating cells should increase exponentially or in a stepwise manner.

Jung and Rothstein (1967) speculated that the observed fluctuations in Na-K content were the means by which the cell regulated its progress through the growth-division cycle. If this speculation be valid, interference with these fluctuations in the intracellular Na/K ratio should affect cell proliferation. This concept appears to be at least partially operational in light of the observations on Kuchler and Orr.

Kuchler (1967) and Orr et al. (1972) observed that cell proliferation and DNA synthesis (as measured by the incorporation of tritiated thymidine into cold acid insoluble material) are inhibited in LM cells

(mouse fibroblasts) and BHK-21 cells (baby hamster kidney) by decreasing the environmental Na/K ratio. The usual ratio of Na/K in tissue culture media is in the neighborhood of 14:1 (14.0), while the inhibition of proliferation and DNA synthesis occured at Na/K ratios between 1;1.9 (0.15) and 1:14 (0.071). This is not surprising in view of Eagle's observation that potassium concentrations greater than 50mM (i.e Na/K ratios less than 3:1) inhibit the proliferation of some types of cultivated mammalian cells (Eagle 1956).

Kuchler (1967) ascribes the reduced DNA synthesis and proliferation in the presence of decreased Na/K ratios to an inhibition of amino acid uptake. The maximum rate of amino acid uptake occurs when the extracellular Na/K ratio is approximately 140mM/10mM (14.0), the usual ratio in standard tissue culture media. Reversal of this ratio completely inhibits amino acid uptake. Orr et al. (1972) believe the altered transmembrane potential to be the cause for the observed inhibition of proliferation and DNA synthesis. These workers also found that cells placed in media with

decreased Na/K ratios increased in volume. Kuchler (1967) showed that the increase in cell volume was directly related to an increase in intracellular potassium and a consequent uptake of water.

Orr et al. (1972) observed that these inhibitory effects of a high potassium environment can be overcome by restoring the normal medium Na/K ratio. This block-and-release procedure also partially synchronized cell proliferation. Most of the cells were found to be in the G1 phase of their cycle (i.e. the interval or Gap between the completion of mitosis and the onset of the S or DNA synthetic phase). The studies of Kuchler (1967) and Orr et al. (1972) show that alteration of the extracellular Na/K ratio and the necessarily altered transmembrane potential may contribute to the observed modulation of cellular proliferation and DNA synthesis. The decreased cellular uptake of amino acids in the presence of high potassium concentrations may also contribute to the general observations.

MacDonald et al. (1972) further examine these effects of altered environmental Na/K ratios. They consider the parameters of ion content, cellular ATP levels, transmembrane potential, and cellular proliferation. An increase in the external potassium concentration from 1.75mM - 128mM elicited a biphasic growth response. Proliferation was stimulated by K concentrations less than, or equal to, 70mM. However, exposure to higher K concentration caused precipitous decline in the rates of proliteration and DNA synthesis (as measured by the incorporation of tritiated thymidine into cold acid insoluble material). Complete inhibition of cellular proliferation occured in the presence of an external K concentration of 128mM. They also observed that cells maintained in medium containing 114mM K gained 44 mMoles of K and lost 14 mMoles of Na per liter of cell water. The intracellular steady state concentrations for both Na and K were established after two hours in the high potassium medium, but the inhibitory effects of this treatment were not manifest until a much later time. These observations imply that the process which eventually results in the observed block (Orr et al. 1972) is most likely a gradual and non-specific series

of events . The membrane potential was shown to respond rapidly to changes in external K levels. Maximal tritiated thymidine incorporation occured at a transmembrane potential of -17 mv, it rapidly declined when the potential difference across the plasma membrane rise above -10mv. No significant alteration in ATP content was observed.

Cone (1970) has suggested that membrane potential may play a significant role in the regulation of cell division. He assumed that there is a functional relationship between transmembrane potential and the ability of a cell to divide. He postulated a complex system in which intracellular ionic ratios would determine membrane potential, which in turn would regulate DNA synthesis and other events required for a cell to proceed through the growth-division cycle. Thus, the cell surface complex and plasma membrane are viewed as a communications link between environmental conditions and intracellular metabolic events.

While increased environmental potassium concentrations are known to rapidly and significantly alter transmembrane potential (MacDonald et al. 1972,

Borle 1968), the removal of environmental calcium ions is also known to cause a dramatic reduction in transmembrane potential (Borle 1968). DNA synthesis and cell proliferation have been shown to be particularly sensitive to environmental calcium levels, and extracellular calcium levels have been shown to regulate the initiation of DNA synthesis in mouse thymic lymphoblasts in vivo (Whitfield et al. 1969) and in vitro (Whitfield et al. 1971), in mouse 3T3 cells in vitro (Boynton et al 1974), in peripheral lymphocytes (Whitney and Sutherland 1972, Whitney and Sutherland 1973, Quastel 1974), in bone marrow cells in vivo (Rixon 1968) and in vitro (Morton 1968) and in a variety of other systems.

Alteration of the transmembrane potential and intracellular Ca^{+2} levels are also involved in muscle contraction (Hoyle 1970, Ashley 1971). When the membrane of a muscle cell is depolarized and Na enters and K subsequently leaks out, calcium is released into the cytoplasm and the muscle cells fibrillar apparatus contracts. If all animal cells store some calcium (in structures such as mitochondria) it is not unreasonable to assume that potassium-induced changes in the

transmembrane potential will change the intracellular Ca^{+2} levels and thereby affect proliferation.

Although few in number, the studies on the relation between extracellular potassium and transmembrane potential provide a reasonable basis for suspecting a regulatory role for potassium in the cell division process, along with the well-established role that calcium plays.

THE CELL CYCLE AND CELL SYNCHRONY: A BRIEF NOTE

The Cell Cycle

The cell cycle was first recognized by Howard and Pelc (1953). Their experiments with growing bean root tips illustrated that only a limited number of cells in a randomly growing population were actively synthesizing DNA at any point in time. They also found that there is a finite period of time between the onset of mitosis and the completion of replicative DNA synthesis. Their experiments also implied that another interval of time must also exist between the onset of DNA synthesis and the completion of mitosis.

The pre- and post-DNA-synthetic periods were designated as Gap 1 (G1) and Gap 2 (G2) respectively. The DNA synthetic period was designated as S phase and the cytologically vivid mitotic period as M. These terms form the operational language of cell cycle experimentation, and the phases which they describe form the frame of reference for cell cycle studies. The cell cycle is schematically represented in Fig. 1-1.

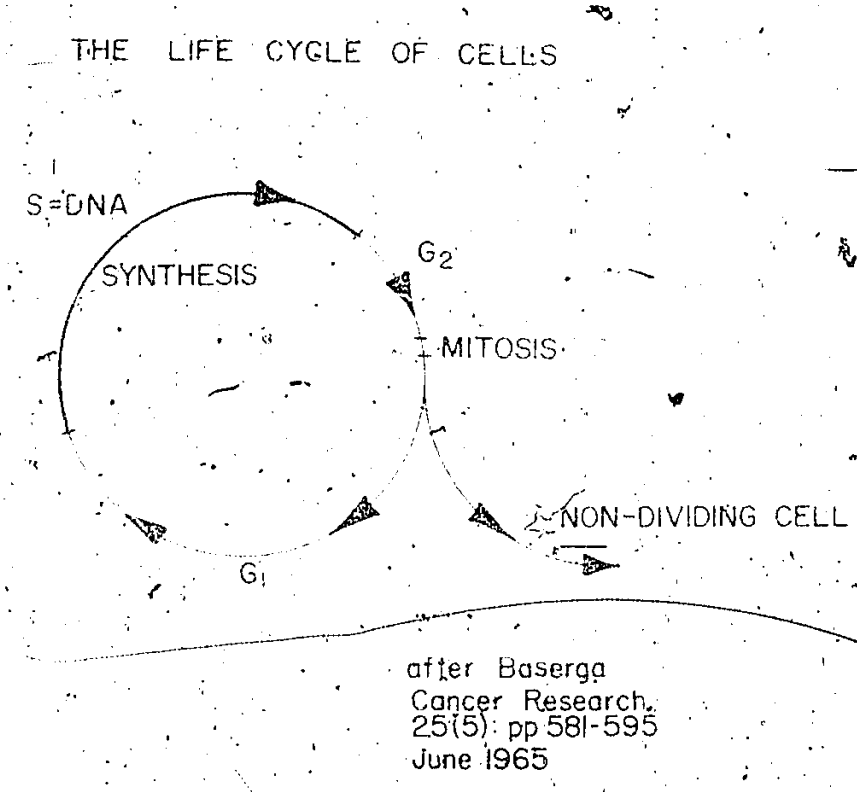

Fig. 1-1 A schematic representation of the cell cycle illustrating the four phases named by Howard and Pelc (1953). M(mitotic), G1 (Gap 1; pre-DNA synthesis), S (DNA synthetic), and G2 (Gap 2, post-DNA synthetic).

The Duration of the Cell Cycle in Mammalian Cells

The duration of the cell cycle of a particular cell type under defined conditions can be considered to be relatively constant from cell to cell. Moreover, each phase of the cycle occupies a constant fraction of this cell type's cycle time. In mammalian cells, the total cycle time (T_C) can vary considerably from cell type to cell type. The basis for most of the variability is the G1 phase. The S phase in most mammalian cell types averages around 6-8 hours, although it can be as short as 2 hours. The G2 phase is always quite short, from 30 minutes to 1 hour, and in some cell types is completely non-existant. Kubitschek (1971) and Mitchison (1971) describe in detail the variations found in cell cycle times.

Synchrony in Mammalian Cell Populations

The normal growth pattern in a continuously proliferating cell population, *in vivo* or *in vitro*, is completely randomnized with respect to the phases of the cell cycle. Such a population is said to be asynchronous. Patterns of asynchronous growth are always associated with cell cultures in which the

cell number is increasing exponentially. It is possible to manipulate an exponentially proliferating cell population in such a way that most of the cells pass through the phases of the cell cycle together.

Synchrony can be induced in mammalian cell populations by either chemical or physical means (Nias and Fox 1971). This is done by temporarily blocking the progression of the cells through the cell cycle. When an exponentially proliferating population of cells is exposed to such a synchronization procedure, the cells progress to a specific point in the cell cycle and accumulate at this point. After a period of time, the blockade is removed and the cells which have accumulated in the blocked phase progress synchronously through the cell cycle. An alternative method for attaining proliferative synchrony is the isolation (or separation) from the population of cells that are all in one phase of the cell cycle (Terisma and Talmach 1961, Terisma and Talmach 1963). The members of this selected subpopulation can then proceed through the remainder

of the cell cycle more or less in phase.

The production of synchronous cell populations by induction or selection is desirable when the process of cell division (i.e. the cell cycle) is being studied. It is clearly advantageous to have large numbers of cells at the same stage of the cell cycle, rather than be limited by techniques based on the examination of individual cells in an asynchronously proliferating population. It should be emphasized that all methods of synchronizing cell proliferation cause the cells to suffer some degree of "unbalanced" growth (Toby et al. 1972). Synchronized cells, once released from a blockade or allowed to proliferate for some time after a selection procedure, will eventually lose their synchrony. All synchronized cell populations will return to an asynchronous state unless randomnization is prevented by a further application of the same or another synchronization procedure (Engleberg 1964).

II MATERIALS AND METHODS

TECHNIQUES OF MAMMALIAN CELL CULTURE

Origin of Experimental Cells

Throughout the experimental work presented in this thesis, a clonally derived line of baby hamster kidney cells (Macpherson and Stoker 1962) was used. The line designated as E4 was obtained from D. Stoltz who isolated this cell line from the BHK (baby hamster kidney)-21 (C 13). It should be noted, that the term "cell line" can be applied to E4 cells only in a tentative way since E4 cells have not been completely characterized. However, these cells have behaved consistantly throughout the period of experimentation covered in this thesis. The partial characterization of E4 cells appears in the results section.

A Brief History of the E4 Cell

The original clone E4 was selected at random from a group of colonies of BHK-21 (C13) cells (Stoltz, unpublished). E4, and other clones were then screened

for their cloning efficiencies. E4 was found to have an efficiency greater than 70% (i.e. the number of clones established in culture was at least 70% of the number of viable cells originally placed in culture). I received these cells from D. Stoltz after they had undergone between 4 and 6 passages.

The cells were maintained on antibiotic-free Minimum Essential Medium (Eagle 1959) containing 10% heat-inactivated Fetal Calf Serum. They were incubated at 37°C in as atmosphere of 5% CO_2 in air. The cells were cultured as monolayers in 250 ml plastic tissue culture flasks (Falcon Plastics, Oxnard, Calif.). After several passages the cultures were found to be severely contaminated with a mycoplasma-like organism. While cultivation of the organism on PPLO agar is the method of choice for confirming the presence of PPLO (mycoplasma), direct observation of the infective agent under negative contrast electron microscopic conditions was sufficient to confirm the presence of an infesting agent that either was, or closely resembled a mycoplasma (PPLO). This infection was

treated according to the procedure outlined on pg. 26.

After the PPLO infection was brought under control, the MEM formulation was modified by reducing the Na_2HCO_3 level and incorporating the non volatile buffer tricine. This modified Eagle's MEM, designated MEM-10-T, was used to maintain the cell line and it formed the basal component of any experimental medium. After twenty passages through the modified medium, large numbers of cells were harvested from actively growing monolayers and some were frozen and stored at $-75^{o}C$ to provide reserves.

Culture Conditions

E4 cells were maintained in monolayers in antibiotic-free MEM-10-T, in air. For passage, the monolayers were always seeded at an initial cell density of 700 cells per cm^2 of flask surface. Plastic disposable tissue culture flasks and tubes (Falcon) were used throughout the experimental work. The cells were passaged every 3½ days.

Medium and Medium Preparation

Eagle's MEM was found to be a suitable culture medium for E4 cells. It was chosen because of its simplicity, low cost, ease of preparation, and most important the ease with which it can be modified. The formulations of the standard MEM and MEM-10-T are shown in Table 2-1.

Tricine (N-Tris (hydroxymethyl) methylglycine) buffer was first used for mammalian cell culture by Gardener (1969). The inclusion of such a non-volatile buffer allows cultures to be incubated in air rather than in a mixture of 5% CO_2 in air. This method of buffering cell cultures stabilizes the medium pH and eliminates the use of expensive CO_2 incubation equipment. These buffers also eliminate fluctuations in medium pH during growth, experimental manipulations, and routine passage procedures which bedevil users of the standard $NaHCO_3$-CO_2 buffer system. Tricine (Calbiochem, Los Angeles) is also believed to be useful in controlling PPLO infections (Spendlove et al. 1971).

Component	Eagle's MEM mg/L	MEM-10-T mg/L
NaCl	6800.00	6800.00
KCl	400.00	400.00
$NaH_2PO_4 \cdot H_2O$	140.00	140.00
$MgSO_4 \cdot 7H_2O$	200.00	200.00
$CaCl_2$ (anhyd.)	200.00	200.00
L-Arginine	105.00	105.00
L-Cystine	24.00	24.00
L-Glutamine	292.00	292.00
L-Histidine	31.00	31.00
L-Isoleucine	52.00	52.00
L-Leucine	52.40	52.40
L-Lysine	58.00	58.00
L-Methionine	15.00	15.00
L-Phenylalanine	32.00	32.00
L-Tyrosine	36.00	36.00
L-Threonine	48.00	48.00
L-Tryptophan	10.00	10.00
Valine	46.00	46.00
Choline Cl	1.00	1.00
Folic acid	1.00	1.00
Inositol	2.00	2.00
Nicotinamide	1.00	1.00
D-Ca pantothenate	1.00	1.00
Pyridoxal HCl	1.00	1.00
Riboflavin	0.01	0.01
Thiamine HCl	1.00	1.00
$NaHCO_3$	2200.00	2200.00
Phenol red	10.00	10.00
Glucose	1000.00	2000.00
Tricine	*******	1792.00

Table 2-1 Formulations for Eagles MEM and MEM-10-T.

The cells were cultured usually in monolayers, but some attempt was made to induce E4 cells to proliferate in suspension. Although E4 cells proliferated in suspension, they tended to aggregate, which in turn caused heterogeneities in the cellular environment from cell to cell as well as difficulties in determining cell concentration. The bulk of the experimental work and all routine maintenance was therefore done with monolayer cultures.

The complete medium, MEM-10-T plus fetal calf serum was prepared in 500 ml batches under sterile conditions. The medium components were either prepared as concentrates in the laboratory or purchased as concentrates from Grand Island Biologicals (Grand Island, New York). The fetal calf serum (Gibco, various lots) was always heat inactivated by heating to 56^{0}C for 30-minutes. This process removes possible cytotoxic elements present in the sera, and moreover is thought to diminish the danger of contaminating cultures with PPLO (Fogh et al. 1971).

The stock solutions were divided into amounts sufficient to prepare one 500 ml bottle of medium,

thus reducing the handling of any individual batch of solutions. This procedure reduced the risk of contamination as well as simplified the preparation of medium. A 25 ml aliquot of each freshly prepared bottle of medium was incubated at 37°C for three days to confirm its sterility.

