ROLE OF IONS IN ACTIVATION OF HUMAN LYMPHOCYTES

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ROLE OF IONS IN ACTIVATION OF HUMAN LYMPHOCYTES

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ABSTRACT

The role of extracellular ions in activation of PHA stimulated lymphocytes was examined. Cells were cultured in media containing varying levels of K^+, Mg^{++}, and Ca^{++}. Lymphocyte transformation was monitored by measuring nuclear diameter, and by evaluating the area of nucleolus which reacted with silver nitrate.

Decreasing extracellular K^+ from normal levels (5.0 mM) to 14% of normal (0.7 mM), and decreasing extracellular Mg^{++} from normal levels (1.0 mM) to 14% of normal (0.14 mM), did not affect nuclear diameter or silver nitrate reactivity of PHA stimulated lymphocytes, nor did it affect the number of AgNORs in metaphase cells.

Chelation of extracellular Ca^{++} with EGTA, during the first 24 hours post stimulation, completely inhibited the increases in silver reactivity, nuclear diameter, and cell division associated with PHA stimulation. Addition of EGTA, 48 hours post PHA, did not inhibit lymphocyte stimulation. Inhibitory effects of EGTA were completely reversed if $CaCl_2$ was added to the medium within 24 hours of PHA stimulation. By 48 hours, the effects were irreversible. Therefore, Ca^{++} is essential for activation of genes coding for proliferation of lymphocytes *in vitro*.

Increasing extracellular Ca^{++} (to 7.8 mM) and/or calmodulin, did not significantly activate the proliferative genes of lymphocytes cultured without PHA.

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INTRODUCTION

I. REGULATION OF GENE ACTIVITY

Only a fraction (2-15%) of the genetic potential of a cell is expressed (Grouse et al., 1972). The fraction expressed fluctuates in response to physiological signals. Thus cells having the same genetic potential may be differentially activated to fulfill different, specialized functions. The mechanics of regulating gene activity is not only of fundamental importance to understanding how normal cells function, but also is paramount to understanding the autonomous proliferation of neoplastic cells, which are not controlled by normal cell regulatory mechanisms.

Little is known of the mechanisms involved in regulation of eukaryote gene expression, though various models have been proposed. Some are patterned after bacterial models, postulating highly specific repressor, derepressor and inducer molecules that interact with specific genes. Others involve the binding of hormone complexes either to chromatin acceptor sites or to cell membrane receptors. These stimulate intracellular activation or inactivation of a biochemical reaction affecting gene expression. The regulation of the highly reiterative rDNA is likely to be different from that of unique sequence DNA.

II. APPROACH TO STUDY

Human lymphocytes were stimulated by phytohemagglutinin (PHA) to reenter the cell cycle. Reentry involves turning on of those repressed genes which code for the proliferogenic components. Stimulation of lymphocyte activity is morphologically characterized by an increase in nuclear and nucleolar size, and by an increase in the ability of the nucleolus to reduce colourless silver nitrate ($AgNO_3$) to black metalic silver (Schwarzacher et al., 1978; Arrighi et al., 1980). These morphological changes are directly related to stimulation of rRNA genes.

The role of ions in regulation of lymphocyte activation was investigated by altering the extracellular concentrations of K^+, Mg^{++} and Ca^{++}. Activity of rRNA genes was monitored by measuring the diameter of the nucleus and the silver reactivity of the nucleolus.

Examination of the model system utilized, and of theories of regulation of rDNA activity will aid in understanding the role of ions in regulating rDNA activity.

LITERATURE REVIEW

I. ACTIVATION OF LYMPHOCYTES WITH PHYTOHEMAGGLUTININ (PHA)

The small peripheral blood lymphocyte was considered to be a fully differentiated cell, incapable of division. Nowell (1960) found that PHA, a mucoprotein extract of the bean plant, Phaseolus vulgaris, caused a high percentage of lymphocytes to undergo mitosis. It is a potent mitogen, stimulating 80 to 90% of small lymphocytes to reenter the cell cycle (Douglas, 1972). Cultured lymphocytes remain in a non-growing state unless exposed to a mitogen which induces cells to transform by enlarging, producing RNA, replicating DNA and undergoing mitosis.

Transformation induced by PHA is initiated by an interaction with membrane receptors (Kornfeld and Kornfeld, 1970). Soon after, membrane-bound adenylate cyclase rapidly increases in activity resulting in an increase in the two primary intracellular regulators, cyclic adenosine 3', 5'-monophosphate (cAMP) (Smith et al., 1971), and Ca^{++} (Whitney, 1972).

After PHA treatment a sequence of morphological changes occurs in human lymphocytes. Nucleus and total cell size, cytoplasmic basophilia and number of vacuoles progressively increase. Mitotic cells are first observed at about 40 hours after the addition of the mitogen, and the number observed peaks at about 72 hours (Ling, 1968).

After as little as 4 hours of exposure to PHA, the nucleolus may begin to enlarge (Douglas, 1972) and the silver reactivity of the nucleolus increases (Arrighi et al., 1980), probably due to the stimulation of nucleolar rRNA synthesis. At about 48-60 hours there are ribosomal aggregates in the cytoplasm and increased amounts of rough endoplasmic reticulum, golgi apparatus and lysosomes. Mitochondria often are swollen and show loss of cristae (Douglas, 1972).

PHA stimulates protein synthesis, initially by increasing the efficiency of pre-existing ribosomes and by preventing wastage, and later by utilizing newly synthesized ribosomes. Prior to PHA, about 30% of ribosomes are engaged in protein synthesis. Soon afterwards about 70% are engaged (Cooper, 1972). Approximately half of rRNA of unstimulated lymphocytes is degraded. This wastage is virtually eliminated after PHA treatment (Cooper and Gibson, 1971).

Waste control may be a mechanism for cells to quickly shift from the resting state to rapid growth upon the appropriate stimulus (Cooper, 1972). Chronic lymphatic leukemia cells are characterized by their inability to reverse the wastage of ribosomes upon stimulation (Rubin, 1971). rRNA production increases as in normal cells but the onset of blastogenesis is delayed.

The increase in efficiency of pre-existing ribosomes and control of wastage is not adequate for normal cell growth and division. Blockage

of rRNA synthesis prohibits DNA synthesis and thus cell proliferation (Kay et al., 1969). In a PHA stimulated nucleolus there is a drastic increase in polymerase A activity (Cooke and Brown, 1973) accompanied by a 10-to 50-fold increase in newly synthesized rRNA (Cooper, 1972) which peaks at around 18 hours (Purtell and Anthony, 1975).

PHA does not stimulate all lymphocytes. Approximately 10 to 20% of lymphocytes are not transformed. Purtell and Anthony (1975) found two rRNA processing pathways in guinea pig lymphocytes, only one of which was stimulated by PHA. This may be explained either by the existence of two major subpopulations of cells, only one of which is preferentially stimulated by PHA, or by all lymphocytes having the potential to use both pathways.

It is not known how PHA activates the genetic components necessary for proliferation. It is known that rRNA synthesis is essential for DNA synthesis and thus for cell proliferation. Regulation of rRNA synthesis may be exercised during transcription, maturation or degradation.

II. RIBOSOMAL RNA (rRNA) PRODUCTION AND PROCESSING

A. General

At least 80% of total cellular RNA is rRNA (Darnell, 1968). Genes coding for 18S and 28S rRNA are located in the nucleolar organizing regions (NORs) of human acrocentric chromosomes (Henderson et al., 1972). During interphase rRNA is transcribed in the nucleolus and modified to produce the precursor molecules of ribosomes. In the cytoplasm, ribosomes translate messenger RNA (mRNA) to produce protein. The activity of the ribosomal cistrons might be controlled at any stage from transcription into RNA to the translation of active proteins.

B. Ribosomal DNA

It has been demonstrated by in situ hybridization that the chromosomal location of genes coding for 18S and 28S rRNA are NORs (Henderson et al., 1972). The NORs of humans are situated at the secondary constrictions of the short arms of the five pairs of acrocentric chromosomes (13, 14, 15, 21 and 22) (Ohno et al., 1961). DNA clusters coding for 5S rRNA are scattered outside the NOR, primarily on the larger chromosomes.

rDNA is highly repetitive. At least 500 sets of genes per haploid

genome, encode for human 18S and 28S rRNA (Jeanteur and Attardi, 1969). Some 2000 genes per haploid genome encode for 5S rRNA (Hatlen and Attardi, 1971). This high degree of repetition allows for rapid production of ribosomes. The capacity for ribosome production in hepatocytes was estimated to be 15- to 50-fold greater than the consumption of ribosomes (Hadjiolov and Nikolaev, 1976). If rRNA is maximally loaded with RNA polymerase, as found by Spring et al. (1974), the control of rRNA processing is critical.

The rDNA is organized into transcriptional units, separated from adjacent regions by non-transcribed spacers (Fig. 1). The transcriptional units are highly reiterative, containing up to several hundred rDNA copies in a cluster. Units from different species vary in length, primarily due to the presence of transcribed spacers. Spacers are segments of gene elements which do not relate to any cytoplasmic products. The evolutionary trend is for transcribed units to increase in size, primarily due to an increase in length of the transcribed spacer units. The lengths of the 18S and 28S RNA sequences has been highly conserved during evolution, indicating it must be under stronger genetic pressure than the rest of the transcribed unit.

Sequences of nucleotides in rDNA are not random. Up to 78% of the base pairs are guanine and cytosine (Hadjiolov and Nikolaev, 1976). Strong conservation of 18S and 28S rDNA nucleotide sequences occurs throughout a wide range of eukaryotes (Evans, 1977).

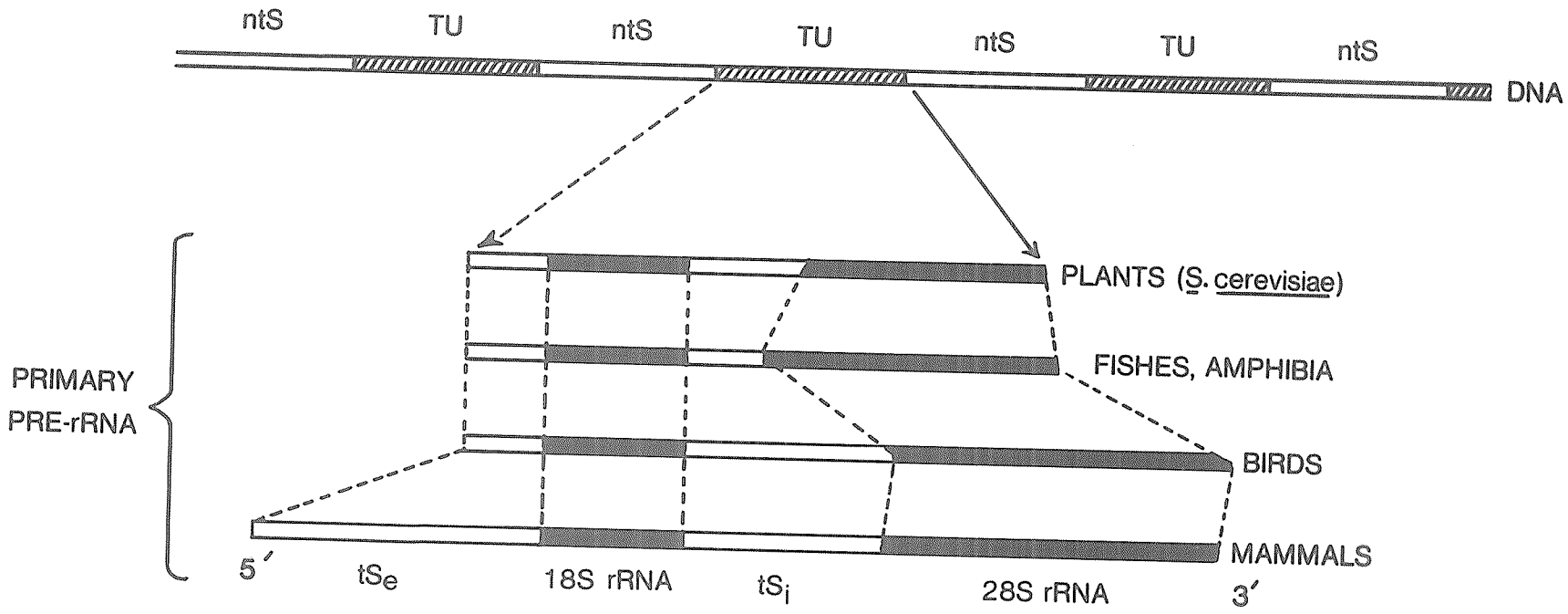


FIG.1 ARRANGEMENT OF SPECIFIC SEQUENCES IN rRNA GENES AND PRIMARY PRE-rRNA OF EUKARYOTES. TU= TRANSCRIPTIONAL UNITS ntS= NON TRANSCRIBED SPACER.

tSe = EXTERNAL TRANSCRIBED SPACER

tS_i = INTERNAL TRANSCRIBED SPACER

(FROM HADJIOLOV,1977)

Spacer sequences differ widely, even between closely related species. In Xenopus laevis and Xenopus mulleri hybrid cells, rRNA of X. laevis is preferentially transcribed (Honjo and Reeder, 1973). Their 18S and 28S sequences are indistinguishable by molecular hybridization. Spacer units are of the same length, but their G-C content has diverged enough to allow separation with cesium chloride (Miller and Hamkalo, 1972), and to be distinguished by molecular hybridization (Honjo and Reeder, 1973). Spacer sequences may play an important role in regulation of transcription or processing.

Silver nitrate is histochemically reduced to form black spheres at the NORs (AgNORs). All NORs do not react. In humans a characteristic number of AgNORs occur, with a mode of 8 per cell (Ray and Pearson, 1979). As RNase and DNase do not eliminate silver reduction, while pretreatment with trypsin or pronase does, it has been concluded that an acid protein is responsible for the silver nitrate reaction (Schwarzacher et al., 1978). Studies of human-mouse somatic cell hybrids showed that the silver reaction detects sites of rRNA activity, rather than the amount of rRNA or DNA (Miller et al., 1976 a & b).

C. Nucleolus

1. Introduction

The nucleolus is the established site of pre-rRNA synthesis and maturation. This involvement in the production of the protein synthesizing machinery of the cell gives the nucleolus a prominent position in cell metabolism.

Nucleolar structure and function is dynamic. It changes with cell cycle variations (Grant, 1972; Grummt et al., 1977), with diurnal rhythms (Glasser and Spelsberg, 1972), and with cell differentiation (Grummt, 1978). The size and activity levels of the nucleolus increase after stimulation with mitogens such as PHA (Schwarzacher et al., 1978), with hormones or serum, and after fusion of cells to form hybrid heterokaryons (Ringertz et al., 1971). A prominent nucleolus characterizes cells engaged in intensive protein synthesis.

Nucleolar hypertrophy is striking in most malignant and virus infected cells (Bernhard, 1966). The increase in size is usually accompanied by structural variations. Inhibition of rRNA synthesis with low doses of Actinomycin D (AMD), reduces the size of nucleolus and has a cytostatic effect on a variety of tumors. Control of nucleolar activity is central to a cell's ability to respond normally to physiological stimuli.

2. Structure and function

The nucleolus was discovered by Fontana in 1781 (Ghosh, 1976). Ruzicka (1891) noted silver impregnation in nucleoli. Association of the secondary constriction regions of the chromosome with the nucleolus was pointed out by Heitz (1931). He termed the regions sine acid thymonucleinico, or SAT regions. Estable and Sotelo (1951) described two components, a filamentous nucleonoma and a structureless pars amorpha, within the nucleolus. Lettré and Siebs (1954) reported the presence of functionally active DNA within the nucleolar filament.

With the advent of the electron microscope, morphological descriptions were refined. The nucleolus is known to be composed of three components: chromatin, the fibrillar component and the granular component (Fig. 2). Approximately 85 to 90% of nucleolar chromatin is located at the periphery of the nucleolus. The remaining 10 to 15% is located within the nucleolus. Only 0.2 to 1% of intranuclear DNA consists of rRNA genes.

Pulse labelling with ^{3}H-uridine and autoradiography demonstrate that the fibrillar component consists of 80S ribonucleoprotein (RNP), which contains 45S pre-rRNA (Karasaki, 1965). The granular component consists of RNP particles containing primarily 28S rRNA and is derived from the fibrillar component (Recher et al., 1971). The relative size of the fibrillar and granular areas is constant for a given cell type (Hardin et al., 1970), implying that maturation is relatively slow