Passage Procedures: E4 Cell Line Maintenance

The growth medium was removed from two, 3½ day old, E4 monolayers and the monolayers were washed once with phosphate buffered saline, pH 7.2, before addition of 8 ml of 0.025% trypsin solution. The trypsin was allowed to act for 2½-minutes then the cells were harvested by gentle shaking and transferred to a flask containing 30 ml of fresh, pre-warmed MEM-10-T. The cell concentration in the resulting suspension was determined by duplicate hemacytometric counts and enough cells were removed from the suspension to provide 5 x 10^4 cells for each 20 ml of final cell suspension. The final cell suspension was divided into 20 ml aliquots each of which was put into a 250 ml tissue culture flask. The cultures were incubated at 37°C.

Control of PPLO Infection

During the course of the studies presented in this thesis, a severe mycoplasma (PPLO) contamination was encountered. An attempt to render the E4 cells PPLO-free was made using the following procedure (Fogh et al. 1971).

The medium of infected monolayers was decanted and the monolayers were washed three times with sterile phosphate buffered saline. The washed monolayers were exposed to sterile distilled water containing 100ug of kanamycin (Bristol Laboratories of Canada, Lot AE-101, Kantrex) per ml for a period of exactly 90-seconds. The kanamycin solution was decanted and fresh pre-warmed MEM-10-T containing 200ug of kanamycin per ml was added. The monolayers were incubated at 37^{0}C for 48 hours; afterwhich the dead cells were removed by gently shaking the cultures, decanting the medium, washing the monolayers with phosphate buffered saline containing 1000ug of kanamycin per ml and adding fresh MEM-10-T containing 200 ug kanamycin per ml. The cultures were incubated at 37^{0}C

until a good growth was achieved. The cells were then passaged into antibiotic-free medium, and examined regularly for any signs of contamination. If the cells seemed to be healthy after the third passage in antibiotic-free medium, they were used for experimental work.

In my hands, this procedure never freed the cells completely from mycoplasma, but the infection was reduced greatly and maintained at an inocuous level.

Prophylaxis for PPLO Infection

Once the PPLO infection was brought under control, the following procedure was added to the routine cell line maintenance schedule. The E4 cells were passaged through MEM-10-T containing 200 ug kanamycin per ml once in every six passages. The cells were never used for experimental purposes during, or immediately after, kanamycin treatment.

Tissue Culture Glassware and Plasticware

Falcon disposable plastic culture products were used exclusively. These culture vessels were used directly from the pre-sterilized package in which they were shipped. All partially used packages were carefully resealed and stored in a dust-free cabinet.

Before use, all glassware was washed in 7X detergent (Gibco), rinsed thoroughly in tap water, and rinsed in triply distilled water. The glassware was then dried in a hot oven (105^{0}C overnight), wrapped and sterilized, and stored in dust-free conditions.

CYTOLOGICAL TECHNIQUES

Preparation of Cells for Light Microscopic Examination

The cells were harvested from the monolayers by mechanical detachment or gentle trypsinization, collected by centrifugation and washed once in PBS. The washed cells were resuspended by vigorous force pipeting directly into fresh Carnoy's solution and fixed for 5-minutes. The fixed cells were centrifuged, the fixative was decanted and 0.5 ml of fresh fixative was added to the cells. A few drops of this cell suspension were placed on a clean microscope slide and ignited immediately. The slides were stained with Giemsa stain (Fisher Scientific).

Chromosome Preparations

E4 chromosomes were prepared according to Hsu (1972). The final preparations were either stained with giemsa stain or nuclear fast red stain.

Preparation of Material for Autoradiography

The cells were handled exactly as described in the first two sections, but the microscope slides

were cleaned before use. The slides were washed first in soap and water, then rinsed in tap water, bi-distilled water, and finally in acetone. Once the slides were cleaned, they were stored in a dust-free area until needed.

Geimsa Staining Procedure

The prepared slides were placed in fresh Geimsa stain for 20-minutes. The stained slides were then dipped three times in bi-distilled water and passed through 3, 5-second changes of 100% acetone. The slides were then cleared in two, 15-minute changes of xylene and mounted in Permount (Fisher Scientific) with a #1 coverslip.

Preparation of Material for Electronmicroscopy

Monolayers were washed twice in phosphate buffered saline and fixed in situ with cold glutaraldehyde solution for 1½-hours. (All operations were carried out at 4^{0}C unless otherwise specified).

The fixed monolayers were gently scraped from the tissue culture flask, the cells are washed twice

with 0.1M phosphate buffer, and then post-fixed in osmium tetroxide for 1½-hours. The cells were then washed thrice in 0.1M phosphate buffer, dehydrated in an acetone series, and finally embedded in Sperrs plastic.

The embedded material was then prepared for examination by thin sectioning, staining in uranyl acetate and counter-staining in lead citrate.

Preparation of Autoradiographs

The following description of the technique, as applied in this thesis, is more or less standard. The use of high specific activity tritiated thymidine (New England Nuclear Canada LTD. Dorval, Quebec. NET 027X) for the labeling experiments allowed the autoradiographs to be processed within 48 hours of preparation.

A portion of the bulk emulsion (NTB-2, Kodak) was melted at 40°C and placed in a dipping jar. The slides were dipped in the emulsion for 3-seconds, stood on end and allowed to dry thoroughly. The dry,

freshly dipped autoradiographs were placed in light-tight boxes and stored at room temperature for 48 hours. After the exposure period, they were developed in D-19 (Kodak), fixed in Fixer (Kodak), and dried at room temperature. The developed autoradiographs were stained in nuclear fast red according to Sams (1967).

CHEMICAL TECHNIQUES

General Discussion of Technique

The extractive and analytical procedures used for the determination of cell protein, DNA, and RNA are all standard methods and were used unmodified.

Nucleic acid extraction was done according to the method of Scott, Fraccastoro and Taft (1956) and the quantities of nucleic acids were determined by UV spectrophotometry. The RNA concentration from the Scott's extract was calculated using the correction described by Fleck and Munro (1962). The protein content was measured according to the Oyama modification (Oyama and Eagle 1956) of the Lowry procedure (Lowry et al. 1951).

CELL CYCLE AND CELL SYNCHRONY METHODS

The Duration of the Phases of the Cell Cycle

An estimate of the duration of the phases of the cell cycle can be obtained by using autoradiography to follow a population of cells which have been pulse-labeled with the radioactive DNA precursor tritiated thymidine (^{3}HTdr), as they pass through the different phases of the cell cycle. Cells in DNA synthesis at the time of the pulse will incorporate label, therefore S phase cells can be traced as they pass into mitosis (M phase), since they can be identified by the autoradiographic labeling that they will produce. When the percentage of labeled cells in mitosis are scored at intervals after the administration of the ^{3}HTdr pulse, a graphical representation of the data can be used to determine the duration of the phases of the cell cycle.

Since mitosis is relatively short in duration compared to the S phase, there are periods of time in which all the mitotic cells will be labeled. A

plot of the data for labeled mitoses vs. time (Fig. 2-2) shows two successive waves of labeled mitotic cells which correspond to the passage of the pulse-labeled S phase population passing through two mitotic periods and one somplete cell cycle. The total cell cycle duration (T_c) is determined from the interval between the mid-point of the two waves of labeled mitotic cells. The duration of the period of time between S phase and mitosis (T_{G2}) is taken as the interval between the first labeled mitosis. T_m, or the duration of mitosis, is determined by the interval of time between the appearance of the first labeled prophase and the first labeled late telophase. The duration of S phase, T_S, is described by the interval of time where 50% of the mitosis are labeled before and after the first wave of labeled mitoses. The duration of the interval between the end of mitosis and the beginning of S phase (T_{G1}) can then be calculated from the difference between the total cell cycle time (T_c) and the sum of T_S, T_M, and T_{G2}. This represents the standard method of determining the duration of the phases of the cell cycle.

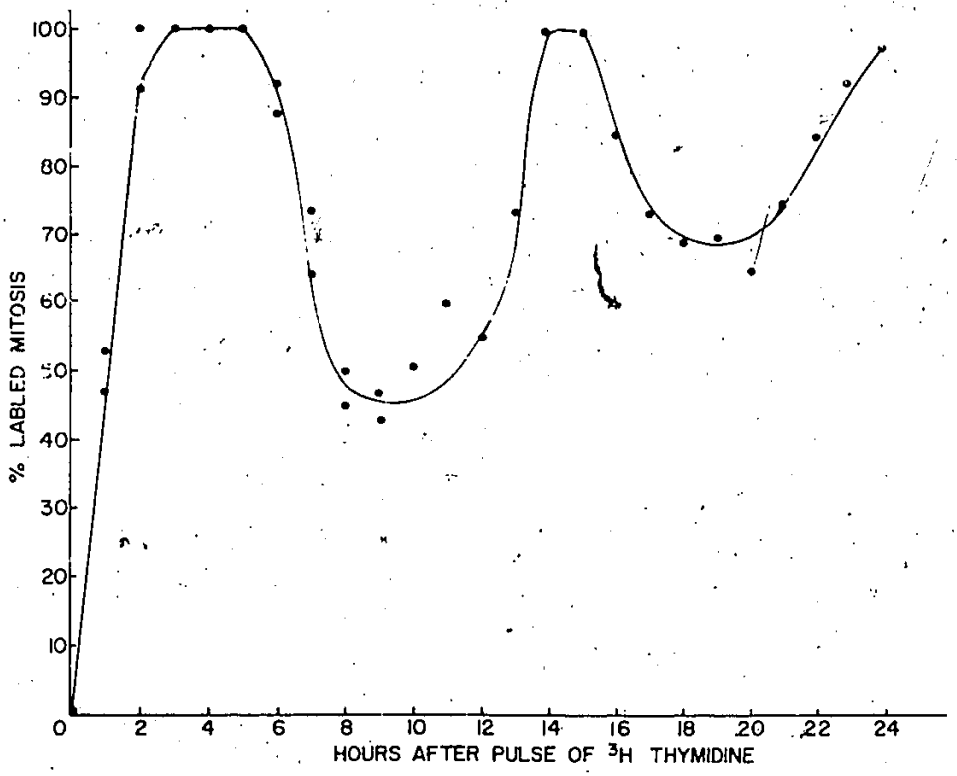

Fig. 2-1 An actual plot of percentage of labeled mitoses data obtained from autoradiographs prepared at intervals after the administration of a 5-minute pulse exposure to tritiated thymidine. This plot is an example of the type used to determine the phases of the cell cycle.

however several other methods are also in use (Lipkin 1971, Miller et al. 1972).

The Engleberg Synchrony Index

A variety of methods exist which allow an estimation of the degree of synchrony in a population of proliferating cells. The Engleberg method (Engleberg 1961) is based on a calculation of the fractional increase in cell number per unit time over any interval of time in which the cell number in a given population exactly doubles. The fractional increase in cell number per unit time, designated as R, is calculated from growth curve of a cell population and compared to the hypothetical plot of R for and exponentially proliferating cell population (R_u).

To facilitate the calculation of the Engleberg Synchrony Index, a computer program, an example of which is provided in Fig. 2-2, was devised in the APL language for use with the IBM 1640 computer. This program converts a growth curve to a plot of the fractional increase in cell number over the interval

```
    SYNC
1   'ENTER DATA: GROWTH CURVE, DOUBLING TIME.'
2   '                    '
3   C←⍞
4   T←⍞
5   DT←T+⍴G⋄N←1⋄M←2⋄K←⍬⋄R←⍬
6   DG←G[M]-G[N]⋄AG←⌊(.5+(G[N]+G[M]))+2
7   R1←⌊(.5+(100x((DG+DT)+AG)))
8   →(M=⍴G)/5⋄N←N+1⋄M←M+1⋄R←R+R1
9   P←⌊(.5+(R+0))⋄0←⍴G⋄R2←⍬⋄N←1⋄M-2
10  →(P<R1/11⋄R1←⌊(.5+(100x((DG+DT)+AG)))⋄AG←⌊(.5+G[N]+G[M]))+2⋄DG←G[M]-G[N]
11  →13⋄R2←R2+R1⋄R1←⍬
12  R2←R2+R1⋄R1←R1-P
13  →(M=⍴G)/10⋄N←N+1⋄M←M+1
14  S←⌊(.5+((R2+R)x100))
15  '                    '
16  '                    '
17  'AREA UNDER CURVE R=';R;'UNITS.  OVERLAP AREA=';R2;'UNITS.   SYNCHRONY=';S;'PERCENT.'
18
```

Fig. 2-2 An APL computer language programme for the calculation of the Engleberg Synchrony Index.

in question and compared it to the appropriate hypothetical curve. The program then calculates the Engleberg Synchrony Index.

III RESULTS

PARTIAL CHARACTERIZATION OF E4 CELLS

Growth of E4 in Suspension and Monolayer Culture

E4 cells proliferated under both monolayer and suspension culture conditions. In all cases the cells were cultivated in MEM-10-T and incubated at 37°C. The cell number in monolayer culture doubled in approximately 12-hours and in suspension culture in about 11-hours.

The viability of the cells was measured at various points in the growth curve by trypan blue exclusion. A persistent and increasing non-viable population was found in the cells cultured in suspension, while the cells in monolayer culture exhibited no loss of viability until stationary growth was achieved. E4 cell cultures in suspension tended to rapidly form and maintain aggregates of 2-15 cells even in the presence of methyl cellulose.

An attempt to adapt E4 to mono-dispersed suspension growth was made by repeated passage in medium containing methyl cellulose. However after 15 passages no significant decrease in cellular aggregation was observed.

The Chromosome Frequency Distribution in E4 Cells

Monolayer cultures of E4 cells were exposed to the mitotic inhibitor colcemid (Grand Island Biologicals, GIBCO, Grand Island, N. Y.) at a concentration of 0.06ug per ml of medium for 2½ hours at 37°C. The accumulated metaphase cells from several monolayers were harvested by gentle shaking (a procedure made possible by the particular sensitivity of mitotic cells to detachment from the monolayer growth surface). Chromosome preparations were made from these cells; 50 randomly selected, well-distributed chromosome groups were photographed at 200X. The chromosome number was determined from suitably enlarged prints of these photomicrographs (Plate 3-1). E4 cells were found to have a mean chromosome number of 63 compared to the 44 reported for BHK-21 (C-13) (Fig. 3-1).

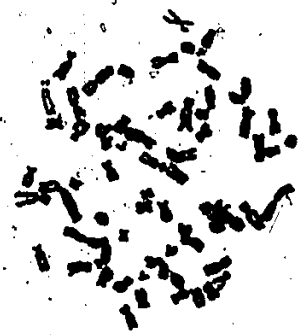

Plate 3-1. Chromosome distribution representative of the type used to estimate the mean chromosome number and chromosome frequency distribution in E4 cells.

CHROMOSOME FREQUENCY DISTRIBUTION FOR E4 CELLS

No.	Frequency
56	
57	
58	
59	
60	**
61	****
62	***
63	**************************
64	**********
65	***
66	*
67	*
68	*
69	
70	*

Average = 63

Fig. 3-1 The chromosome frequency distribution in E4 cells. The above distribution is based on direct counts made from 50 different photomicrographs of well distributed chromosome preparations.

The Karyotype of E4

The karyotype for E4 was not definitely established but an attempt was made to compare superficially the chromosomes of E4 to the parental BHK cell line. While a much more detailed study is required to properly assess the karyotype of E4, however crude karyograms (Plate 3-2) do demonstrate a striking similarity between the chromosomes of E4 and BHK-21 and suggest that E4 is triploid with respect to BHK-21, the parental cell line.

The Growth Morphology of E4 Monolayers

E4 and BHK-21 monolayers in late exponential growth were photographed in situ in phase contrast optics. The E4 cells were epithelioid while the parental BHK-21 cells were spindle-shaped (Plate 3-3).

The Cell Cycle of E4

The cycle of E4 cells, cultured in MEM-10-T at 37°C, was determined by the labeled mitosis technique. Labeled prophase cells (Plate 3-4) were seen within 5 minutes of a 5-minute pulse of ^{3}HTdr, which indicated that E4 cells have no G2 phase under these conditions.

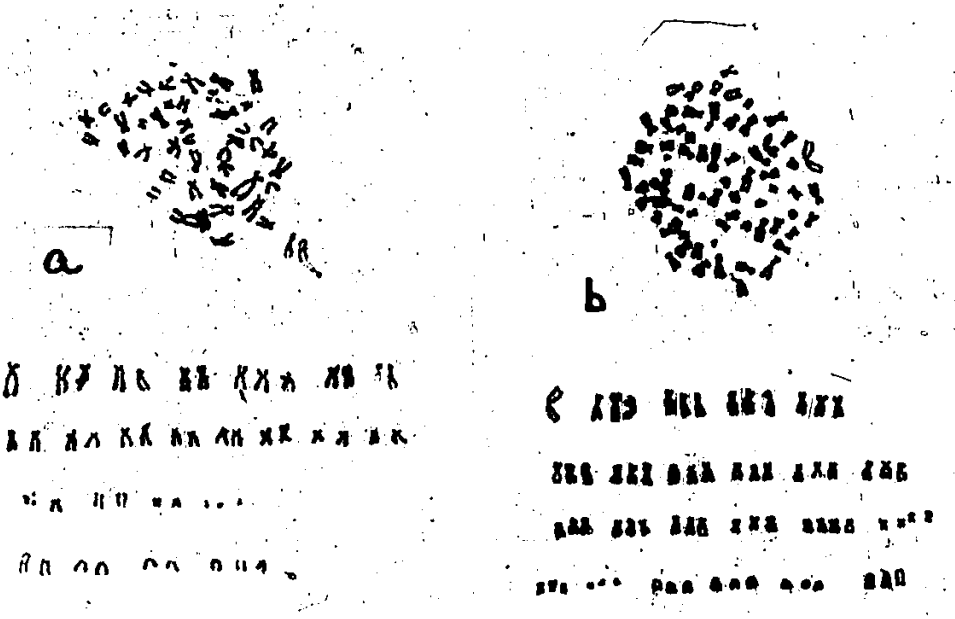

Plate 3-2. Karyograms of (a) BHK-21 parent cell line and (b) clone E4.