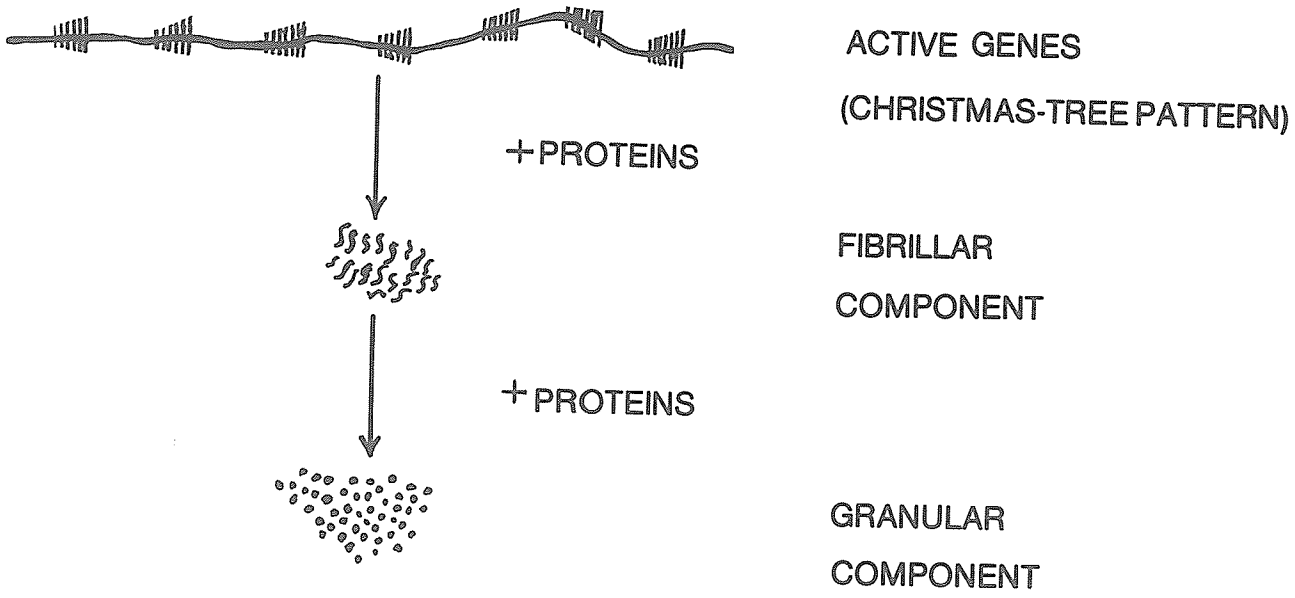
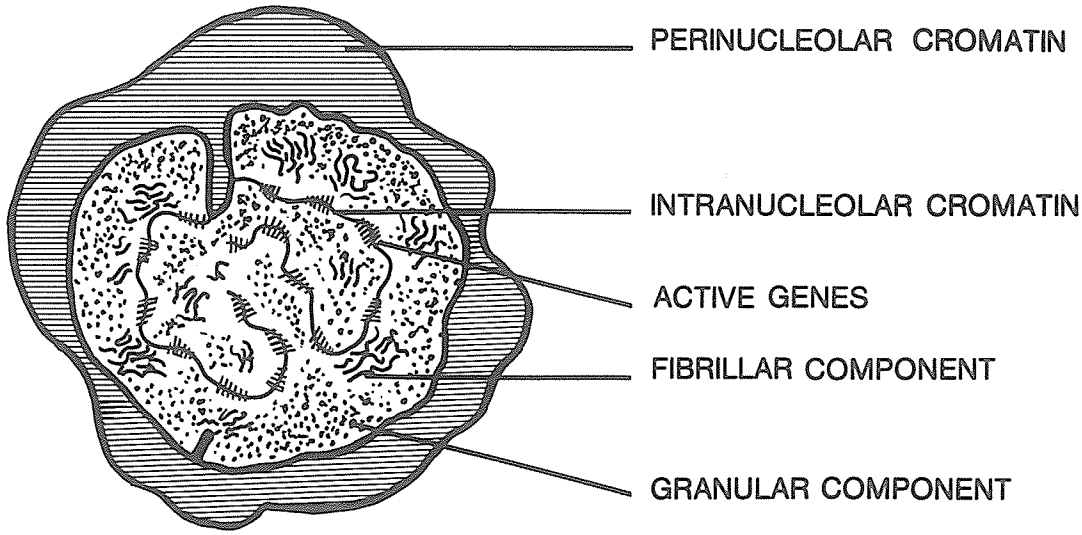


FIG.2 SCHEMATIC PRESENTATION OF NUCLEOLUS.
(FROM HADJIOLOV AND NIKOLAEV, 1976)



and subject to precise regulation.

Nucleoli appear during telophase and increase in size as the cell produces more RNA. Chromatin from the NORs uncoils and is dispersed within the nucleolus. During prophase the nucleolus disintegrates. If disintegration is late, there is insufficient time for complete condensation of chromatin and the NOR of the metaphase chromosome is usually large (Ghosh, 1976).

Solitary nucleolar DNA fibrils may be visualized after implementing the spreading techniques of Miller and Hamkalo (1972) (Fig.2). The axial fibril of DNA is coated at regular intervals with gradients of short to long matrix fibres, perpendicular to the axis. All units have the same polarity of gradients and are separated by matrix-free segments. This is the classic "Christmas-tree" pattern of active rRNA genes (Miller and Hamkalo, 1972). DNA intervals with matrix fibres are transcribed units and intervals without fibres are non-transcribed spacer units.

Transcribed rRNA units undergo a complex maturation process during which transcribed spacer units are eliminated and numerous RNA-protein interactions occur. Heavy rRNA, sedimenting at 45S, is transcribed at the NOR and interacts with proteins to form the fibrillar component of the nucleolus. This is subsequently degraded via intermediate steps to produce the granular component containing 28S and 18S rRNA.

3. Regulation of rRNA production and maturation

The capacity for rRNA production is genetically determined. It has been estimated that this capacity is 15- to 50-fold higher than the consumption of ribosomes (Hadjiolov and Nikolaev, 1976). Critical sites for control of rRNA production in the nucleolus are during transcription, maturation and transportation.

Transcription may be limited by the availability of purine nucleotides or by polymerase A concentrations or activity. A close parallel between the intracellular level of ATP and GTP and the amount of RNA polymerase active in transcription has been reported (Grummt, 1978). As ATP and GTP levels are related to many cellular reactions, the parallel may not be related to the availability of nucleotide pools.

Changes in nucleolar RNA synthesis were correlated with changes in RNA polymerase A activity during transcription (Cooke and Brown, 1973; Grummt, 1978). In contrast, Roeder et al. (1970) found no correlation between levels of enzyme activity and amount or type of RNA synthesis. Spring et al. (1974) estimated that rDNA is maximally loaded with RNA polymerase, suggesting that post-transcriptional control mechanisms are critical.

Maturation may be controlled at one or more of the RNA-protein interactions. In a given cell type one major pre-RNA maturation pathway occurs, but the RNA cleavage sites may be flexible, resulting in other

maturation pathways, involving different pre-rRNA molecules. PHA preferentially stimulates one of two maturation paths in lymphocytes (Purtell and Anthony, 1975). This channelling may be a critical site for qualitative and quantitative regulation of RNP.

Maturation pathways may be influenced by the secondary structure of RNP (Hadjiolov and Nikolaev, 1976). Secondary structure consists of numerous hairpin loops which correspond to G-C rick areas of the transcribed spacer units. Loops could physically direct protein interactions and, as even closely related species have great variability in spacer sequences and secondary structure, cause inter-species and possibly intraspecies variability in ribosome structure.

Inspite of the fact that equal amounts of 28S and 18S RNA are produced, the bulk of the granular component of the nucleolus is 28S rRNA. This discrepancy suggests either a great difference in maturation time and/or in speed of migration to the cytoplasm.

The migration of RNP from the nucleolus to the cytoplasm is poorly characterized, though the passage of RNP through the nuclear pore complex has been observed (Bernhard, 1971). In heterokaryons, rRNA passage was inhibited until a nucleolus was present and synthesizing RNA. Since the migration of both rRNA and tRNA to the cytoplasm was inhibited after irradiation of the nucleolus (Sidebottom and Harris, 1969), the nucleolus may control the passage of all RNA to the cyto-plasm.

The synthesis of DNA, often triggered by physiological stimuli, may be coordinated in the nucleolus. Blockage of rRNA synthesis with low doses of AMD, prohibit DNA synthesis by indirectly regulating DNA polymerase production (Cooper, 1972). The period of greatest sensitivity is early during activation, long before DNA polymerase synthesis begins.

D. Ribosomes

Ribosomes are large complex organelles, which probably change in shape and protein content at different stages of protein synthesis (Brimacombe, 1976). Each ribosome consists of a large and a small RNP subunit. In eukaryotes, the smaller subunit contains 20 to 30 proteins, dependent on the species, and a 18S rRNA molecule. Larger subunits contain 30 to 40 proteins, one 28S RNA, and one 5S RNA molecule. Few of the proteins are present in multiple copies.

In every cell, ribosomes translate mRNA to produce proteins. The concept that an excess of stable ribosomes is involved in translating a fluctuating level of mRNA is oversimplified. In eukaryotes, the control of ribosome biogenesis is more rapid and versatile than mRNA or tRNA formation. Serum growth stimulation of contact inhibited mouse fibroblast resulted, within 2 hours, in a 2-to 3-fold increase in protein synthesis and formation of new ribosomes, while mRNA synthesis increased only 20 to 30% (Rudland et al., 1975). During metaphase, protein synthesis decreases to 20-40% that of interphase. This cannot be explained by a decrease in mRNA, but is associated with a rapid disappearance of polyribosomes. Synthesis of new RNA is not necessary for protein synthesis to increase in interphase (Hodge et al., 1969). Changes in cell biosynthetic pathways often result in old ribosomes being degraded and new ribosomes being formed (Grasso and Woodard, 1966; Cocucci and Sussman, 1970). This suggests that

ribosomes are heterogeneous and that special classes of ribosomes may fulfill different functions.

Crick (1968) suggested that the primitive apparatus for protein synthesis might have originally consisted only of RNA, and that the addition of proteins during evolution refined the machinery. No essential differences have been found in the basic functions of ribosomes, however the greater complexity of architecture of eukaryote ribosomes supports the idea that the realization of genetic information may be at the ribosomal level.

Existing ribosomes respond to requirements for increased protein synthesis by increasing their efficiency and by decreasing ribosome degradation. Approximately 70% of resting lymphocytes are not engaged with mRNA in protein synthesis. After PHA addition, only 30% are not engaged (Cooper, 1972). The degradation of significant portions of rRNA is reversed after PHA stimulation of normal lymphocytes (Cooper and Gibson, 1971). Wastage of rRNA may be a mechanism by which resting cells may quickly shift to rapid growth.

The increased utilization of pre-existing ribosomes is inadequate for normal cell growth and mitosis. Blockage of rRNA synthesis prohibits DNA synthesis and thus cell division.

E. Silver Nitrate Reaction

1. History

The reaction of the nucleolus to silver nitrate was observed almost 100 years ago by Ruzika (1891). Cajal (1910) (Schwarzacher et al., 1978) described the silver stained granula of the nucleolus. Estable and Sotelo (1951) described a nucleonema of silver positive fibrils. Lettré and Siebs (1954) and Lettré et al. (1966) described this network as silver positive granules around a backbone of the chromosomal NORs. This view was confirmed with electron microscope observations (Schwarzacher et al., 1978). Cellular reactions with silver nitrate have recently gained interest because it was found that NORs react (Goodpasture and Bloom, 1975).

2. Location and molecular basis of reaction

Colourless silver nitrate is differentially reduced at the chromosomal locations of rDNA to form a black precipitate. In maximally activated cells of a given type, the degree of silver nitrate precipitation is characteristic of an individual chromosome, and this characteristic is an inherited property (Markovic et al., 1978; Mikelsaar et al., 1977). Silver nitrate reactivity is associated with actively transcribing rDNA cistrons in the fibrillar region of the nucleolus. As it is not located in the granular component, the reactive

substance is not part of the RNP particle.

Silver nitrate reacts with chromosomal proteins rather than rDNA. Digestion of DNA or RNA does not inhibit the reaction, whereas treatment with trypsin or pronase does (Goodpasture and Bloom, 1975). Hubbell et al. (1979) isolated the protein involved, and found it associated with actively transcribing cistrons of the fibrillar region. Histochemical studies indicate that the interactions of silver ions with carboxyl groups of acidic proteins are primarily responsible for the selective silver staining (Olert et al., 1979).

3. Silver reactivity represents rRNA activity

Silver reactivity is indicative of activity rather than the presence of rRNA genes. This is supported by the following three observations.

In mouse-human somatic hybrid cells, where expression of human rRNA genes was suppressed, only mouse NORs reacted with silver nitrate (Miller et al., 1976a). In mouse-human hybrid cells, where expression of mouse rRNA genes was suppressed, only human NORs reacted with silver nitrate (Miller et al., 1976b).

The pattern of rRNA synthesis during male gametogenesis (Tres, 1975) and during PHA induced transformation of lymphocytes (Cooper, 1972) followed that of silver precipitation (Engel et al., 1977).

In Xenopus embryos, rRNA synthesis was demonstrated after early gastrula (Brown and Littna, 1964) at which time silver precipitation was first detected (Engel et al., 1977).

The coefficient of correlation between the intensity of silver reactivity and uridine incorporation after inhibition of rRNA with low doses of AMD was 0.99 and 0.96 (Hofgärtner et al., 1979). This almost precise correlation demonstrated that the amount of silver reactivity in the nucleolus is a measure of the functional activity of rRNA genes.

III. ROLE OF IONS IN REGULATION OF rDNA ACTIVITY

A. General

Ions are known to exert a profound influence on such varied cellular processes as cell motility, muscle contraction, nerve impulse transmission, endocytosis and exocytosis. The stringent control under which ionic levels are maintained, emphasizes their importance to the cell. Ions dynamically respond to physiological stimuli and either inhibit or activate biochemical reactions. A high degree of specificity of action can be achieved if ionic species are combined or modulated by proteins.

B. Potassium (K^+)

Potassium is a universal requirement for the growth of cells and is closely monitored by all cells. Levels are influenced by corticosterone, cortisol, deoxycorticosterone, and aldosterone.

A dynamic equilibrium exists between Na^+ and K^+ fluxes. Within minutes after the addition of PHA to cultured lymphocytes, K^+ influx doubles (Quastel and Kaplan, 1970b). The concentration of cellular K^+ does not change with stimulation since an increased eflux balances the influx (Hamilton and Kaplan, 1977). As mitogens cause human lymphocyte membranes to be extremely leaky to ions in general, it is not surprising that K^+ influx doubles.

The extracellular concentration of K^+ affects the intracellular level. Negendank and Shaller (1979) equilibriated human lymphocytes in a range of external K^+ levels, and found that as external K^+ levels increased linearly, internal K^+ levels increased rapidly in a sigmoidal fashion.

Low cellular K^+ inhibits protein synthesis by limiting the transfer of amino acids from aminoacyl soluable RNA to polypeptides (Lubin and Ennis, 1964). When cell K^+ fell below 60 to 80% of control levels, protein synthesis decreased in proportion to further decreases of K^+ (Ledbetter and Lubin, 1977). Such dramatic changes are not known to occur during normal cellular processes.

Low cellular K^+ does not inhibit RNA synthesis (Lubin and Ennis, 1964; Pollack and Fisher, 1976). Oubain, a specific inhibitor of the K^+ site of membrane Na^+ K^+-ATPase, inhibited PHA stimulation of lymphocytes. K^+ reversed this inhibition (Quastel and Kaplan, 1970a). Thus K^+ may be implicated in activation of lymphocytes.

The degree of chromosome swelling is affected by Na^+ and K^+ levels, and this is correlated with changes in the capacity of chromosomes to support RNA synthesis (Lezzi, 1970). Mg^{++} and Ca^{++} amplify the difference between the effects of Na^+ and K^+.

C. Magnesium (Mg^{++})

Free Mg^{++} in tissues of rat remains relatively constant at about 1 mM. There is little or no concentration gradient between extracellular and intracellular compartments (Bygrave, 1978b). Increasing extracellular $MgCl_2$ from 1.2 to 2.5 mM rapidly stimulated DNA synthesis and mitotic activity of rat thymocytes (Whitfield et al., 1969a). Reducing Mg^{++} to 20% of normal caused considerable change in growth rate of lymphoblasts, while a reduction to less than 2% of normal Ca^{++} was necessary to affect the growth rate (Owens et al., 1958).

The lack of hormones and other mechanisms to control the levels of Mg^{++}, make it unlikely that Mg^{++} plays a prominent role in cell regulation. Mg^{++} is important in maintaining the functions of many enzymes including RNA polymerase A, adenylate cyclase, and Ca^{++}-transport ATPases. Since the function of Ca^{++} and Mg^{++} are intertwined, Mg^{++} deprivation can inhibit responses to a Ca^{++} surge (Whitfield et al., 1979).

D. Calcium (Ca^{++})

Calcium has long been known to influence many biological processes including cell motility, muscle contraction, endocytosis, exocytosis, chromosome movement, neurotransmitter release, microtubule assembly and disassembly, cellular adhesion, cytoplasmic streaming, metabolism of carbohydrates and fats, and others. More recently, Ca^{++} and the hormones which control its levels were found to initiate DNA synthesis and mitosis in the bone marrow, liver and thymus cells of the rat (Whitfield, et al., 1979). Tumor cell proliferation is characterized by not being influenced by Ca^{++}.

According to the three messenger concept, the major regulators of cell activity are hormones, cyclic nucleotides and Ca^{++} (Cheung, 1980). These function as messenger molecules which transduce chemical messages into messages which control one or more critical biochemical reactions. Messengers are interrelated and complement each other with respect to the distance the message travels, the speed and the duration of response. Hormones are the intercellular regulators while cyclic nucleotides and Ca^{++} are the intracellular regulators.

Hormones serve as the first messenger, cAMP the second and Ca^{++} the third. Many hormones, such as epinephrine, PGE, vasopressin, and parathyroid hormone interact with receptor sites on the cell membrane and elicit rapid increases in intracellular cAMP and stimulation of DNA synthesis and mitosis. Almost every hormone system

has an absolute requirement for exogenous Ca^{++} (Rasmussen, 1970). Hormones which regulate Ca^{++} (Calcitonin; 1α , $25(OH)_2$ vitamin D_3; parathyroid hormone) are also major regulators of DNA synthesis and mitotic activity. The existence of such hormones emphasizes the importance of Ca^{++} levels in biological systems.

Stimulation of cell membrane adenylate cyclase by a hormone leads to an increase in cellular cAMP. In turn, cAMP functions by stimulating various protein kinases. A striking parallel exists between extracellular Ca^{++} concentrations, cellular cAMP, level of DNA synthesis and cell proliferation (Whitfield et al., 1973). Since Ca^{++} inhibits adenylate cyclase (Whitfield et al., 1973), Ca^{++} stimulation of cAMP was not understood until the intimate relationship of Ca^{++} and calmodulin was discovered (Reviewed by Cheung, 1980).

Calcium exists in many forms. These include exchangeable, non-exchangeable, free (ionized), diffusable but complex, and bound and precipitated in mineral form. The ionized form is biologically the most active and this is a small portion of the total calcium (Bygrave, 1978a).