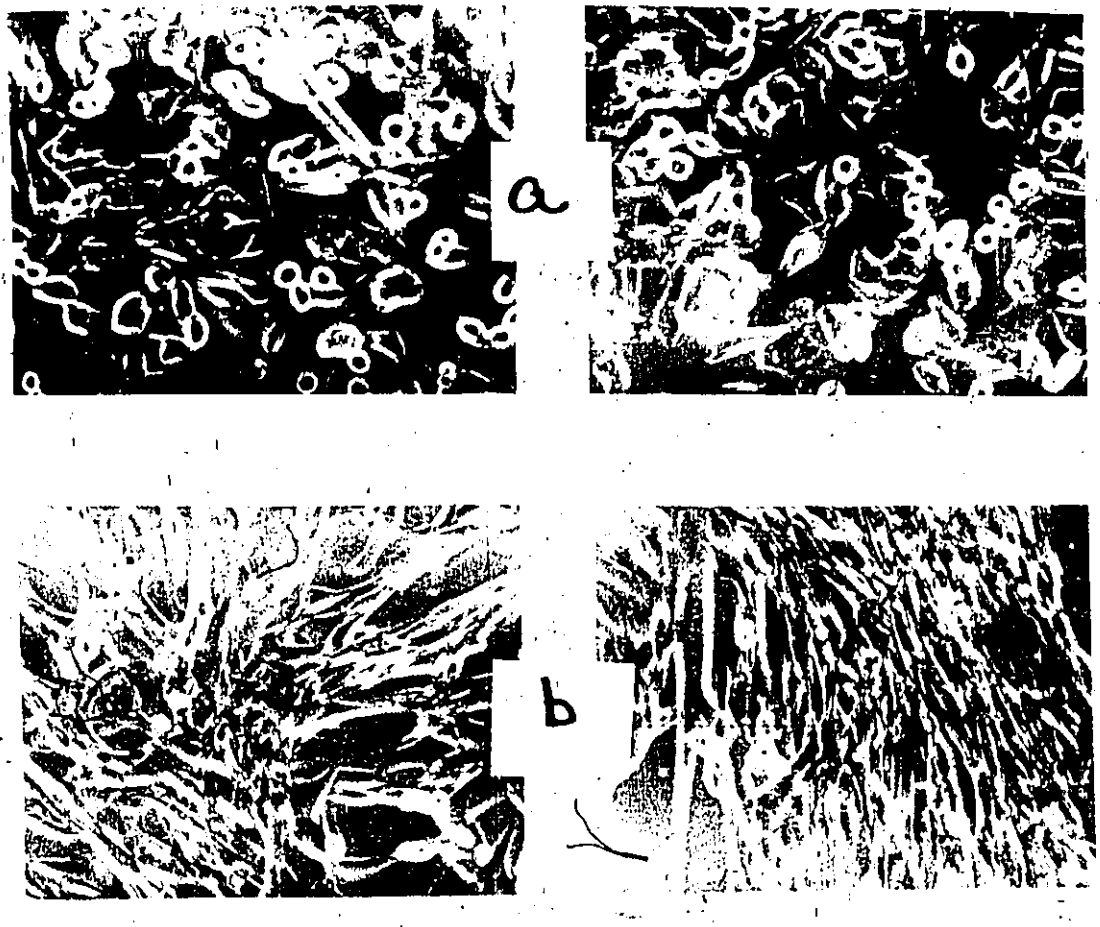

Plate 3-3. The pattern of growth in monolayer culture of (b) BHK-21, the parental cell line, and (a) the E4 clonal derivative. These monolayers were photographed under Vari-Color Phase Contrast optics (Wild, Heerbrugg) at 100X when they were in late exponential growth.

Plate 3-4. An example of labeled prophase figures observed in autoradiographs prepared from samples taken within 5 minutes of the 5-minute pulse exposure to tritiated thymidine (spec. act. of 18 ci/mMole in a concentration of 2uci/ml of medium).

The pulse-labeled cells were sampled at the intervals specified in the plot of the percentage of labeled mitoses (Fig. 3-2) and the duration of the phases of the cell cycle were determined from that plot. The total cell cycle time (T_C) is approximately 12 hours and is comprised of a 1-hour M phase, a 5-hour G1, and a 6-hour S phase.

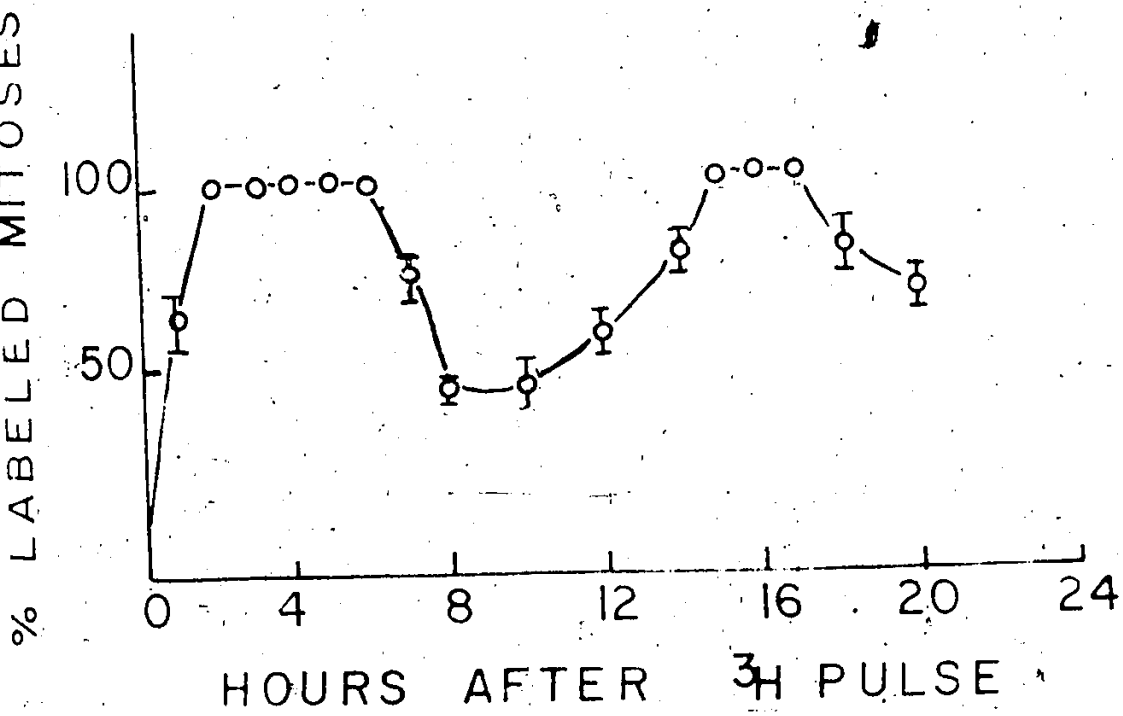

Fig. 3-2. The plot of % labeled mitoses derived from the 5-minute pulse label of an exponentially proliferating population of E4 cells with tritiated thymidine (specific activity of 18 ci/mMole at a concentration of 2uci/ml medium). The points represent the mean ± S.D. of four samples in two separate experiments.

THE EFFECT OF A HIGH POTASSIUM ENVIRONMENT ON THE MULTIPLICATION OF E4 CELLS

The Inhibition of Cell Proliferation by High Potassium Medium

The cell number and viability of E4 cells cultured in MEM-10-THK (high potassium medium) was followed for 96 hours. The high potassium treatment began 24 hours after seeding fresh monolayers. High potassium medium has exactly the same composition as MEM-10-T except that the sodium and potassium concentrations were changed from 118mM and 5.4mM respectively to 10mM and 114mM respectively. The total cell number was estimated by counting all the cells within a representative area of the growth surface which appeared healthy, and showed a normal adherance to the growth surface. This can also be taken as a measure of apparent viability.

Both the multiplication and viability of E4 cells were grossly affected by exposure to high potassium medium (Fig. 3-3). The loss of viability was accompanied by a detachment from the growth surface and a visible degredation of nuclear structure (i.e the complete loss of nuclear structure followed by the formation of clumps of nuclear material.

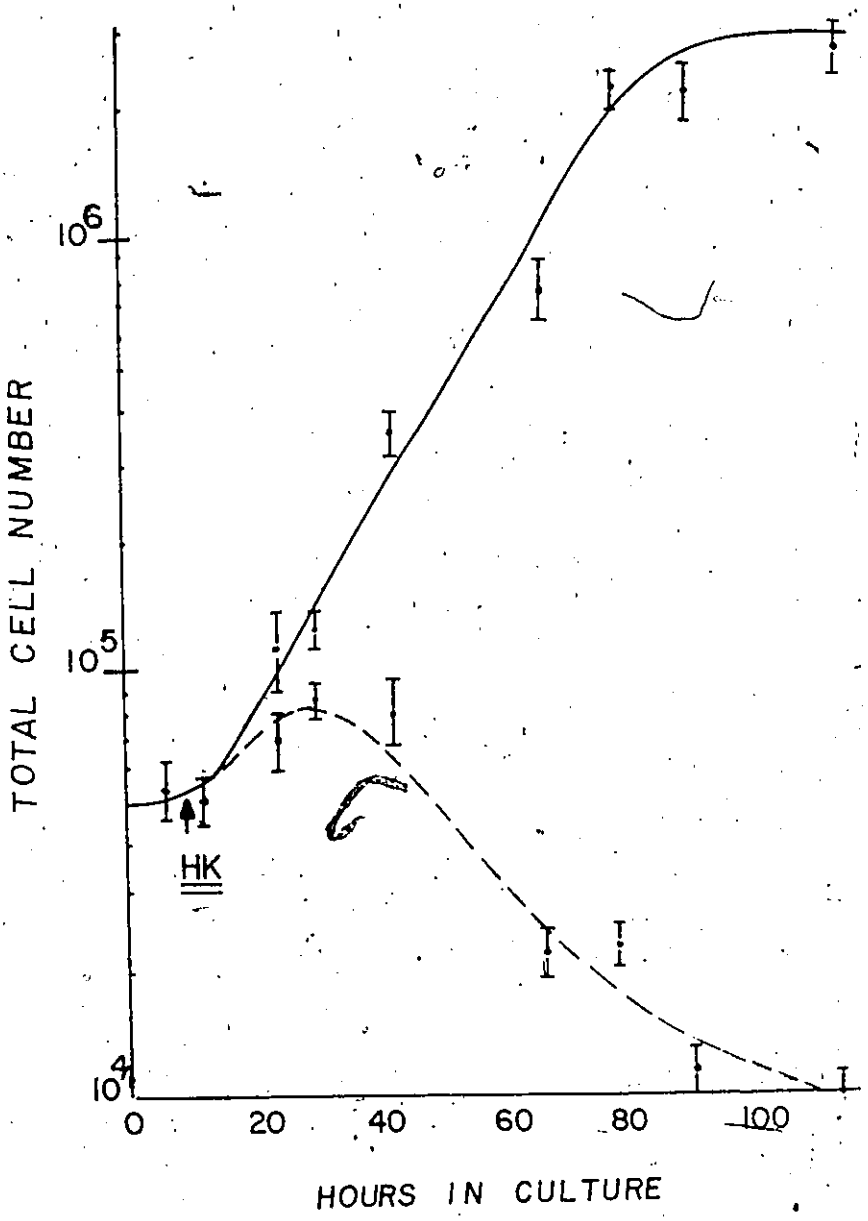

Fig. 3-3. The effect of high potassium medium on proliferation and viability of E4 cells in monolayer culture. The solid line (——) represents the control cultures grown up in MEM-10-T and the dotted line (---) shows the response of the high potassium treated cells. Each point in the mean $\pm$ S.D. of 6 samples from 2 experiments.

Cell Multiplication in the Presence of Normal Sodium Levels and Varying Potassium Levels

Eagle's MEM-10-T was modified by the addition of sufficient KCL to yield concentrations of 25, 50, 75, and 114 mM. Appropriate amounts of choline chloride were added to each of the different media, as well as to MEM-10-T, to ensure osmotic consistency between them.

A series of E4 monolayers, 12 hours post-seeding, were divided into 5 groups and the medium was replaced in each group by one of the 5 modified media. The total cellular protein content per culture was determined 36 hours later. The cultures were incubated at 37°C over this period. A concentration of 50 mM K produced a small, but significant ($P < .01$), decrease in the growth of E4 cultures. The effect on growth becomes increasingly pronounced as the K concentration is raised to 75 and 114 mM respectively (Fig. 3-4).

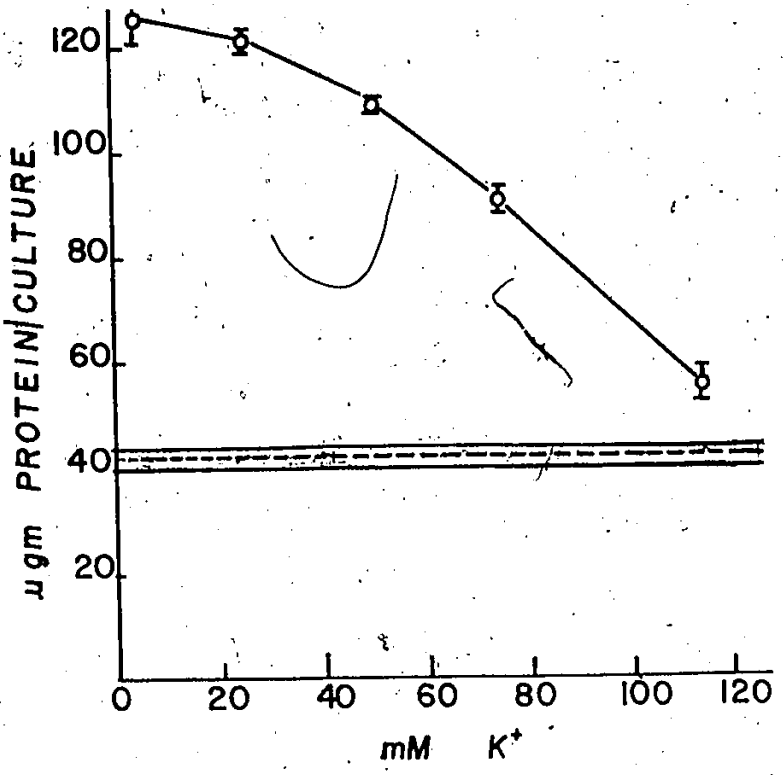

Fig. 3-4. The growth of E4 cells in 118mM Na medium with varying concentrations of K. The concentrations used were 5.4, 25, 50, 75, and 114mM respectively. Each point represents the mean±SEM of the total protein content of 8 cultures from 2 individual experiments. The shaded bar shows the average protein content ±SEM for the cultures at the start of the experiment.

Cell Multiplication in High Potassium Medium with Varying Amounts of Additional Sodium

MEM-10-THK medium was modified by the addition of sufficient NaCl to yield Na concentrations of 25, 50, 75, and 118mM. Appropriate amounts of choline chloride were added to each of the modified media as well as to MEM-10-THK to compensate for the osmotic differences among them. The experimental design was the same as for the previous experiment. The addition of Na to high potassium medium permitted growth of E4 cells at a reduced rate. The magnitude of the effect depended on the Na concentration. No effect on growth was noted at concentrations less than 50mM (Fig. 3-5).