There is a steep, 10^4-fold gradient between intracellular (0.1μM)(Sulakhe and St. Louis, 1980) and extracellular (> 1.0mM) (Whitney and Sutherland, 1972) Ca^{++} levels. Cells work unceasingly to keep this gradient. Passive influxes of Ca^{++} are determined by

the concentration gradient and by membrane permeability. At least 3 separate "gates" by which Ca^{++} enters, exists. One of which is independent of membrane potential and possibly dependent on hormone activation (Sulakhe and St. Louis, 1980). Ca^{++} eflux is active and probably consists of two separate mechanisms: a Ca^{++} regulated ATPase and a NA^{+}-Ca^{++} exchange system. Basal rates of transmembrane fluxes do not vary greatly with cell type (Claret-Berthon et al., 1977).

The distribution of Ca^{++} is heterogenous and fluxes are a vital component of physiological responses of cells. Uneven distribution is maintained by specific calcium transport systems, and by the specificity of Ca^{++} binding to oxyanions, such as carboxylates and phosphates (Bygraves, 1978a). Claret-Berthon et al. (1977) analysed rat liver cells and found the following three major exchangeable pools of Ca^{++}.

The first compartment contained rapidly exchangeable Ca^{++} (4 min.) and was identified as extracellular Ca^{++} bound loosely to the cell membrane. The Ca^{++} chelator, EGTA, which does not penetrate the cell membrane, completely displaced Ca^{++} from this compartment.

The second compartment contained less rapidly exchangeable Ca^{++} (16 min.) and was identified as intracellular Ca^{++}. It was comprised of Ca^{++} pools in the mitochondria, endoplasmic reticulum and associated with proteins. Since 30% of intracellular Ca^{++} was sequestered in

the mitochondria, it is evident that these organelles play an important role in cellular Ca^{++} homeostasis.

The third compartment consisted of slowly exchangeable Ca^{++} (223 min.), which was identified as consisting at least partly of extracellular Ca^{++} in intimate contact with the cell membrane. EGTA displaced about 85% of this Ca^{++}. With the addition of the Ca^{++} displaced from the first compartment, EGTA displaced 63% of total exchangeable Ca^{++}.

Calcium ions are able to regulate many diverse and complicated cellular processes by activating Ca^{++}-binding proteins, such as troponin C and calmodulin. Proteins bind Ca^{++}, selectively and reversibly, at their carboxylate groups causing conformational changes, which offer great potential for regulating a wide variety of cellular processes.

Most regulatory roles previously attributed to free Ca^{++}, require its association with calmodulin (Means and Dedman, 1980). Calmodulin itself is not active. Binding with Ca^{++} results in an active, more helical molecule (Liu and Cheung, 1976; Dedman et al., 1977) which binds to an apoenzyme to form a holoenzyme. The specificity of Ca^{++} action is through calmodulin binding with other proteins.

Calmodulin is an important link between Ca^{++} and cAMP. It regulates Ca^{++}-dependent adenylate cyclase, which explains why

exogenous Ca^{++} could cause a cAMP surge, while Ca^{++} alone inhibits adenylate cyclase (Whitfield et al., 1979). Another link exists controlling Ca^{++} efflux. Membrane associated Ca^{++} pump acitivity is likely modulated by calmodulin and cAMP-dependent protein kinase(s) (Sulakhe and St. Louis, 1980).

Calmodulin has a striking lack of tissue or species specificity. It has been structurally conserved throughout the animal and plant kingdoms (Cheung, 1980). The protein shares a large homology with troponin C. It is characterized by thermal stability and abundant acidic amino acids which furnishes carboxylate groups for the reversible binding of Ca^{++} (Cheung, 1980).

Harper et al. (1980) reported labelling of the nucleus of rat liver and adrenal cortex cells with antibody for calmodulin. The amount of labelling varied as a function of hormonal activity and was in a pattern consistent with nucleolar organization.

Calcium is essential for the mitogenetic effects of PHA (Alford, 1970; Whitney and Sutherland, 1972). The removal of Ca^{++} with citrate inhibited transformation (Alford, 1970). This inhibition was totally reversed by restoring Ca^{++} within 18 hours after the addition of PHA, before DNA synthesis began. As the removal of Ca^{++} after DNA synthesis began, did not alter nucleic acid synthesis, the action of Ca^{++} is not directly on DNA synthesis. Whitney and Sutherland (1972) added EGTA at progressively later times after PHA,

and found that for the first 12 hours, the amount of DNA synthesis progressively increased as the time lapse between adding EGTA to PHA stimulated cells increased. Later additions of EGTA did not produce any inhibition.

EGTA also inhibited PHA stimulation of RNA synthesis, an event which occurs early during transformation. Increasing concentrations of EGTA from 0 to 1.48 mM progressively inhibited RNA and DNA synthesis in PHA stimulated lymphocytes (Whitney and Sutherland, 1972). Cooper (1971) proposed that the direct effect of Ca^{++} on early RNA synthesis could control transformation.

Within five minutes after PHA stimulation of lymphocytes a brief influx of Ca^{++} occurs, along with other ions. The influx is not thought to initiate transformation as lowering extracellular Ca^{++} levels long after this surge subsided still prevented the initiation of DNA synthesis (Whitfield et al., 1979). Also, under conditions which ionophore A23187 did not cause Ca^{++} to enter the cell, DNA synthesis was triggered (Rosenstreich and Blumenthal, 1977; Gemsa et al., 1979).

Exogenous Ca^{++} directly stimulates DNA synthesis of rapidly responding thymic lymphocytes (Whitfield et al., 1980). Raising the Ca^{++} concentration to 1.5 mM for 5 minutes to 5 hours, stimulated an increase in synthesis of cAMP and DNA synthesis. Pretreatment with parathyroid hormone enabled small increments (0.02 to 0.6 mM) of Ca^{++} to stimulate both cAMP and thymic lymphocyte proliferation.

Similarly, increasing extracellular Ca^{++} of liver cells from 2.3 to 14 mM resulted in increased protein, RNA and DNA synthesis (Anghileri and Heidbreder, 1978).

Reducing blood Ca^{++} levels of rats by removing the parathyroid gland and by reducing dietary intake, almost immediately reduces cell proliferation in the bone marrow and thymus. Proliferation can be induced by injection of parathyroid hormone or $CaCl_2$ (Perris et al., 1967).

The proliferation of neoplastic cells is distinguished by not being controlled by Ca^{++}. Such cells can proliferate in low Ca^{++} media (0.001 to 0.1 mM) (Whitfield et al., 1979 & 1980) and their mitochondria tend to sequester and retain Ca^{++}, resulting in low cytoplasmic levels (Owens et al., 1958). The key to understanding and controlling autonomous proliferation of cancerous cells could possibly be found after there is a better understanding of the role of Ca^{++} in non-cancerous cells.

MATERIALS AND METHODS

I. MATERIALS

A. Lymphocytes

Human peripheral blood, collected in heparinized vacutainer tubes was utilized.

B. Culture Media

Cells were cultured in McCoy's 5A medium (modified) with 10% fetal calf serum (FCS), except where otherwise indicated. Custom formulated McCoy's 5A (modified) media without KCl, and without $MgSO_4$ were utilized for K^+ and Mg^{++} reduced experiments.

C. EGTA (Ethyleneglycol-bis-(B-amino-ethyl ether) N, N^1 - tetra-acetic) (MW-380.4)

A stock solution of 7.2 mM/cc was prepared by adding enough medium to 136.8 mg EGTA, to produce 10cc. This was neutralized to pH 7 with NaOH to dissolve the EGTA. The solution was sterilized with a millipore filter.

D. Calmodulin (CDR)

Calmodulin was gratefully received from Dr. M.S. Nijjar, Department of Pathology, University of Manitoba. The solution was produced from bovine brain and contained 0.15 mg CDR/cc.

II. METHODS

A. Culturing

Human peripheral blood was collected in heparinized vacutainer tubes and erythrocytes allowed to settle at room temperature for 1 to 2 hours. Plasma (0.2cc) was added to sterile culture tubes containing 5 cc of McCoy's 5A medium (modified) with 10% FCS. This medium contains 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and other essential growth elements. Tubes were incubated at 37^0C in a CO_2 incubator for periods up to 72 hours.

Custom formulated McCoy's 5A (modified) media without KCl and without $MgSO_4$ were purchased. Constant osmolality was maintained by adding NaCl to K^+ reduced medium and $MnSO_4$ to Mg^{++} reduced medium. Both formulations contained 10% FCS, therefore K^+ reduced medium contained approximately 0.5 mM K^+, and Mg^{++} reduced medium contained approximately 0.1 mM Mg^{++}. Since 0.2 cc of human plasma with lymphocytes was added to 5 cc of culture medium for each treatment, the final serum concentration was approximately 14%. K^+ reduced treatments contained approximately 0.7 mM K^+, and Mg^{++} reduced treatments contained approximately 0.14 mM Mg^{++}.

Lymphocytes were stimulated with 0.05 cc of phytohaemagglutinin (PHA) per 5 cc of medium.

B. Harvesting

When mitotic spreads for NOR examination were required, colcemid (5.0×10^{-3} µg/ml) was added to prevent spindle formation. Two hours post colcemid, culture tubes were centrifuged at 600 rpm for 5 minutes. The supernatant was discarded and 5 cc of hypotonic KCl (0.075 M) added to the cell suspension. After 10 minutes, the suspension was centrifuged at 600 rpm for 5 minutes. The supernatant was discarded and cells fixed in 3:1 parts methanol-acetic acid for a minimum of 20 minutes. Slides were prepared by dropping the cell suspension from a pasteur pipette held approximately one metre above a wet microscope slide, which had been cooled in ice water. This effectively burst the cell membrane, allowing chromosomes to spread.

Cells used for measuring the nuclear diameter and nucleolar silver reactivity were harvested without the addition of colcemid. Tubes were centrifuged at 600 rpm for 5 minutes, the supernatant discarded and cells fixed in 3:1 methanol-acetic acid for a minimum of 20 minutes. Slides were prepared by gently spreading the cell suspension on slides.

C. Silver Nitrate Treatment (modified from Bloom and Goodpasture, 1976)

Slides were treated in a humid environment. The environment was prepared by lining the bottom of a petri dish with absorbant towelling to hold moisture. Small rubber rings supported the slides and prevented them from touching the moist bottom. Each slide was flooded with 50% silver nitrate, covered with a coverslip and the petri dish placed in a 55 ^{o}C oven for 16 to 18 hours. After treatment, slides were washed in running tap water, air dried, placed in xylol for 1 to 2 minutes and mounted in neutral buffered mounting medium.

D. Evaluation of Nuclear Size and Silver Reactivity

The first 100 lymphocytes, from 3 different slides of each treatment were evaluated. The nuclear diameter was measured with the aid of an eyepiece micrometer disc and classified as follows:

	Nuclear Diameter (μ)
Small:	$\leq$ 5.0
Medium:	5.5 - 8.5
Large:	$\geq$ 9

Quantification of silver reactivity was more difficult. A combination of measuring the size of the reactive area, counting

the number of reactive areas and subjective evaluations was used to classify the cells as follows (Figs.3 & 4):

0	-	no reaction
1^+	-	1 to 2 small reactive areas
2^+	-	several small reactive areas or one area with a diameter greater than 1.0 μ
3^+	-	5 or more small reactive areas or one area with a diameter greater than 1.5 μ
4^+	-	a cluster of reactive areas or one area with a diameter greater than 2 μ.

E. Metaphase Examinations

Twenty metaphase spreads from each of the K^+-reduced, the Mg^{++}-reduced and the EGTA treated cultures were analysed. The latter was treated for the last 1/3 (24 hours) of a 72 hour culture period. Cells in metaphase were not observed after longer periods of treatment with EGTA. A total of 526 control metaphase cells were examined. The number of silver reactive NORs (AgNORs) was recorded.

Figs.3 and 4: Silver reactivity of lymphocytes. The silver reactivity of lymphocytes was graded from 0-4^+, based on the size of the reactive areas. Unstimulated lymphocytes were small and had small reactive areas (Fig. 3). PHA stimulated lymphocytes were larger and had larger reactive areas (Fig. 4).

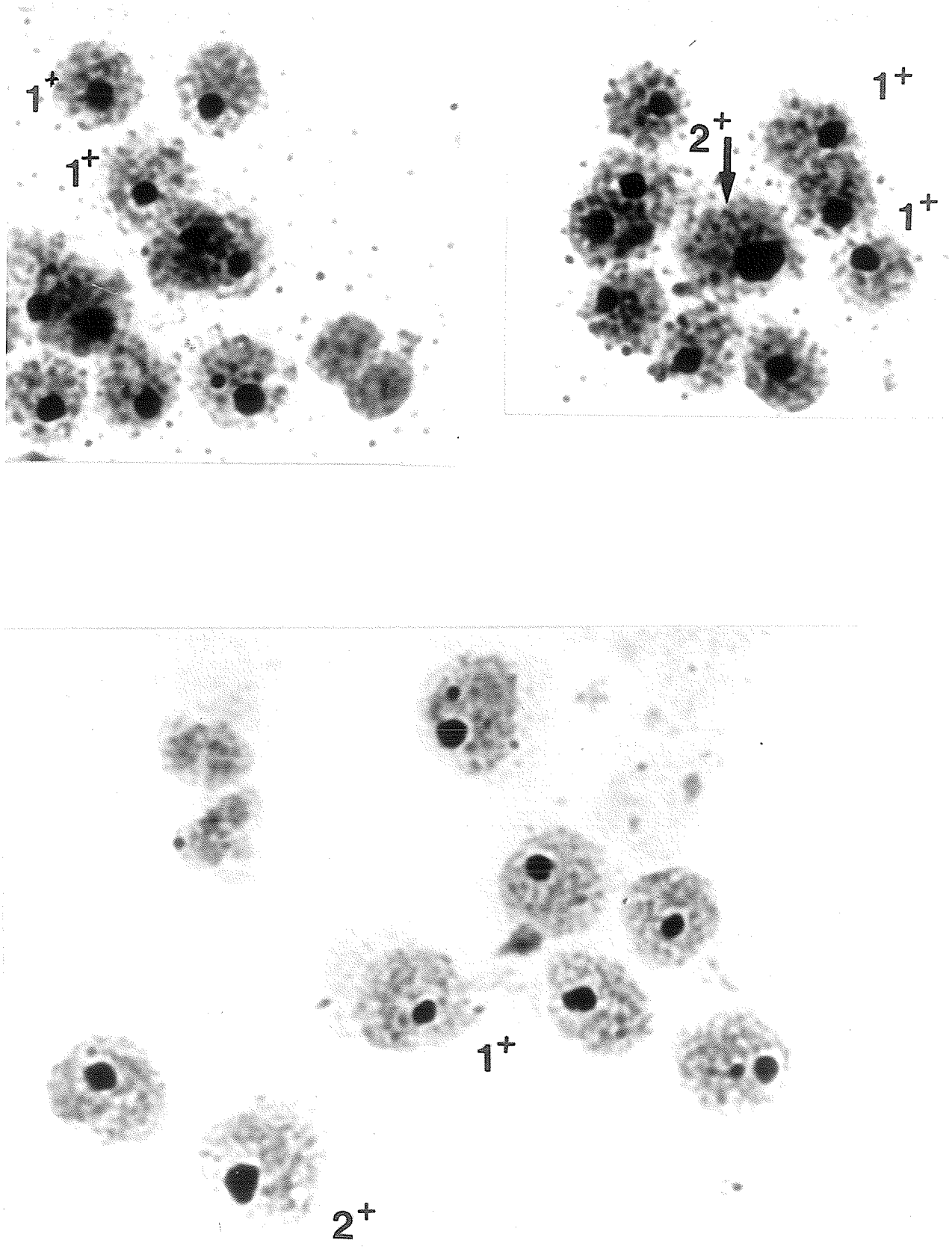
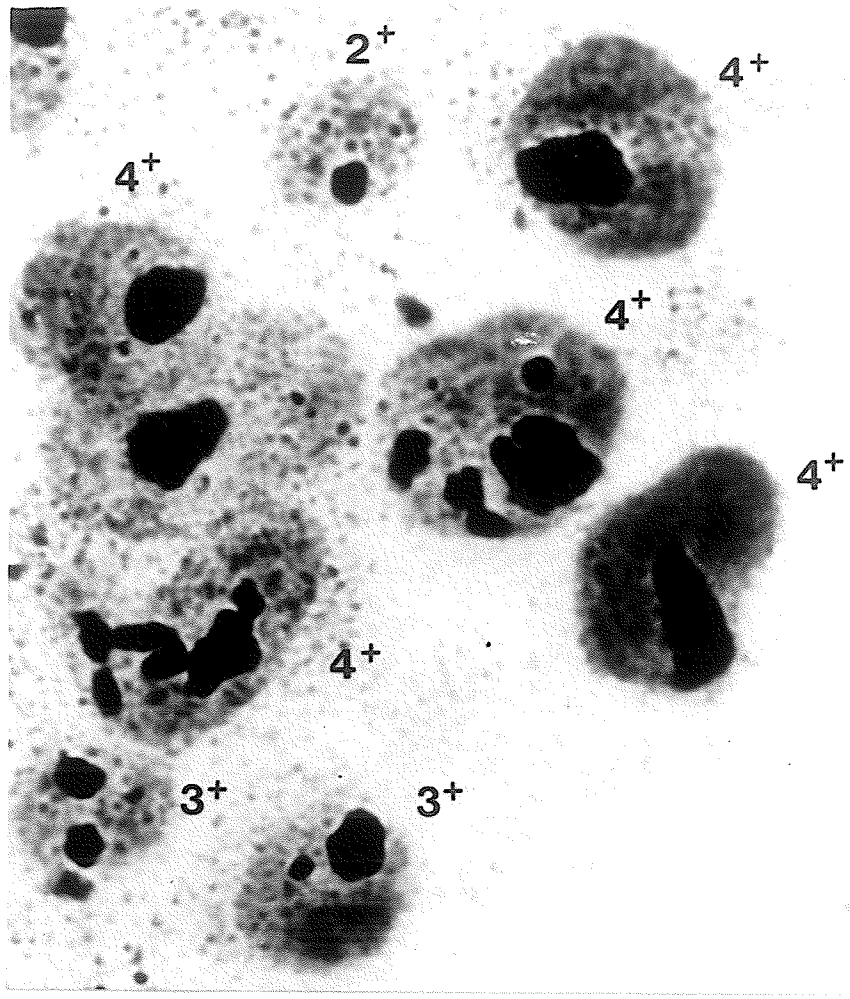