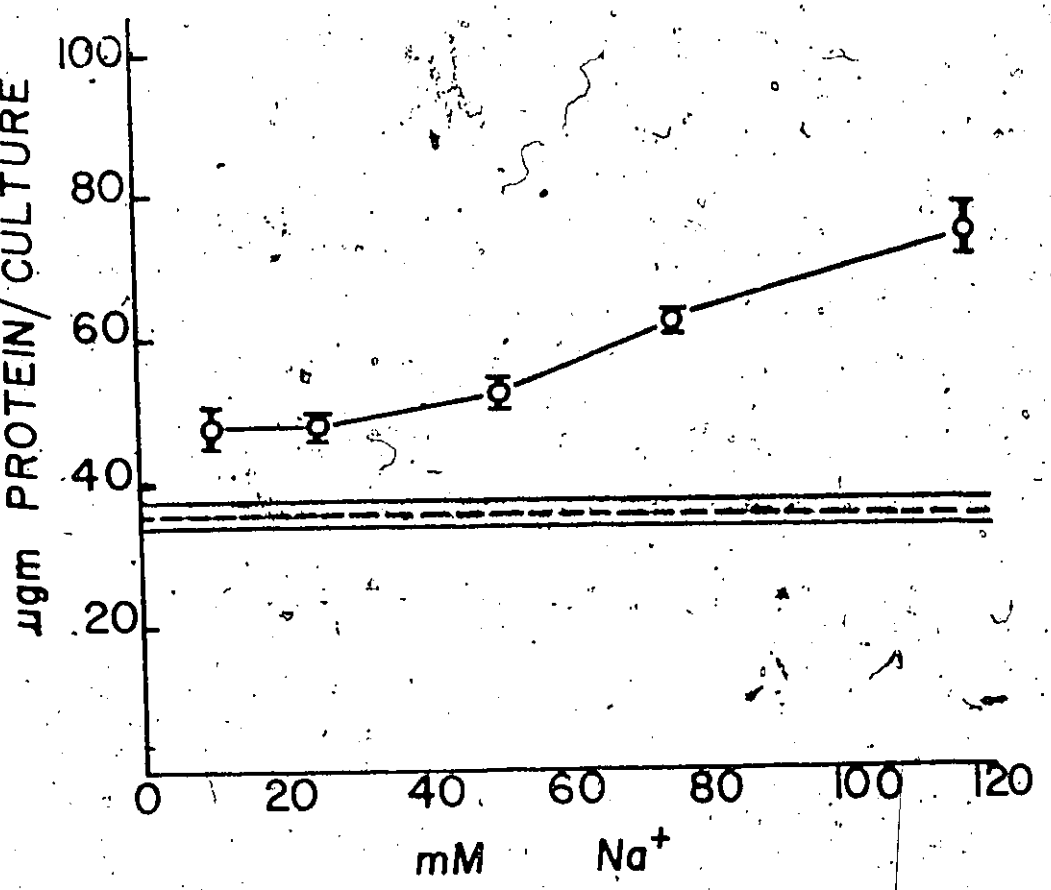

Fig. 3-5. The growth of E4 cultures in 114mM potassium medium with varying concentrations of Na. The Na concentrations used were 10, 25, 50, 75, and 118mM. Each point represents the mean±SEM of the total protein content of 8 individual cultures from 2 separate experiments. The shaded bar is the average protein content for all the cultures at the start of the experiment.

THE MORPHOLOGY AND FINE STRUCTURE OF E4 CELLS IN RESPONSE TO HIGH POTASSIUM TREATMENT

Experimental Design for Morphology Studies

A series of monolayers in exponential growth phase (40 hours post seeding) were divided into 2 groups of 3 and 21 cultures respectively, the smaller group of cultures were controls. At 0 time, the medium was replaced in each of the experimental cultures with pre-warmed MEM-10-THK and then incubated at 37^{0}C. The control cultures received fresh, pre-warmed MEM-10-T, and were fixed at 0 time. The experimental cultures were sampled three at a time at 1, 2, 3, and 24 hours during high potassium treatment. At 24 hours, the medium in the remaining experimental cultures was replaced with fresh pre-warmed MEM-10-T and re-incubated at 37^{0}C. These cultures were also sampled in groups of three at 1, 2, and 3 hours after the second medium replacement.

The monolayers were handled in the following

way at each sampling point. The selected monolayers were washed once with sterile Earle's Salts Solution and immediately fixed in cold glutaraldehyde solution. Within two minutes of fixation the monolayers were photographed in phase contrast optics, after which the fixed cells were scraped from the growth surface and prepared for electromicroscopic examination.

Light Microscopy: Sequential Changes in the Morphology of E4 Cells Before, During, and After Exposure to a High Potassium Environment.

The morphological response of E4 cells during the first three hours of exposure to high potassium medium (114mM K) is marked by cytoplasmic vacuolization, nucleolar condensation, an apparent increase in cell size, and a noticeable increase in cytoplasmic projections. Twenty-four hours of exposure to the high potassium environment does not substantially enhance the morphological changes present after the first three hours. After the cells were returned to MEM-10-T medium (5.6mM K), the morphological appearance gradually returned to normal over the

gradually returned to normal over the next three hours (Plate 3-5).

The degree of nucleolar condensation was estimated by scoring the number of nucleolar masses present in 25 cells selected at random from the photomicrographs representative of each experimental and control group. The data show that significant nucleolar condensation occured during the course of HK treatment and that the condensation of nucleoli was reversed by restoration of the normal Na/K ratio (Fig. 3-6).

The Fine Structure of E4 Cells Before, During, and After Exposure to High Potassium Medium

Electronmicrographs of cells from each control and experimental group were prepared. Generally, an increase in cytoplasmic organelle number and a decrease in the electron density of the cytosol were observed. The cytoplasm was filled with microfibrils and microtubules after 3 hours in high potassium medium, but the number of these structures in the cytoplasm after 24 hours of treatment was greatly reduced relative to the earlier sample (Plate 3-6).

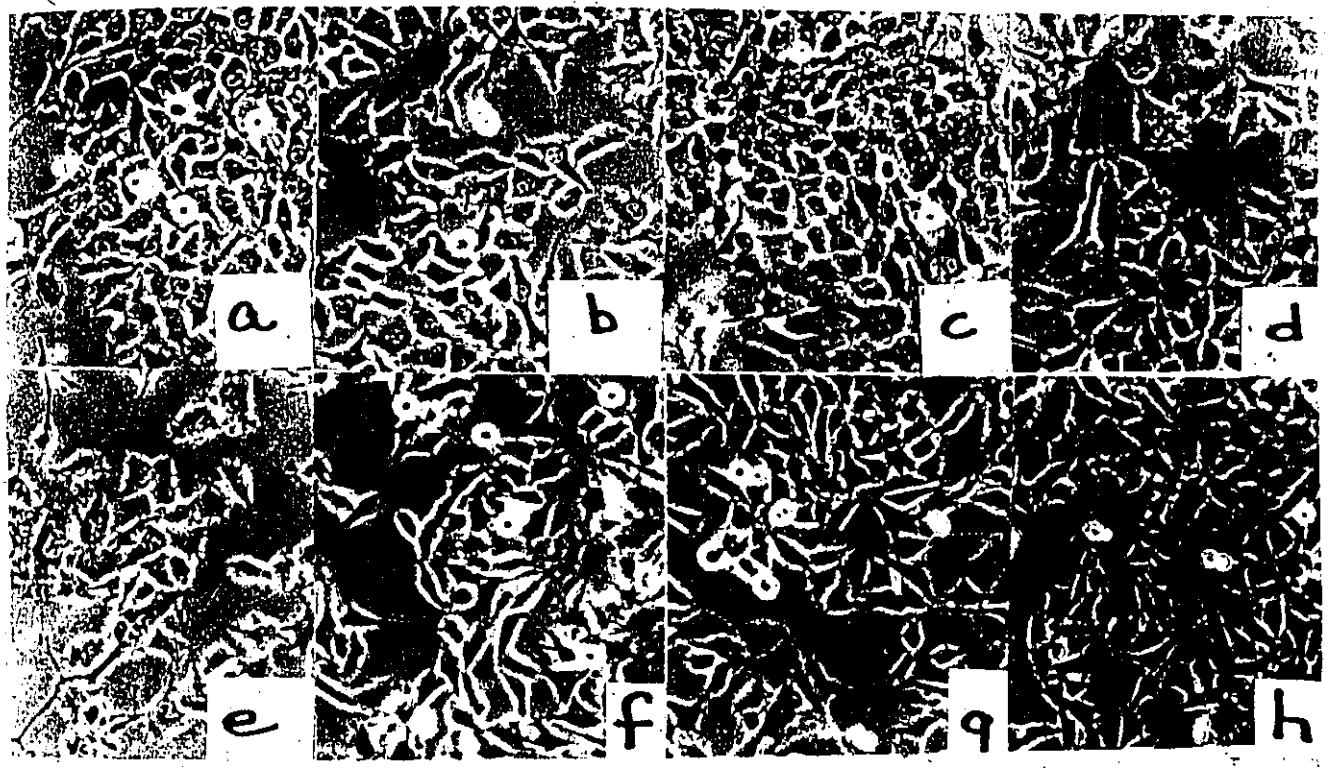

Plate 3-5. An illustration of the morphological changes induced in E4 cells exposed to high potassium medium. Control cells are shown in panel (a). Panels (b-e) represent 1, 2, 3, and 24 hours of high potassium treatment. Panels (f-h) show the recovery of normal morphology 1, 2, and 3 hours after the monolayers are returned to MEM-10-T medium. The highly refractile cells are in mitosis. The photomicrographs are of glutaraldehyde fixed monolayers and are taken under phase contrast optics on a Zeiss inverted microscope at 160X.

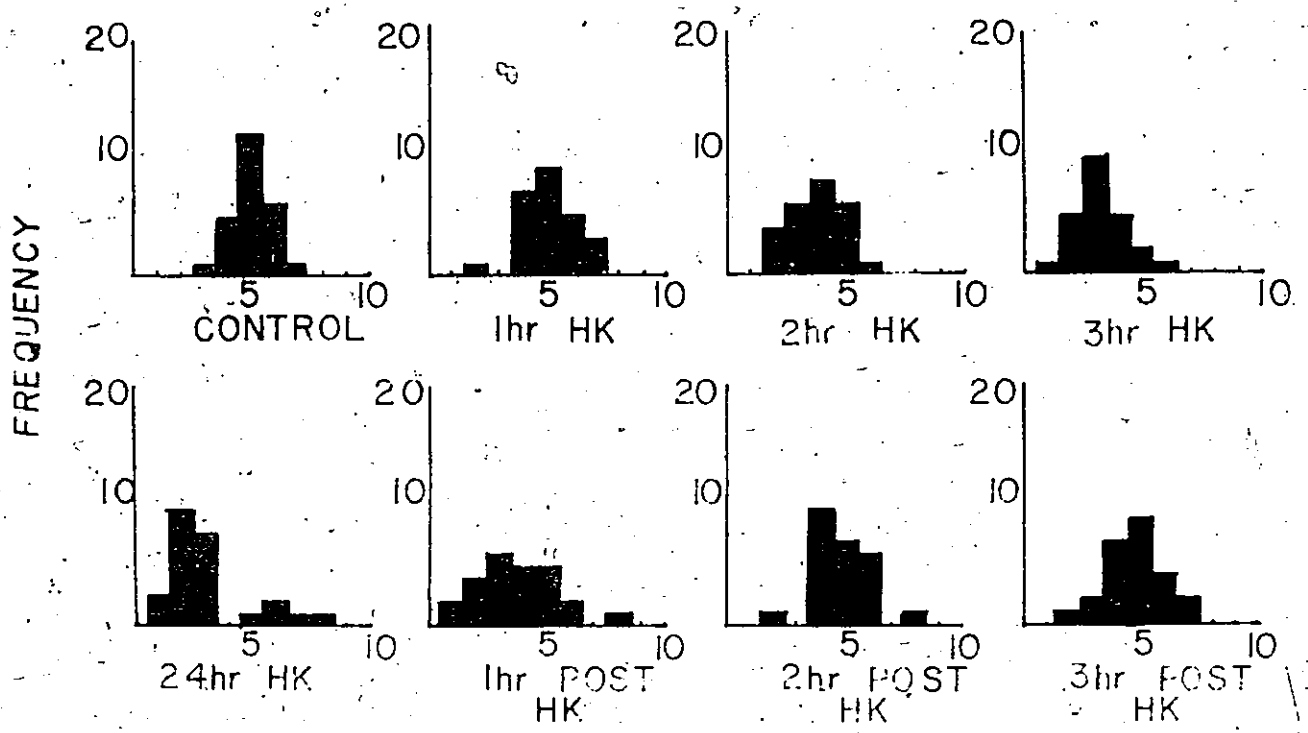

Fig. 3-6. Nucleolar condensation in E4 cells exposed to high potassium medium. Each frequency distribution is based on the number of nucleolar masses in each of 25 cells selected randomly from photomicrographs of glutaraldehyde fixed monolayers. The counts were made on samples taken before, during, and after high potassium treatment as indicated under each frequency distribution.

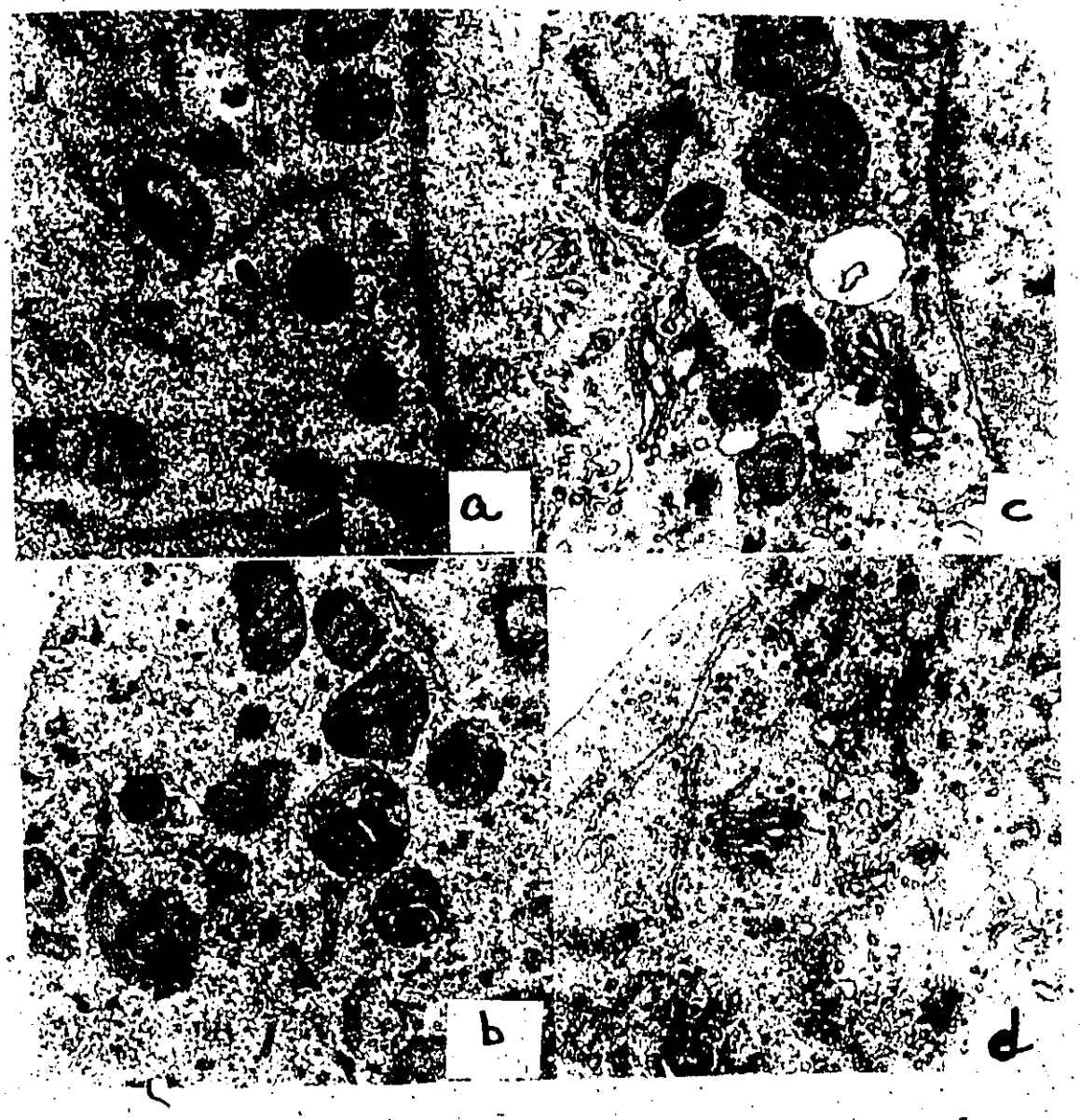

Plate 3-6. Alterations in the fine structure of E4 cells exposed to high potassium medium. Control cells are represented by (a), (b-c) show the effects of high potassium 3 and 24 hours respectively after initiation of treatment and (d) shows the fine structure of a cell that has been released from the high potassium medium for 3 hours. The original magnification of the electronmicrograph is 30,000X and the photo enlargement factor is approximately 2½X.

The distribution of ribosomes was also affected by high potassium treatment. Three hours after the cells were placed in high potassium medium the ribosomes became loosely aggregated, as opposed to the more or less even distribution seen in the controls (Plate 3-7). After 24 hours of treatment, the ribosomes were clearly aggregated into rosettes. When the cells were returned to a normal Na-K environment, the rosettes gave way to numerous, evenly distributed polysome groupings. The amount of endoplasmic reticulum increased above the control in response to high potassium treatment as well. After 3 hours of exposure to high potassium medium, the endoplasmic reticulum appeared to lose ribosomes, but it never completely lost its ribosomes even after 24 hours in high potassium medium. When the cells were released into MEM-10-T the endoplasmic reticulum became heavily studded with ribosomes. Poorly formed golgi-like structures were visible in the cytoplasm of the 24 hour high potassium sample in conjunction with increased amounts of endoplasmic reticulum. These golgi-like structures became very well organized when the cells were returned to MEM-10-T (plate 3-6).

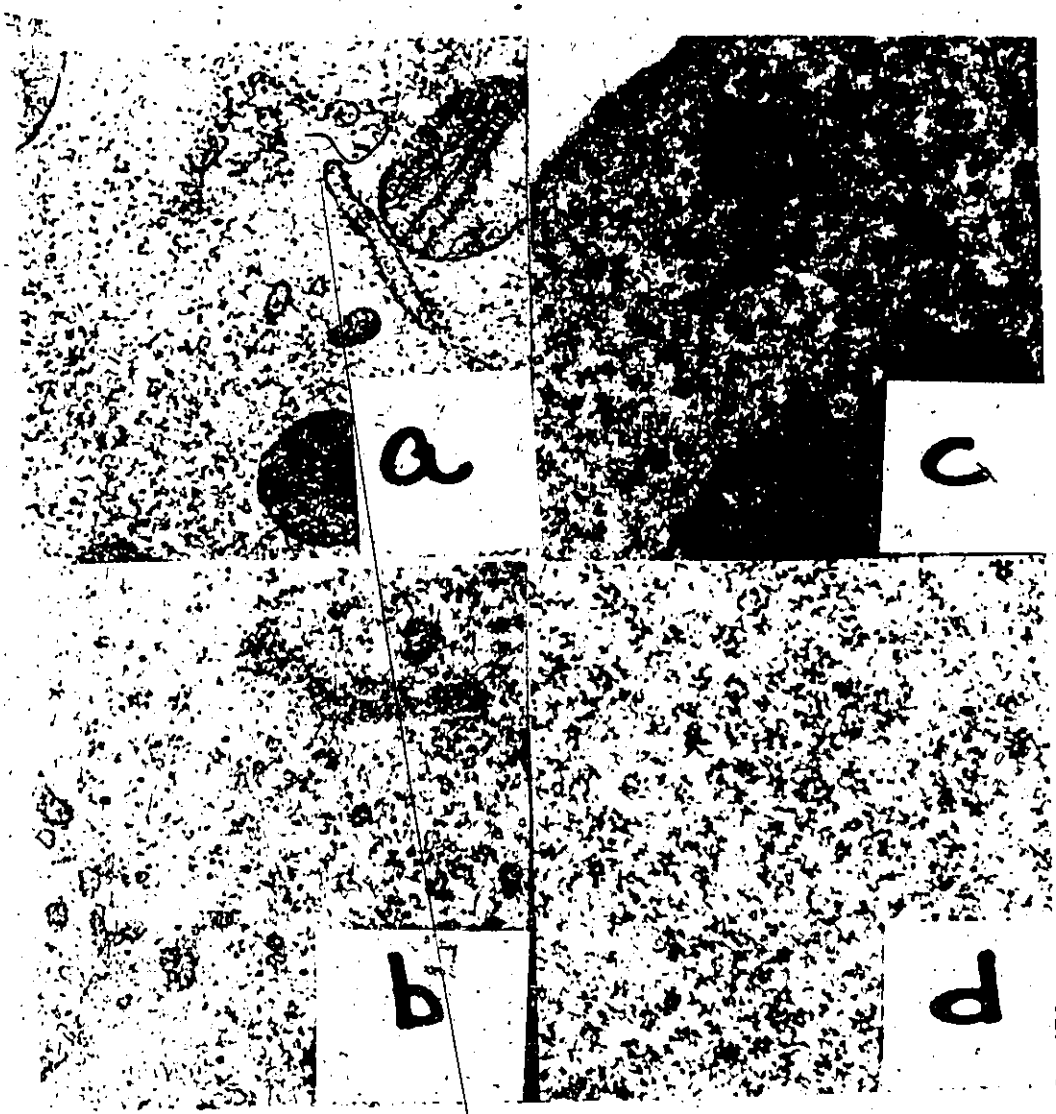

Plate 3-7. The distribution of ribosomes in the cytoplasm of E4 cells before (a), during (b and c, 3 and 24 hours in high potassium respectively), and after restoration of a normal Na-K environment (d, 3 hours post release). The original magnification of the electronmicrographs was 30,000X and the photo-enlarging factor is 5X (N.B. ribosomal rosettes in panel-c).

THE EFFECT OF HIGH POTASSIUM TREATMENT ON MACROMOLECULAR CONTENT

DNA, RNA, and Protein Content in Cells Exposed to High Potassium

A number of E4 monolayers in exponential growth phase were divided into two groups. The medium in one group was replaced with MEM-10-THK, and maintained in MEM-10-T. After 24 hours the RNA, DNA, and protein content per cell were determined according to Scott (1956) and the protein content was determined by Oyama's method (Oyama and Eagle 1962). The RNA and protein contents of high potassium treated cells were significantly greater than the controls, $P<.01$ in each case (Fig. 3-7). The difference in average DNA content per cell between high potassium treated groups and controls is not significant, $P<.20$ but $>.10$. Cells that have been returned to MEM-10-T after 24 hours in MEM-10-THK show a definite return to normal cellular macromolecular levels.

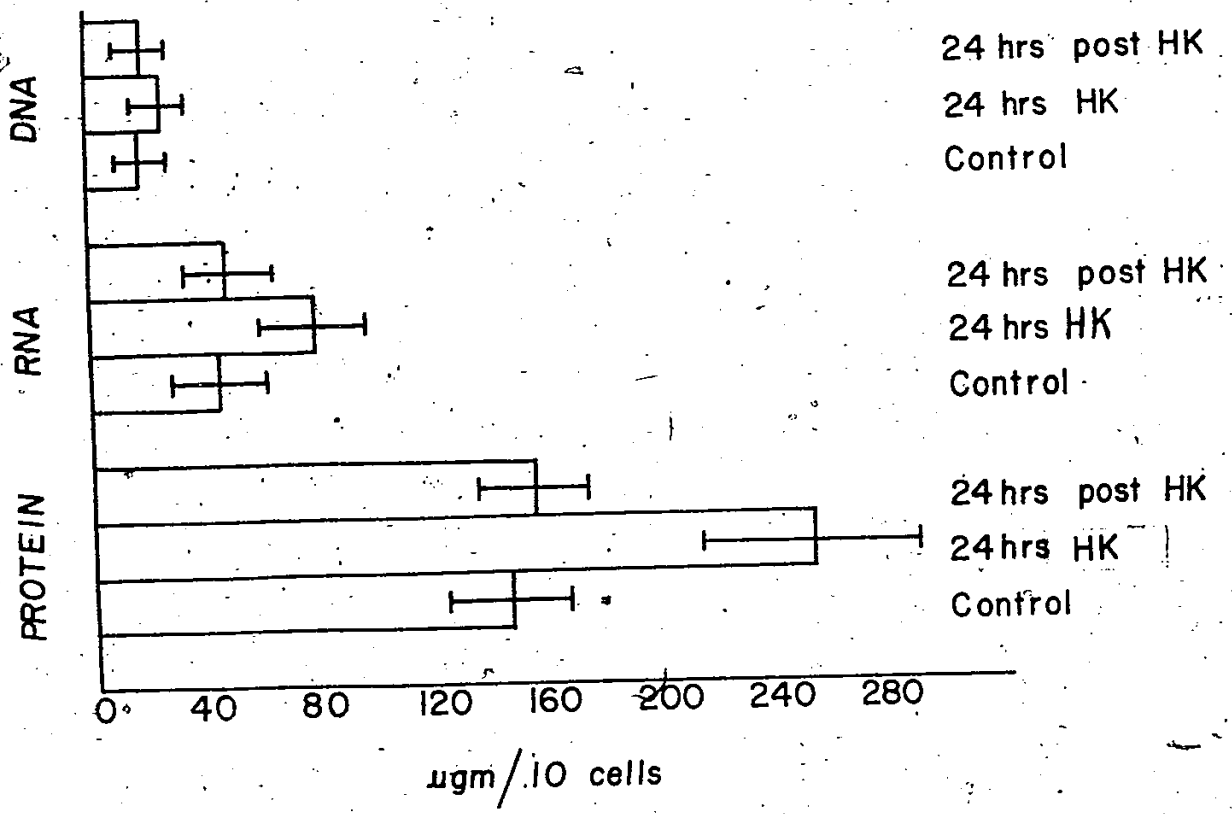

Fig. 3-7. The macromolecular content of E4 cells before, during, and 24 hours after high potassium treatment. The DNA, RNA, and protein contents were expressed on a per cell basis. The data is based on 8 separate determinations from 2 different experiments and is expressed as the mean ± SD at the times indicated on the figure.

THE EFFECT OF HIGH POTASSIUM ON DNA SYNTHESIS

Experimental Design for Uptake and Incorporation Studies

E4 monolayers, either control or high potassium treated, were continuously exposed to radioactively labeled DNA precursor (high specific activity tritiated thymidine - 18 ci/mMole) for a period of 90 minutes. Control monolayers were always studied in parallel with high potassium treated cultures. The tritiated thymidine was added to the medium to give a final concentration of 2uci/ml. The cultures were sampled in triplicate by random selection at 1, 15, 30, 45, 60 and 90 minutes after addition of isotope. The "hot" medium was decanted, and the monolayers immediately washed with "cold" Earle's salts solution containing 2mM thymidine and 0.1M Na-azide. The cells were then scraped from the surface of the culture flask and suspended in 2.5-ml of distilled water. The resulting suspension was placed into a test tube and 0.5-ml was removed for protein analysis. The remainder of the suspension was used for nucleic acid and scintillation analysis.

The Immediate and Prolonged Effect of High Potassium Treatment on the Accumulation and Incorporation of Tritiated Thymidine

The accumulation and incorporation of tritiated thymidine in exponentially proliferating E4 cells that have just been shifted to MEM-10-THK (114mM K) and cells that have been exposed to high potassium conditions for 24 hours were measured over a 90 minute period. The levels of isotope incorporation into the DNA fraction (cold acid precipitable material) and its accumulation in the cytoplasmic precusor pools (cold acid soluble material) were combined to yield an estimate of the total uptake of tritiated thymidine by E4 cells. The uptake curves (Fig. 3-8) show a lag of about 30 minutes before any appreciable uptake is evident in high potassium treated cells. The uptake of the isotope then proceeds at a reduced rate compared to that of the control cells. Since the isotopic content of the cells is largely a function of the incorporation of tritiated thymidine (Fig. 3-9) the resulting incorporation curves are similar to the uptake curves (Fig. 3-10).

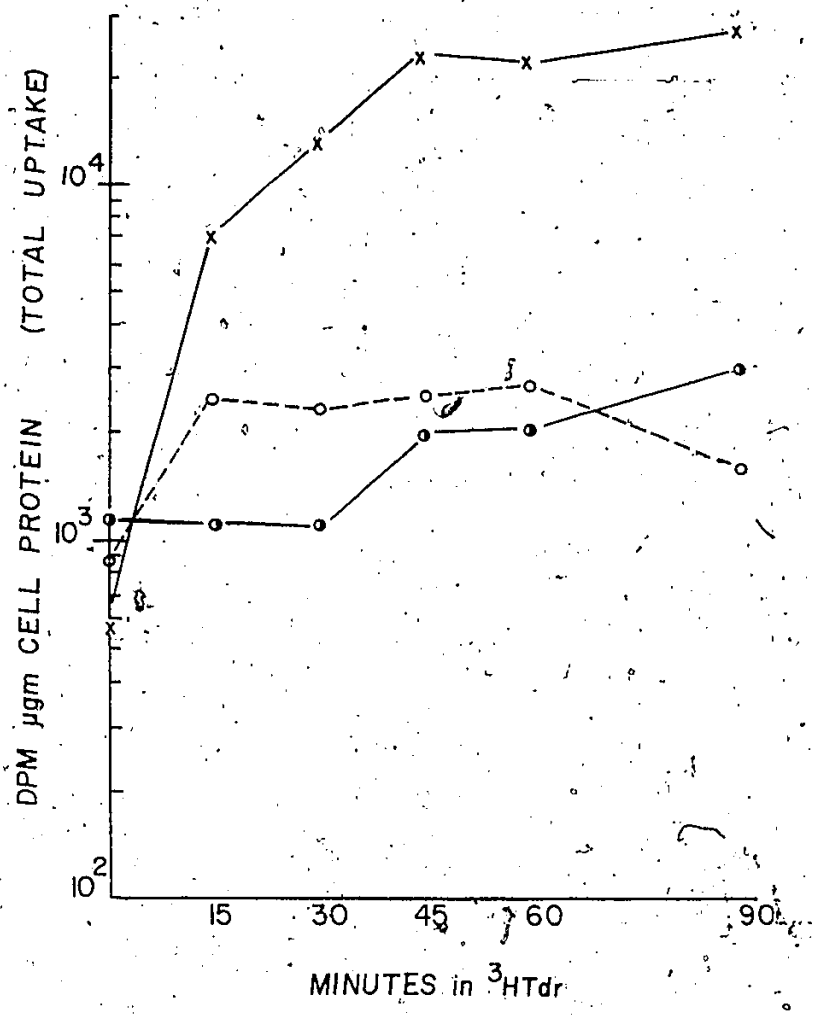

Fig. 3-8. The total uptake of tritiated thymidine by E4 cells in high potassium medium. Controls are represented by x——x, the cells that have just been exposed to high potassium medium by ●——●, and the cells that have been exposed to high potassium for 24 hours prior to labeling by o——o. The points were derived from the sum of the mean values for the incorporation and accumulation of radioactive precursor.

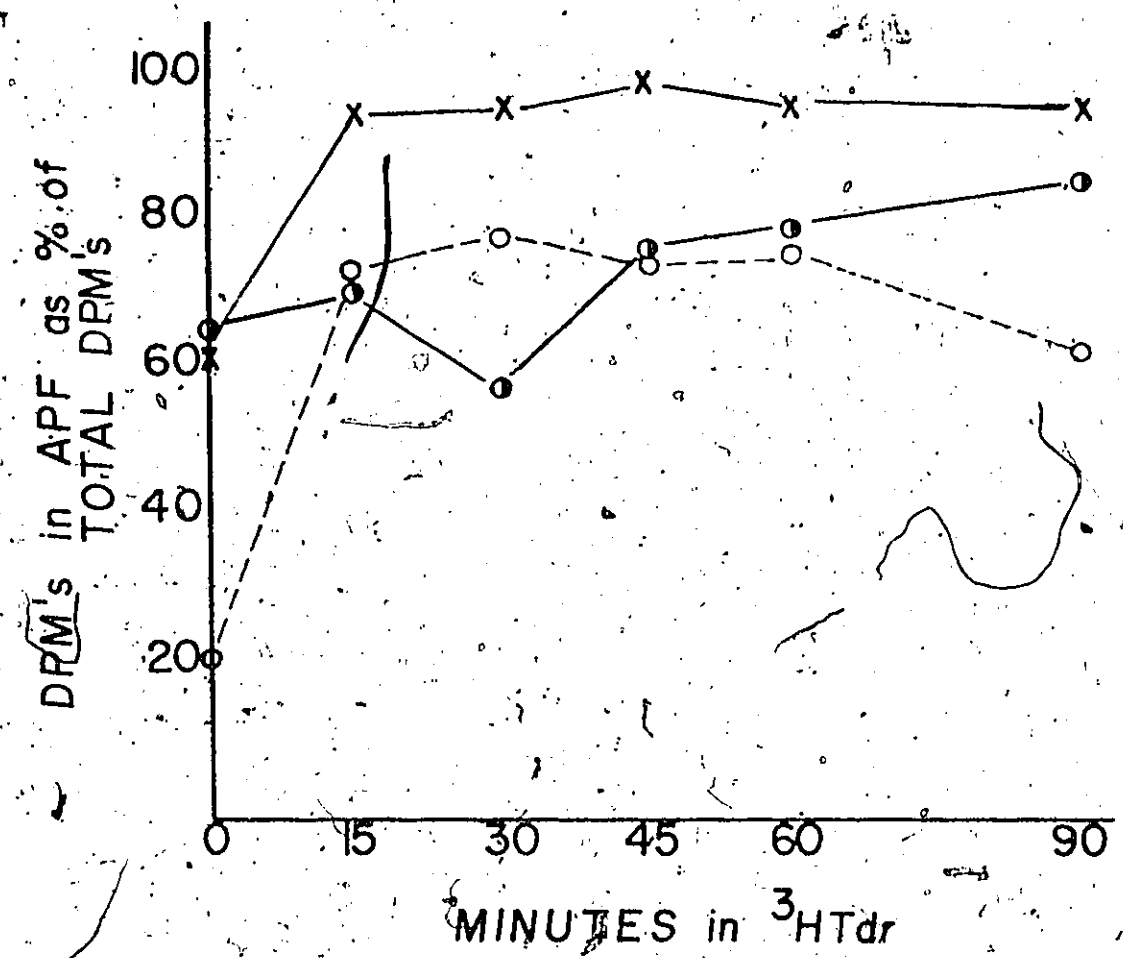

Fig. 3-9. The proportion of the total radioactivity that can be accounted for by the isotopic content of the cold acid precipitable material in control (x——x), cultures recently exposed to high potassium medium (0——0), and cultures that have been exposed to high potassium medium for 24 hours prior to labeling (0——0).