FIG. 3



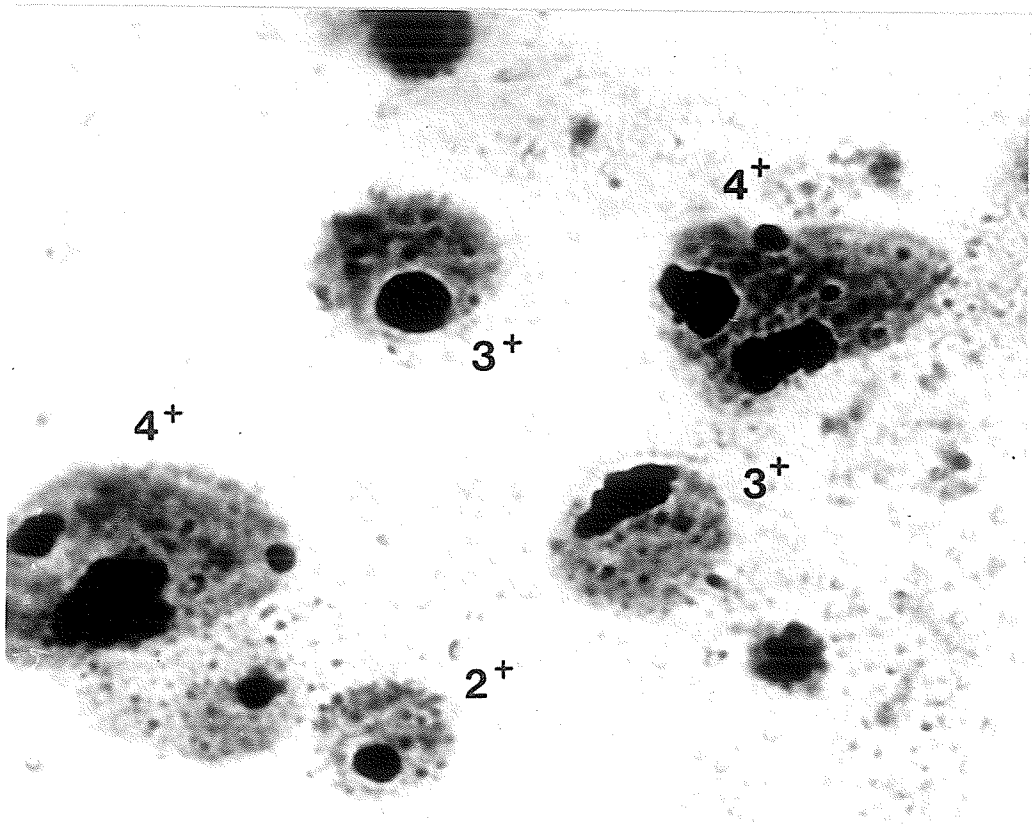


FIG.4

RESULTS

Unstimulated cultured lymphocytes always had a small nuclear diameter and a small silver reactive area (Fig. 3), neither of which increased with time (Fig. 5). After stimulation with PHA the nuclear diameter and nucleolar silver reactivity greatly increased (Figs.4 & 5). The peak period of increase was 18 to 48 hours after the addition of PHA. The increase in silver reactivity was linearly related to the log of the nuclear diameter (Fig. 6). Both parameters are indicators of lymphocyte activation.

Lymphocytes were cultured in medium containing a graduation from normal levels of K^{+} (5.0 mM) and Mg^{++} (1.0 mM) to 14% of normal K^{+} (0.7 mM) and Mg^{++} (0.14 mM). Removal of up to 86% of the extracellular Mg^{++} and K^{+} did not affect the silver reactivity, nuclear diameter (Fig. 7), or the mean number of AgNORs (Table I) of PHA stimulated lymphocytes.

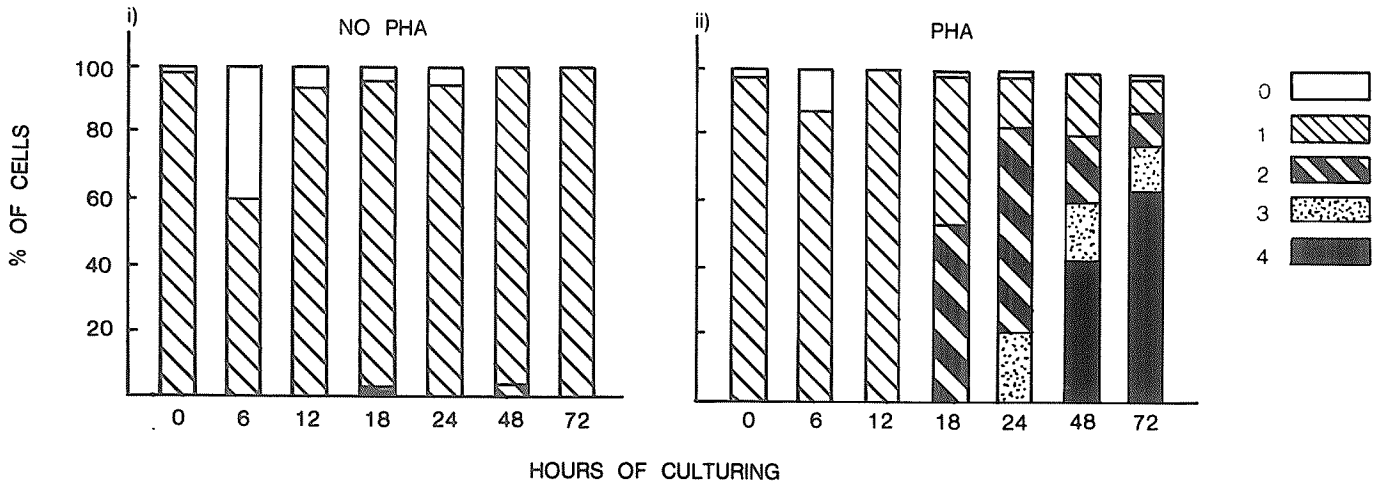
Ca^{++} was reduced in the culturing media by chelation with EGTA. Since 1M EGTA binds 1M $CaCl_2$, and human McCoy's growth medium has 1.8 mM $CaCl_2$, all Ca^{++} should be bound when the final EGTA concentration is 1.8 mM.

When Ca^{++} was chelated with EGTA for the last 24 hours of a 72 hour culturing period, a slight decline in nuclear diameter and silver reactivity was observed (Fig. 8). The decrease was correlated with an increased concentration of EGTA and did not plateau at 1.8 mM

Fig. 5: Histograms showing changes in a) silver reactivity and b) nuclear diameter of lymphocytes at various times after culturing i) with and ii) without PHA. PHA was added to treated cultures at 0 hour.

FIG. 5

a) SILVER REACTIVITY



b) NUCLEUS DIAMETER

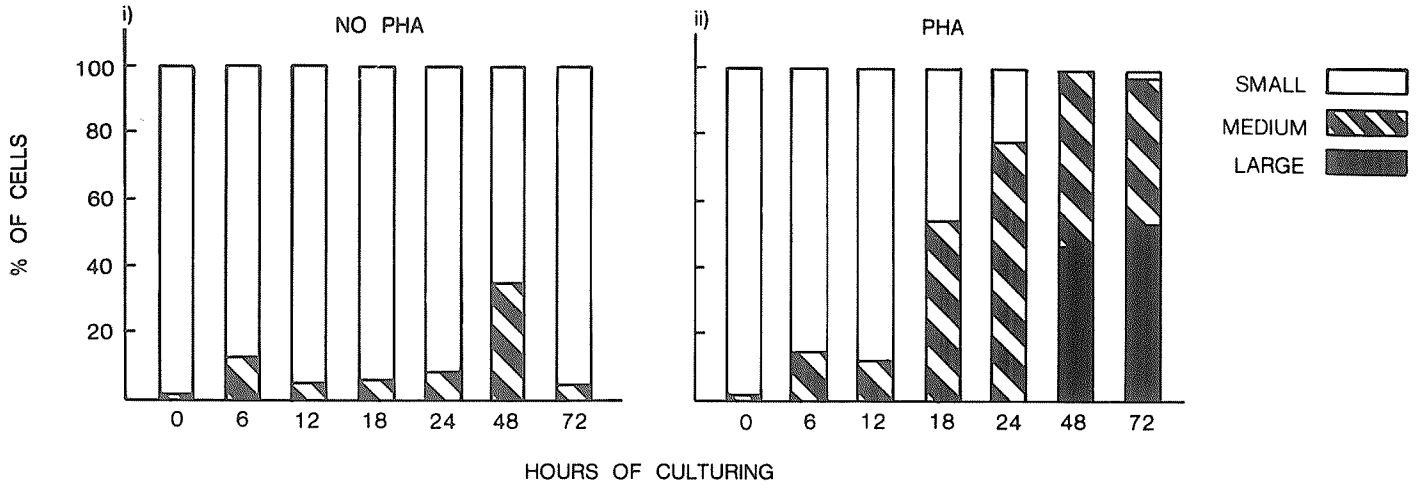


Fig. 6: Relationship of silver reactivity and nuclear diameter of lymphocytes.