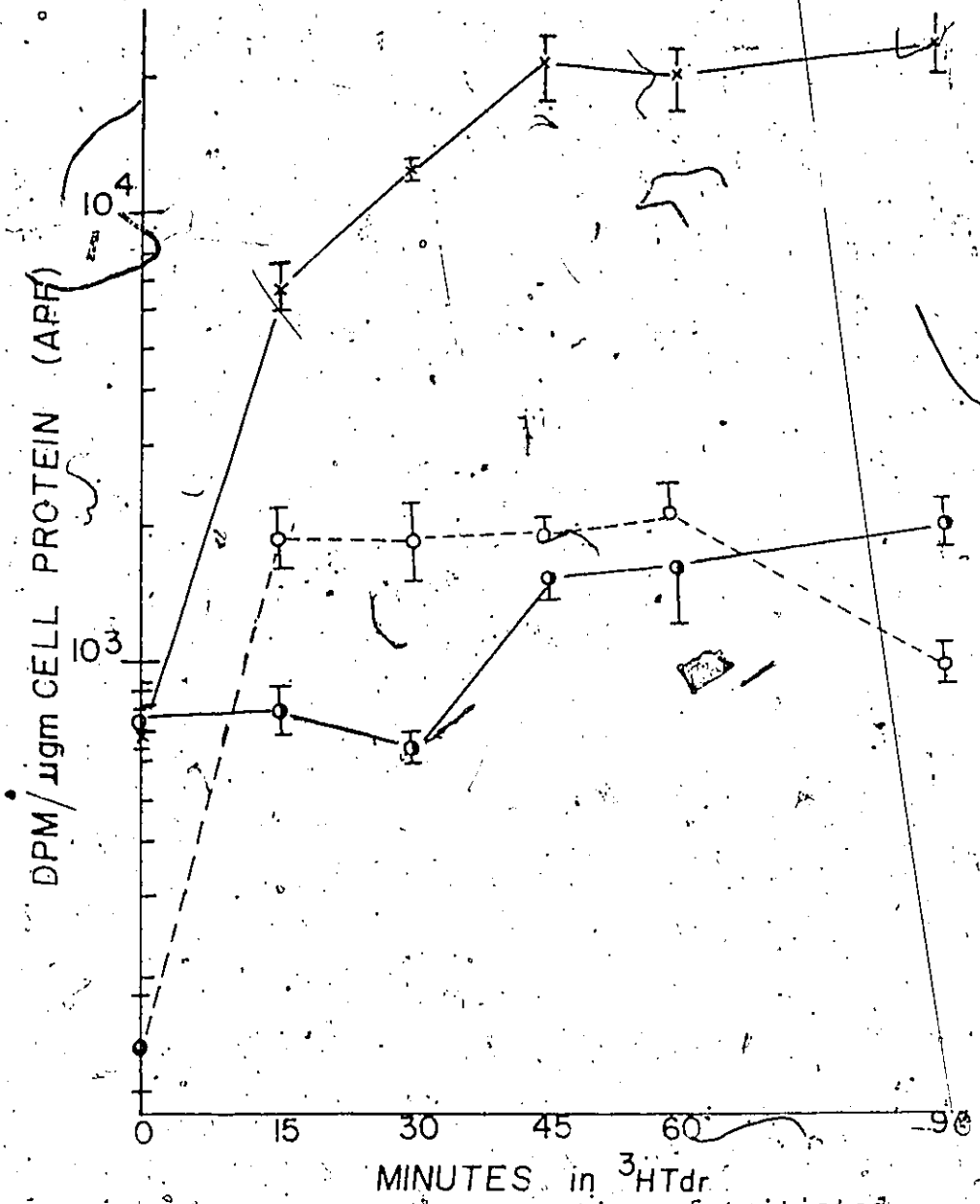

Fig. 3-10. The incorporation of tritiated thymidine into the cold acid precipitable material of E4 cells. The control curve is represented by (x——x), (●——●) represents the cultures over the first 90 minutes of exposure to high potassium medium, and (○——○) indicates the response of cells that have been exposed to high potassium medium for 24 hours prior to labeling. Each point represents the mean ± SD for 6 cultures from 2 separate experiments.

The accumulation of tritiated thymidine into the cold acid soluble material of control and high potassium treated cells shows that high potassium treatment causes an inhibition of precursor accumulation (Fig. 3-11). Both the incorporation and accumulation of precursor by cells that have been exposed to high potassium medium was greatly reduced as illustrated by the appropriate plots in Figs. 3-8 through 3-11.

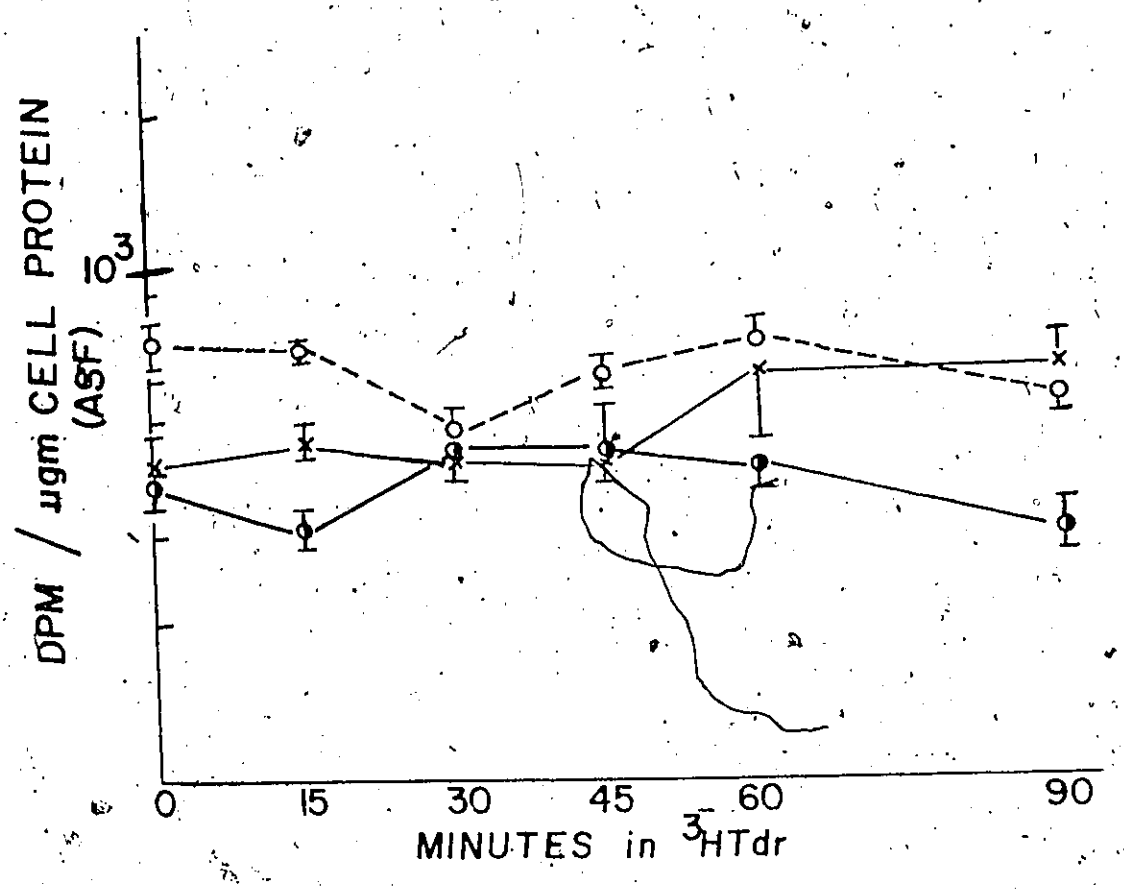

Fig. 3-11. The accumulation of tritiated thymidine in the cold acid soluble material extracted from E4 monolayers that were untreated (x——x), just exposed to high potassium medium (●——●), and also cells that have been cultivated in high potassium medium for 24 hours prior to labeling (o——o). Each point represents the mean ± SD for 6 cultures from 2 experiments.

The Effect of High Potassium Treatment on S Phase in E4 Cells

The progress of cells into and through S phase was determined from the labeling index of autoradiographs prepared from high potassium treated E4 cells. A 5-minute pulse label of tritiated thymidine (specific activity of 18ci/mMole at a concentration of 2uci/ml of medium) followed by a one-minute chase with medium containing 2mM thymidine was given to E4 cells exposed to high potassium medium at 0, 7, 12 and 18 hours during high potassium treatment. Exponentially proliferating control cells were pulse-labeled at each sample point.

The plot of labeling index vs. time (Fig. 3-12) indicates that the proportion of cells that are in S phase increases significantly above the controls 12 hours after the cells were introduced into high potassium medium ($P<.01$). The size of the S phase population then decreased until the proportion of the cells that were in S phase was not significantly different from the controls at 18 hours ($P<.1$ but $>.05$).

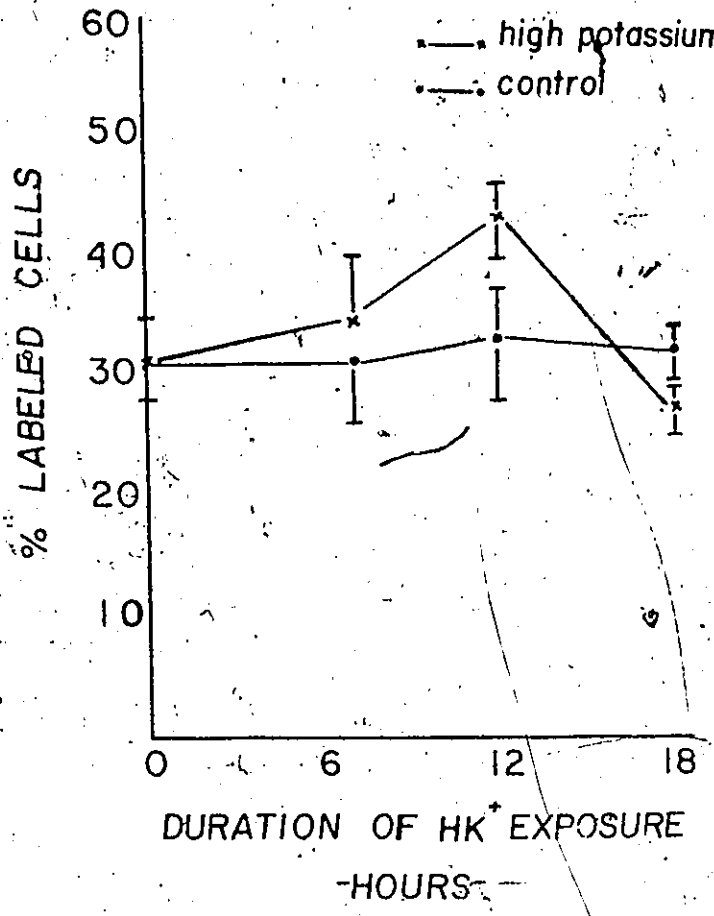

Fig. 3-12. The proportion of a high potassium treated cell population that is in S phase is illustrated by this plot of labeling index over the period of high potassium treatment. Each point represents the mean ± SD of 12 cultures in 2 separate experiments.

The Effect of High Potassium Treatment on Membrane Permeability

Exponentially proliferating E4 cells were pre-labeled with tritiated thymidine (18ci/mMole at a concentration of 2uci/ml of medium) for a period of 3 hours. The monolayers were washed once in pre-warmed MEM-10-T containing 2mM "cold" thymidine (before the wash medium was decanted, the monolayers were allowed to stand at room temperatures for 30-seconds), and then washed again with warm Earle's salt solution. Fresh pre-warmed MEM-10-THK was added to half the cultures while the other half received MEM-10-T and were used as controls. At 20-minute intervals the medium was decanted from the control and experimental cultures and the medium was again replaced with either MEM-10-T or MEM-10-THK. At each sampling point, a sample of medium was taken from each culture and analyzed by scintillation counting. The data (Fig. 3-13) show that significantly greater amounts of tritiated thymidine leak out of the cells treated with high potassium medium than from the controls. Two pulses of leakage are evident, one occuring in the period between 20 and 40 minutes of high potassium

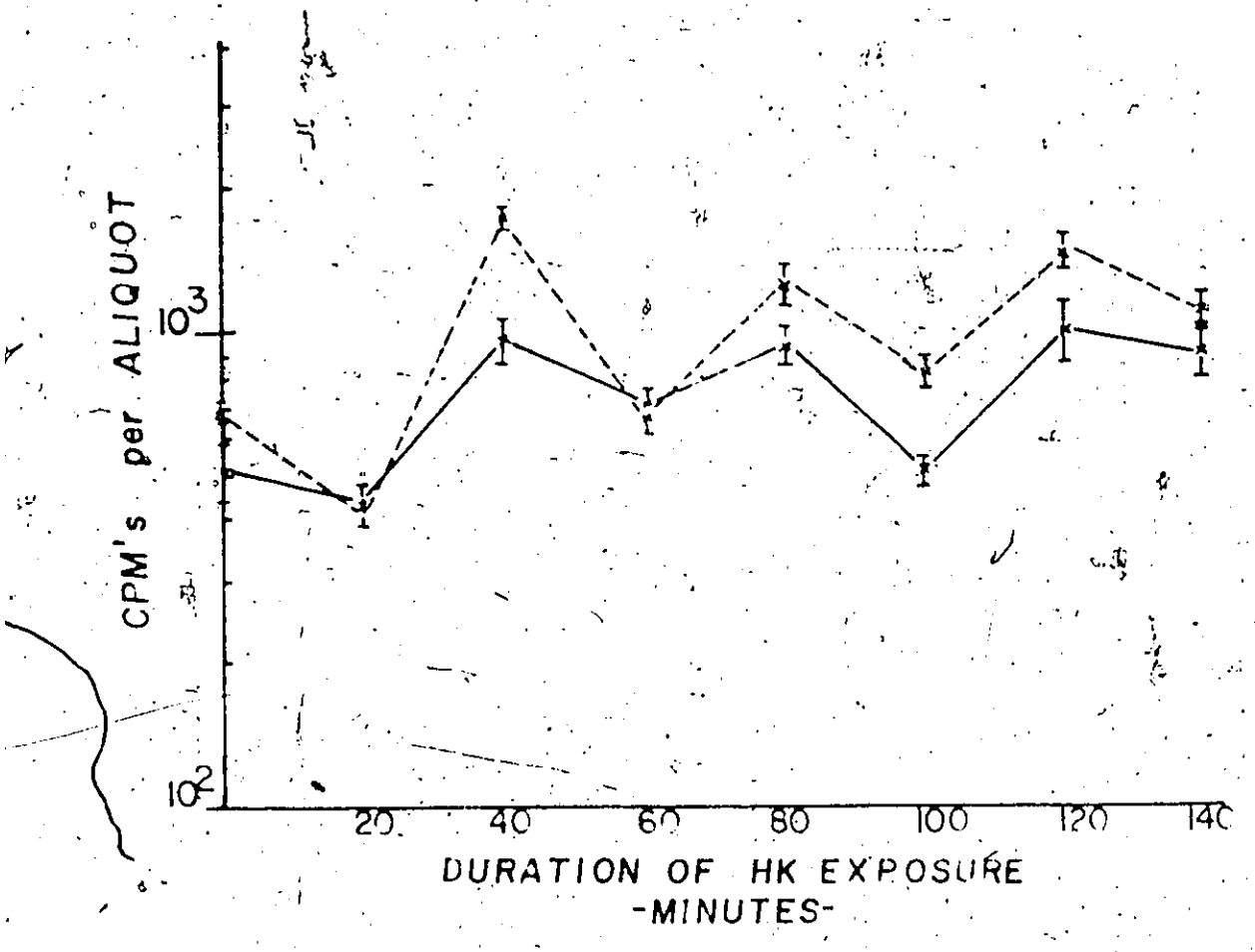

Fig. 3-13. The effect of high potassium medium on the permeability of the plasma membrane of E4 cells expressed as the amount of tritiated thymidine that washes out of prelabeled cells in a series of 20-minute intervals over a 140 minute period. Each point in the mean ± SEM of 6 or 12 samples from two separate experiments. The control curve is represented by (x——x) and the high potassium treated cells by (x——X).

treatment and the other occuring throughout the period between 80 and 120 minutes.

The Effect of high Potassium on Cytokinesis

E4 cells were accumulated in metaphase in the presence of colcemid (0.06mgm/ml of medium) for a period of 2½-hours. The cells were harvested by gentle shaking (detaching the mitotic cells selectively due to their reduced adherence to the growth surface). The cells were then washed free of colcemid and divided into two lots, centrifuged and resuspended again. One group was resuspended in MEM-10-THK while the other group, used as controls, was resuspended in MEM-10-T. Each suspension was divided into aliquots of 5 ml each which were placed into plastic tissue culture flasks. Control and high potassium treated metaphase cells were photographed under phase contrast optics of a Zeiss inverted microscope at 160X at 0, 15, 30, 45, 60, and 90 minutes.

The ability of the cells to complete mitosis

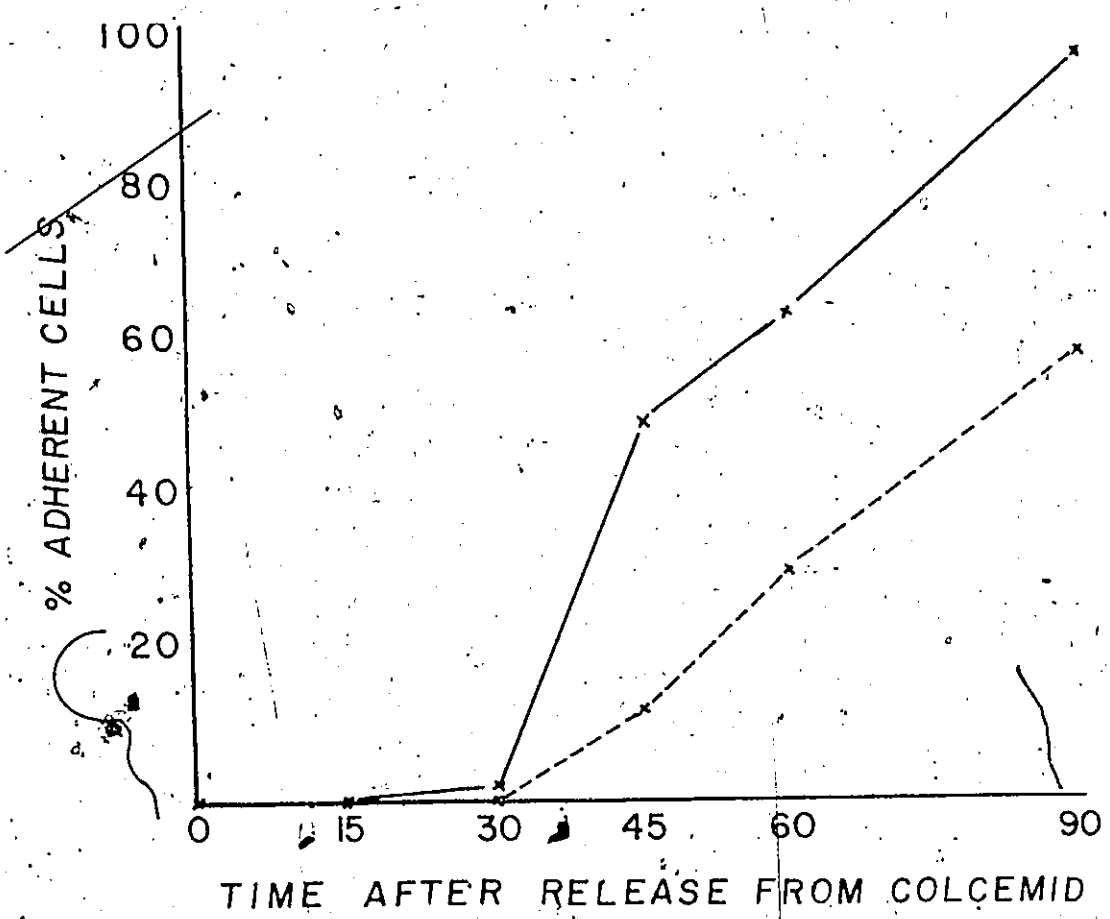

Fig. 3-14. The percentage of total cells which are able to proceed through mitosis and flatten out against the growth surface. Controls released from colcemid block into MEM-10-T are shown by (x——x) and the high potassium treated cells by (x---x).

was measured (directly from the photomicrographs) by scoring the percentage of cells which proceed to flatten against the growth surface. The data (Fig. 3-14) show that relative to the control, there is approximately a 15 minute delay before high potassium treated cells flatten out against the growth surface. A large number of bi-nucleate cells (at least 50% of the adherent cells) were seen in the 90-minute high potassium sample indicating that cytokinesis was likely affected. Plate 3-8 illustrates the bi-nucleate cells.

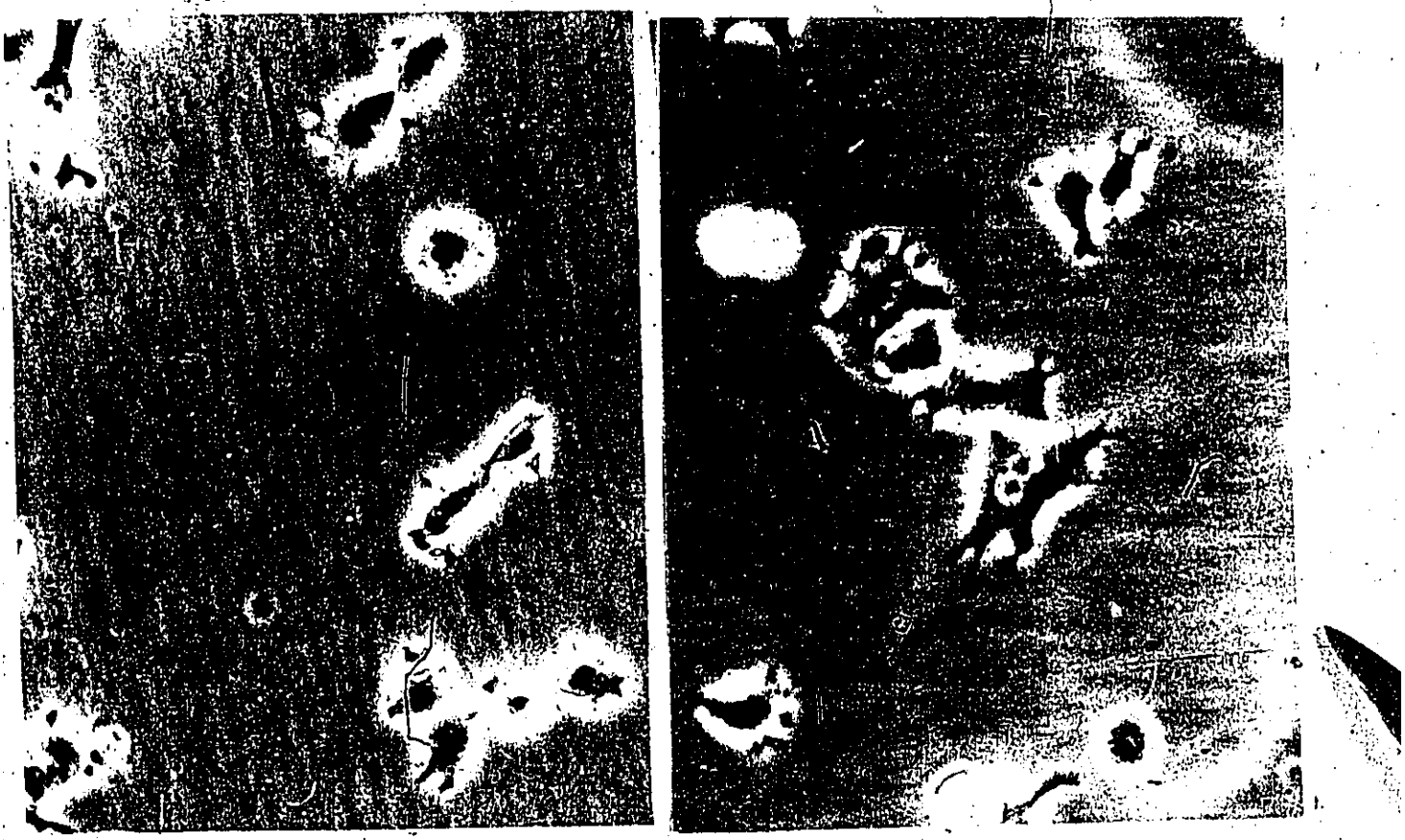

Plate 3-8. A comparison of the control cells (left) and the high potassium treated cells (right) as they appear 90 minutes after release from colcemid block. Note the bi-nucleate cells in the high potassium treated sample.

THE INDUCTION OF SYNCHRONY IN E4 Cells

Protocol for Block-and-Release Experiments

E4 cultures in mid-exponential growth phase were used in all experiments unless otherwise specified. The medium of control and experimental cultures was replaced with fresh pre-warmed MEM-10-T and MEM-10-THK respectively. The cultures were incubated at 37°C for 24 hours, and the medium in all cultures was replaced with fresh pre-warmed MEM-10-T. The cultures were always sampled in triplicate and were sampled at various times during, and after, high potassium treatment.

Increase in Cell Number After Release From High Potassium Block

The total cell number in four monolayers cultures and five suspension cultures was determined at hourly intervals for 25 hours following release from high potassium block. The cell number in the suspension cultures was determined by hemacytometric counts, and the number of cells in the monolayers was determined from a measured and representative area of the growth

surface by direct count. There was little or no increase in cell number until about 9 hours post release (Fig. 3-15). At this time, there was a wave of proliferative activity. The appearance of this wave of proliferative activity indicated that a certain degree of synchrony had been induced in the population. The degree of synchrony (i.e. the efficiency of the induction method) was estimated by Engleberg's method (1961) and was found to range from 35 - 65%.

Tritiated Thymidine Incorporation After Release From High Potassium Block

The proportion of cells in S phase (i.e. actively synthesizing DNA) just prior to, and after, release from high potassium block was assessed by analysis of the labeling indices of autoradiographs prepared from pulse-labeled E4 cells. The pulse-chase protocol is the same as previously described for the cell cycle analysis. The cultures were sampled just prior to medium replacement, at 30-minute intervals for the next 7 hours and at 60-minute intervals between 7 and 9 hours post-release. Exponentially proliferat-

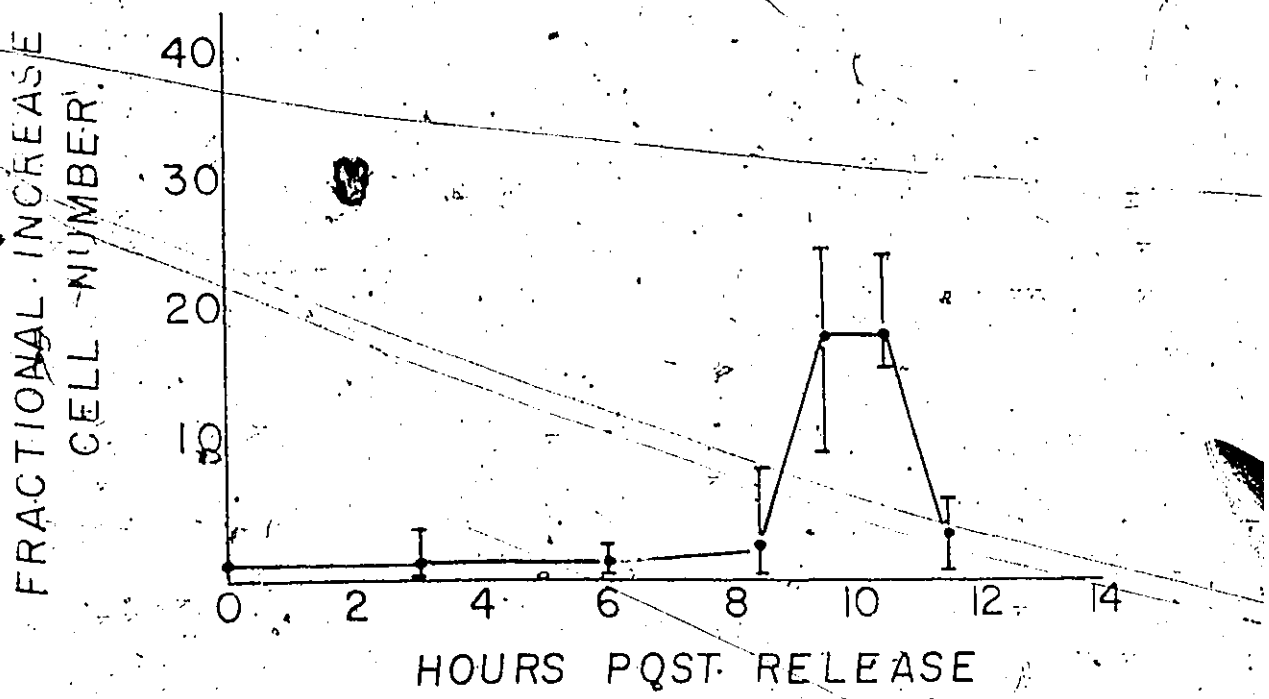

Fig. 3-15. High potassium induction of synchrony in E4 cells as indicated by the pattern of the increase in cell number after a release from a 24 hour high potassium blockade. The plot is constructed from the normalized growth curves (based on the % of the maximal cell number in each curve) of five suspension cultures and four monolayer cultures. Each point on the graph represents the mean ± SD of 7 cultures.

ing cells were sampled at 0, 6, and 9 hours to obtain a control level.

The labeling index of high potassium treated cells just prior to release from 18 hours was not significantly lower than the control value (P .1 but .05). The pattern of labeling indices after release (Fig. 3-16) indicates that at least one population is poised at the G1-S interface while the bulk of the cells pass synchronously through S phase between 6 and 9 hours post-release.

The Enhancement of High Potassium Induced Synchrony by Colcemid

The synchrony induced by high potassium block-and-release was variable and moderate in degree. The degree of synchrony was enhanced by blocking the high potassium generated synchronous population, which passes through mitosis between 9 and 12 hours post-release, with the metaphase-blocking agent colcemid (GIBCO, Grand Island, N.Y.). Colcemid was added to the medium of high potassium synchronized cells at a concentration of 0.06ugm/ml of culture

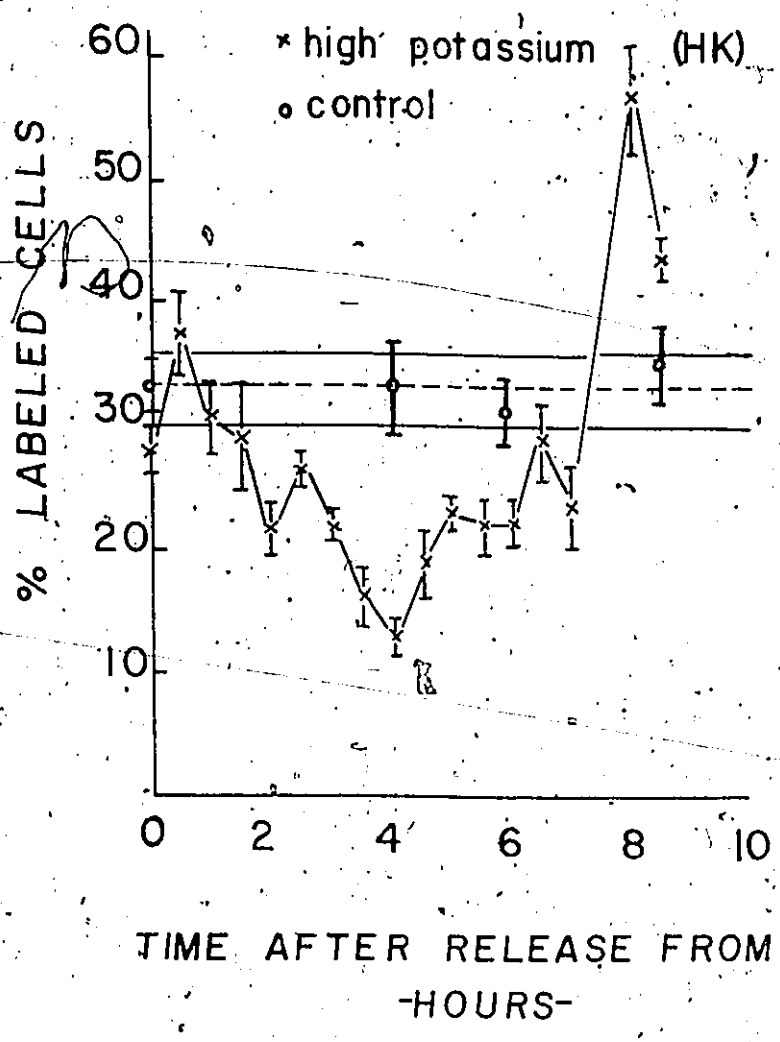

Fig. 3-16. High potassium induction of synchrony in E4 cells expressed as the proportion of cells synthesizing DNA (S phase cells) at points in time after restoration of normal environmental Na-K concentrations. The percentage of labeled cells after a 5-minute pulse exposure to high specific activity tritiated thymidine (18 ci/mMole at a concentration of 2uci/ml of medium) in autoradiographic preparations were scored. Each from two separate experiments. The controls are shown by (o—o) and the high potassium synchronized cells by (X——X).

medium for a period of three hours commencing at nine hours post-release. The accumulated metaphase cells were harvested by gently shaking the flasks, collected by centrifugation, washed free of colcemid, and released into colcemid-free medium. The total cell number per culture and the mitotic index were assessed by direct observation of the cultures. The initial mitotic index was determined from slides prepared from the colcemid-selected population just prior to washing the cells. Over the first hour, the mitotic index decreased from 92% to 2% (Fig. 3-17). The cell number did not increase at all until about 10 hours after removal of the colcemid blockade and then increased rapidly at about 11 hours post-release, peaking at 12 hours. The mitotic index rose from 6 to 70% in the interval between 10 and 11 hours. The degree of synchrony initially present in the cell population following the high potassium block-and-release and the subsequent colcemid selection was approximately 92%.

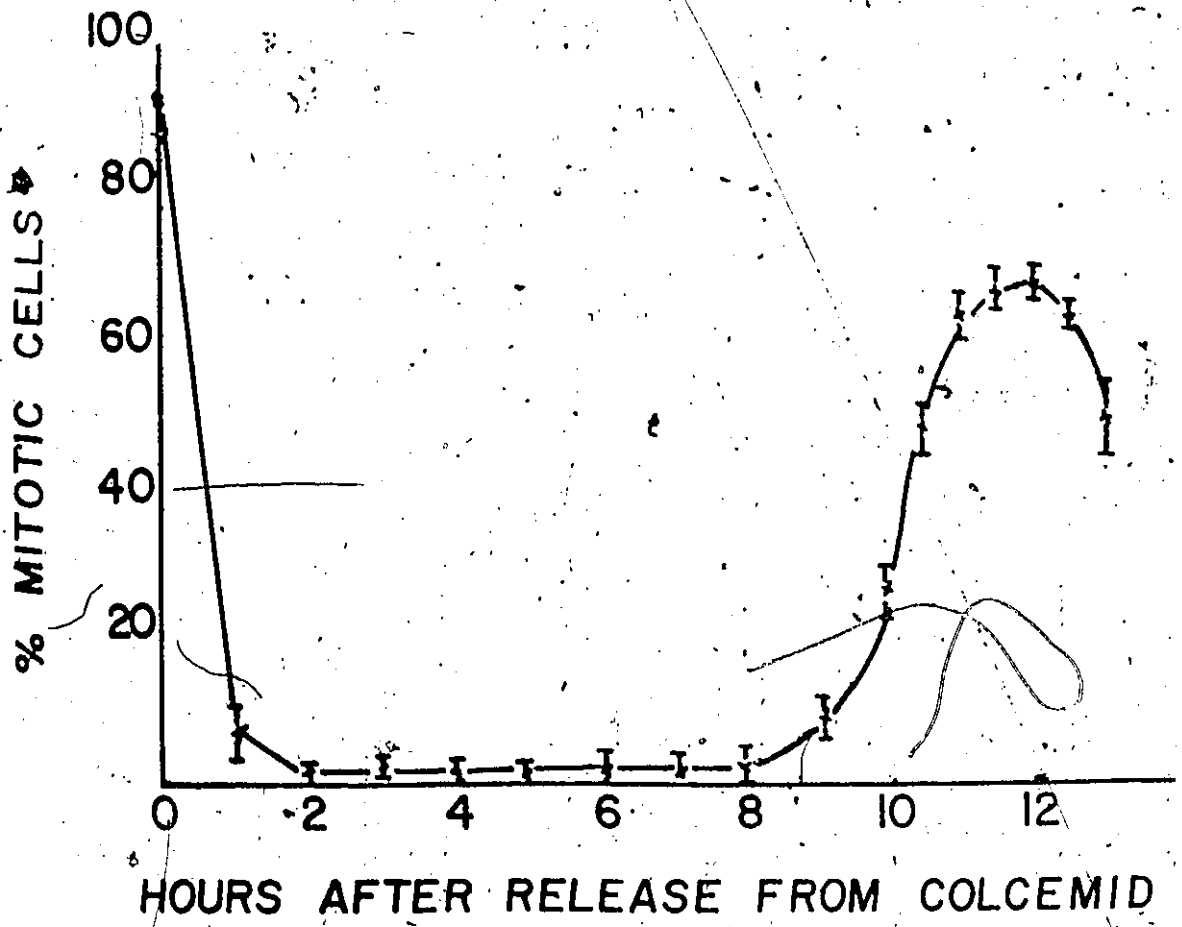

Fig. 3-17. The synchrony induced in E4 cell populations after release from a 24-hour high potassium blockade and a 2½-hour accumulation of mitotically synchronous cells by colcemid treatment (0.06 mgm/ml of medium) is indicated by the pattern of mitotic indices in these synchronized populations. Each point represents the mean ± SEM for 5 cultures.

IV DISCUSSION

Elevated extracellular potassium levels in combination with reduced extracellular sodium levels affect the proliferation and fine structure of E4 cells in culture. When E4 cells are exposed to high potassium medium, the morphology, fine structure, and DNA synthetic rate are immediately affected; subsequently the proliferation, the macromolecular content, and the viability of these cells are affected. Restoration of the normal Na-K ratio in the medium reversed many of the observed effects of high potassium treatment, and induced partially synchronous proliferation (parasynchronous) in the E4 populations. The current explanation of the effects of high potassium medium suggest that altered transmembrane potential either directly or indirectly affects a nuclear response which is reflected in the observed phenomena (MacDonald et al. 1972). Some of the observations reported for E4 cells are not novel, but E4 does represent a new subject

cell line.

The temporal relationships between the decrease in the transmembrane potential resulting from an elevation of extracellular potassium concentrations (MacDonald et al. 1972, Borle and Loveday 1968) and the inhibition of the uptake and incorporation of exogenous tritiated thymidine by E4 cells as well as BHK-21 (Orr et al. 1972) suggest that there is a fairly close relationship between the two events. The pattern of tritiated thymidine accumulation into the cold acid soluble fraction of cells exposed to high potassium medium compared to the controls (Fig. 3-11) indicates that the accumulation of thymidine is inhibited and that there may be a net loss of thymidine from the cytoplasmic precursor pool. The pattern of tritiated thymidine incorporation into the cold acid precipitable material over the first 90 minutes of exposure to high potassium medium (Fig. 3-10) shows that there is an apparent inhibition of nucleoside incorporation for about 30 minutes followed by the resumption of incorporation at a reduced rate. It is not clear from these data whether

the inhibited thymidine incorporation and accumulation are related events, but since there is some tritiated thymidine in the acid soluble material at all times, an inhibition of tritiated thymidine incorporation would indicate an inhibition of DNA synthesis. Since the amount of tritiated thymidine in the acid soluble material begins to decrease at approximately the same time as the incorporation of precursor resumes, the assumption can be made that DNA synthesis was inhibited and has now recovered partially from the inhibition while the influx of thymidine remained inhibited.

The inhibition of thymidine accumulation could be caused by inhibited Na-K transmembrane flux, but there is no evidence that thymidine accumulation is coupled to the transmembrane movements of sodium and potassium in the same way as amino acids and sugars. The sodium-potassium gradient theory of amino acid and sugar transport (Crane 1962, Crane 1965, Kuchler 1967, Eddy et al. 1967, Schultz and Warren 1970) states that the movement of sodium and potassium ions down their respective gradients, across the plasma membrane, drives the accumulation of amino acids.

Kuchler (1967) has observed that extracellular Na-K ratios similar to the Na-K ratio of MEM-10-THK medium do inhibit the accumulation of amino acids by mammalian cells, and that the mechanism for amino acid transport depends on Na-K movements. It is unlikely that the inhibition of DNA synthesis observed in the minutes following exposure to high potassium medium is caused by inhibited sodium and potassium coupled transport mechanisms, since in spite of the continued inhibitory conditions for Na-K flux, DNA synthesis resumes after intervals of ½ and 2 hours in E4 cells and BHK-21 (Orr et al. 1972) respectively.

An increase in plasma membrane permeability was observed in high potassium treated cells as demonstrated by the increased leakage of tritiated thymidine from pre-labeled cells (Fig. 3- 13). Decreased transmembrane potential is believed to cause a decrease in the calcium binding capacity of the plasma membrane and the permeability of the membrane is in part dependent on the amount of calcium bound to it (Rasmussen 1970). Borle (1969) has described the calcium bound to the plasma membrane-glycocalyx complex as a large and

readily exchangeable cellular calcium compartment. This calcium stockpile would be ideal to supply increased intracellular Ca^{+2} under circumstances where the cell required that ion. It is possible that this calcium compartment is analagous to the sarcoplasmic reticulum in muscle cells (Ashley 1971, Hoyle 1970), adding and removing calcium for the intracellular environment in response to changes in the transmembrane potential. Considering the established role for calcium in the regulation of DNA synthesis (Whitfield et al, 1973), a high potassium induced release of calcium into the cytosol in response to a rapid decrease in transmembrane potential could explain the effects of high potassium on DNA synthesis as observed in E4 cells. The elaboration of microtubular and microfibrillar components in the cytoplasm of treated cells can also be related to calcium.

The aggregation of ribosomal and nucleolar material (Plate 3-7 and Fig. 3-6 respectively) probably results from the increased ionic strength of the cytosol. The net intracellular sodium and potassium content,

according to the data of MacDonald et al. (1972) and Kuchler (1967), increases after cells are exposed to high potassium medium. This increase in ionic strength would act to "salt out" ribonuclear protein inside the cell. Increased ionic strength might also act to stabilize the microtubules and microfibrils that have formed in response to high potassium treatment.

The macromolecular content of E4 cells is significantly altered with respect to RNA and protein ($P<.01$ in each case) after 24 hours in high potassium medium while the DNA content is not significantly different than for the exponentially proliferating control cells ($P<.2$ but $>.1$), see Fig. 3-7. These data show that the metabolism of E4 cells is unbalanced by high potassium treatment. A normal macromolecular level is re-established in E4 cells sampled 24 hours after the high potassium medium was replaced with medium containing the usual sodium and potassium concentrations.

The growth experiments show that E4 cells lose

viability, contrary to Orr's observation (Orr et al. 1972), after a 24-hour exposure to a high potassium environment (Fig. 3-3). The difference in response could be due to inherent differences between E4 cells and BHK-21, or to differences in the high potassium medium used in each case. Orr's medium was richer with respect to amino acid content and vitamin supplement than MEM-10-THK and it has a Na concentration of 60mM compared to only 10mM in MEM-10-THK. The increased environmental amino acid concentration and vitamin supplement may partially counteract nutrient depletion due to the inhibited Na-K coupled nutrient accumulation. The data for E4 cells suggests that Na concentrations greater than 50 mM in high potassium medium do permit proliferation of cells at a reduced rate (Fig. 3-5); therefore, the relatively high Na concentration in Orr's medium probably protects the cells from the high potassium toxicity. If amino acid accumulation is inhibited by high potassium medium as suggested by Kuchler (1967), then the intracellular amino acid pools would become depleted after some time. The amount of tritiated thymidine in the acid soluble

fraction of cells continuously labeled with isotope decreased in treated cells, suggesting that there is a net depletion of nutrients from the cytoplasm (Fig. 3-11). Cell death would then result from a literal starvation process.

The autoradiographic data (Fig. 3-12) indicates that high potassium treatment caused a transient inhibition to cell cycle traverse by a retardation of the rate of movement through S phase. The cells appear to proceed through only one mitotic period after the completion of S phase and there was no further increase in cell number (Fig. 3-3). When E4 cells were returned to a normal ionic environment they appear to be partially synchronized with respect to the phases of the cell cycle (Fig. 3-15 and Fig. 3-16). Orr suggests that high potassium block-and-release might provide a useful way to synchronize the proliferation of mammalian cells in culture (Orr et al. 1972).

The synchrony induced after release from high

potassium blockade is characterized by the presence of several synchronous subpopulations (Fig. 3-16) and a reasonable variation in the efficiency of synchronization from experiment to experiment (35 - 65% synchrony). The majority of the cells were observed to pass through mitosis between 9 and 12 hours after restoration of the normal Na-K ratio. Based on the timing of the E4 cell cycle, Fig. 3-2, it appears that this population of cells is blocked over a large portion of the G1 phase, confirming Orr's observation of partial synchronization of BHK-21 by high potassium medium (Orr et al. 1972) by a G1 blockade.

As previously stated, the synchrony is induced by the transient retardation of cell cycle traverse caused by the pile up of cells in S phase (Fig. 3-12) and the eventual blockade to cell cycle traverse in G1 as a result of high potassium treatment. The synchrony is detectable in the patterns of increase in cell number and the proportion of cells in S phase when the cells were returned to normal Na-K environmental conditions. If the inhibitions of Na-K flux by high potassium medium (Kuchler 1967)

inhibits the accumulation of amino acids in E4 cells, then the intracellular pool of amino acids would be exhausted and become limiting for protein biosynthesis. Therefore, it is likely that the S phase synchronized population is inhibited from proceeding through the cell cycle due to a nutritional deficit and not a block to a specific G1 reaction or process. The observation that increased sodium concentration in the high potassium medium permitted some proliferation, and the degree of proliferative activity was dependent on the amount of sodium added (Fig. 3-5), strongly suggests that a sodium-potassium coupled mechanism is involved. These data argue against, but do not eliminate, the possibility that the physical forces that have caused the formation of ribosomal rosettes and the condensation of nucleolar material might mechanically interfere with the machinery of protein synthesis as well.

The multiple synchronous populations induced by high potassium treatment may be an artifact of the timing of release from high potassium blockade, since the autoradiographic data show that a small

proportion of the population is at the G1-S boundary. These cells may not have had time to enter S phase before the cells were released from the potassium blockade or they may have been trapped at the G1-S boundary due to the depletion of nutrients. It is also possible that these cells represent a rapidly cycling sub-population or a sub-population of cells that is specifically blocked by high potassium at the G1-S boundary.

The high potassium induced synchrony was enhanced by selecting out the major population of synchronized cells as they passed through mitosis with the metaphase arresting agent colcemid. Large numbers of highly synchronous cells were obtained with this procedure and could be released into colcemid-free medium to progress through the cell cycle synchronously (Fig. 3-17). All synchronization procedures do induce some perturbation to cell cycle traverse (Toby et al. 1972) and most methods result in unbalanced growth (Mitchison 1971). The total cell cycle time for double synchronized E4 cells (i.e. high potassium followed by colcemid) does not seem to be altered as indicated by the 12-

hour interval between release from colcemid and the subsequent wave of mitosis (Fig. 3-17). The DNA synthesis data (Fig. 3-16) are consistent with the timing of the E4 cell cycle, namely a 9 hour interval between the peak activity and the time of release if the blockade is at mid G1, but the duration of the wave of activity in the population of cells that pass through S phase at 9 hours post release and the small population that pass through S within the first hour after the cells are returned to normal Na-K environmental conditions is remarkedly short, lasting only one hour in each case. The duration of the S phase in a non-synchronous population (Fig.3-2) is 6 hours. In spite of the anomalous S phase, this high potassium-colcemid synchronization procedure does provide a rapid method of obtaining a high yield of synchronized cells from monolayer cultures, and in light of the rapid passage of cells through S phase after synchronization, might provide a good system to study the initiation of DNA synthesis in synchronized mammalian cells.

A model for the regulation of the cell cycle by transmembrane potential can be constructed by integrating some of the high potassium induced phenomena and the established calcium-cyclic AMP hypothesis for the control of DNA synthesis. The mitogenic action of calcium is closely associated with the cellular cyclic AMP levels (Whitfield et al. 1973). Without detailing the intricate cyclic AMP-calcium interactions, or the body of evidence which supports the cyclic AMP-calcium hypothesis, it is useful to consider the basic interaction of the system. Essentially, calcium can stimulate cyclic AMP formation which in turn has a mitogenic effect believed to be centered around the activation or initiation of the S phase in the cell cycle. When the calcium concentration is elevated above a certain level, it feeds back to inhibit the mitogenic component of the system. This work suggests that a natural cyclic AMP-calcium regulation may exist and regulate the initiation S phase in proliferating cells. This regulation would require "... a brief endogenously generated increase in the cellular permeability to calcium at an appropriate point in the

cell cycle which would provide the internal calcium surge needed to trigger DNA synthesis". (Whitfield et al. 1973), or an endogenously generated release of Ca^{+2} into the cytoplasm from a readily exchangeable cellular calcium compartment. The interrelationships between transmembrane potential decrease, membrane permeability, calcium binding, and the cyclic AMP-calcium hypothesis would comprise a regulatory mechanism within the cell cycle if there was evidence that alterations in transmembrane potential occur during the cell cycle in proliferating mammalian cells. Jung and Rothstein (1967) have observed periodic shifts in the intracellular potassium and sodium content over the mammalian cell cycle in synchronously proliferating L5178Y mouse lymphoblasts. While no direct demonstration of decreased transmembrane potential associated with these shifts in cellular ion content has been reported, the loss of 20% of the cellular potassium content just prior to S phase would certainly cause a decrease in the transmembrane potential, and thereby cause an increase in the membrane permeability or a release of Ca^{+2} due to the decreased binding capacity for

that mineral. Although the relationship between the observed behavior of E4 cells in response to high potassium treatment, and the DNA synthetic regulatory action of cyclic AMP and calcium, appear opposite in effect, the apparent toxicity of high potassium medium (Fig. 3-3) may mask any positive response.

Cyclic AMP and calcium can also be related to the appearance of the microtubular and microfibrillar components observed in the cytoplasm of treated cells (Plate 3-6). Borisy et al. (1972) have shown that exogenous cyclic AMP stimulates the assembly of microtubules in cultured chick dorsal root ganglia. Since calcium can elevate cyclic AMP levels in mammalian cells (Whitfield et al. 1973), any increase in the intracellular calcium level due to high potassium treatment could possibly stimulate the formation of microtubules and microfibrils. Weisenberg (1972) has reported that microtubular assembly is inhibited by a calcium concentration of 50mM, however the environmental calcium concentration

in high potassium medium is only 1.8mM, therefore even if plasma membrane permeability to calcium is maximal and the intracellular and extracellular calcium concentrations are in equilibrium, the concentration of calcium would be far below the inhibitory level reported by Weisenberg. The large amount of microtubular and microfibrillar structures in the cytoplasm of high potassium treated cells may also account for the inhibition of cytokinesis (Fig. 3-14) since such a network of structures might mechanically restrict the process.

In summary then, it can be stated that high potassium treatment affects DNA synthesis and cytoplasmic fine structure; and results in an inhibition of cytokinesis and proliferation. If E4 was exposed to high potassium medium (114mM K) for an extended period of time the cells died. When the cells were returned to medium with a normal potassium concentration (5.6mM K) after a 24-hour exposure to high potassium medium, DNA synthesis and proliferation proceeded synchronously. The synchrony induced in E4 populations by high potassium treatment was moderate

in degree and rather variable. The quality of the synchrony was enhanced by a subsequent colcemid selection-and-release timed to segregate the largest synchronous potassium induced population as it moved through mitosis. This combination of procedures reliably yielded a large number of highly synchronous cells and may prove useful in the study of G1 and particularly S phase cell cycle events.

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