Bars represent $\pm$ 1 S.D.

FIG. 6

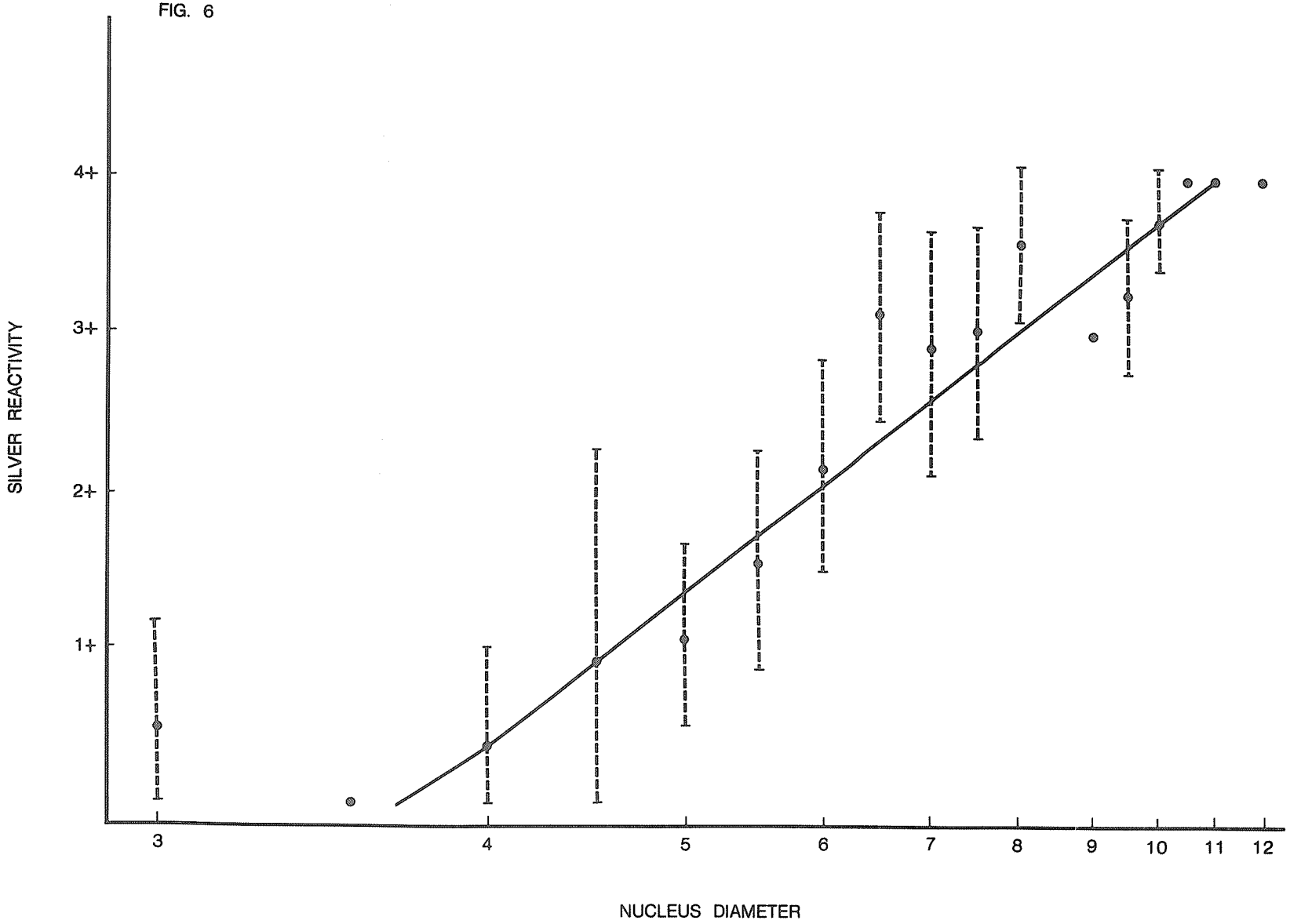


Fig. 7: Histograms showing a) silver reactivity and b) nuclear diameter of lymphocytes after 72 hours of culturing with various concentrations of i) K^+ and ii) Mg^{++}.

FIG. 7

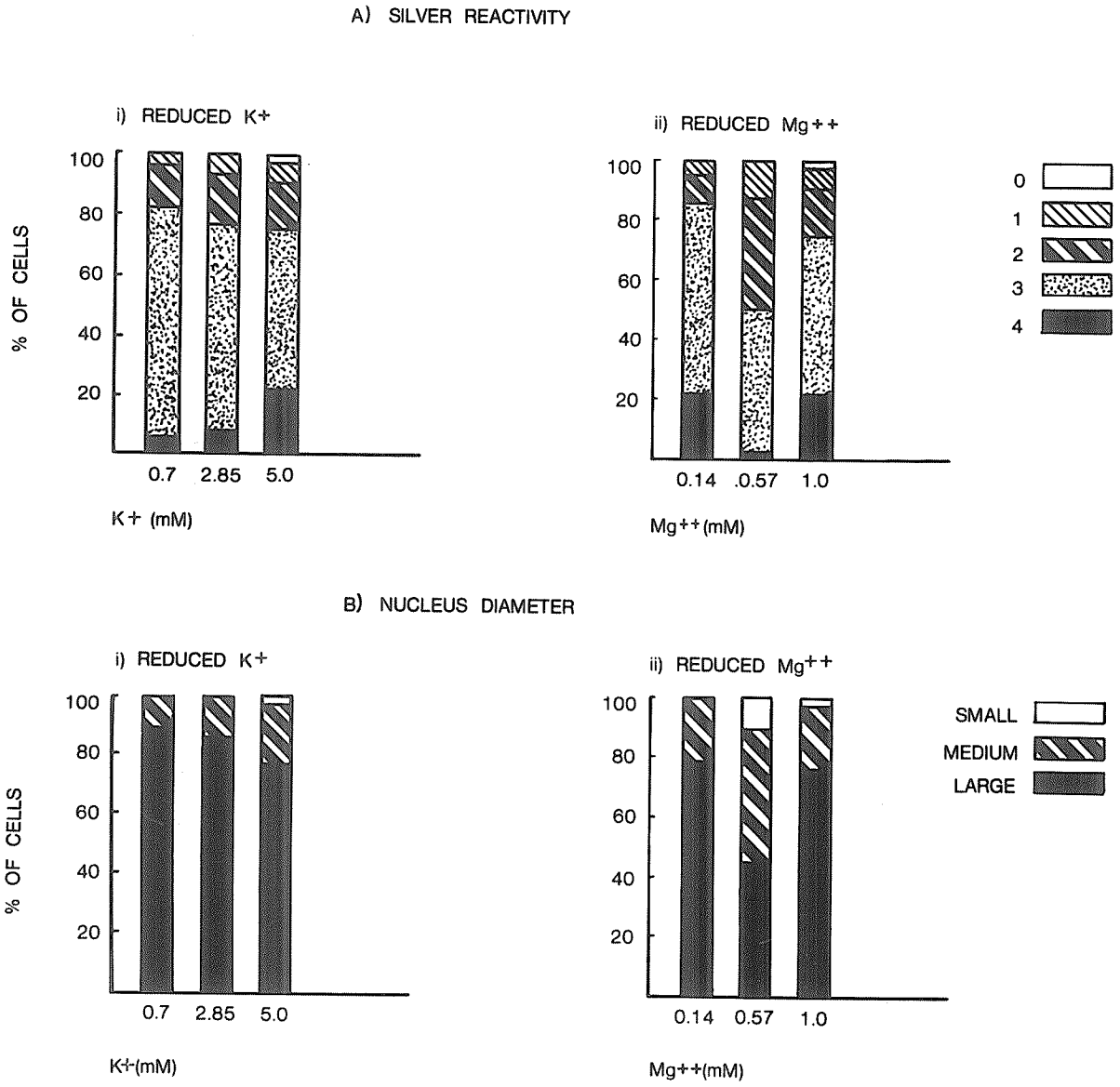

A) SILVER REACTIVITY
i) REDUCED K+
ii) REDUCED Mg++
% OF CELLS
100
80
60
40
20
0.7
2.85
5.0
K+ (mM)
0.14
.0.57
1.0
Mg++(mM)
0
1
2
3
4
B) NUCLEUS DIAMETER
i) REDUCED K+
ii) REDUCED Mg++
% OF CELLS
100
80
60
40
20
0.7
2.85
5.0
K+(mM)
0.14
0.57
1.0
Mg++(mM)
SMALL
MEDIUM
LARGE

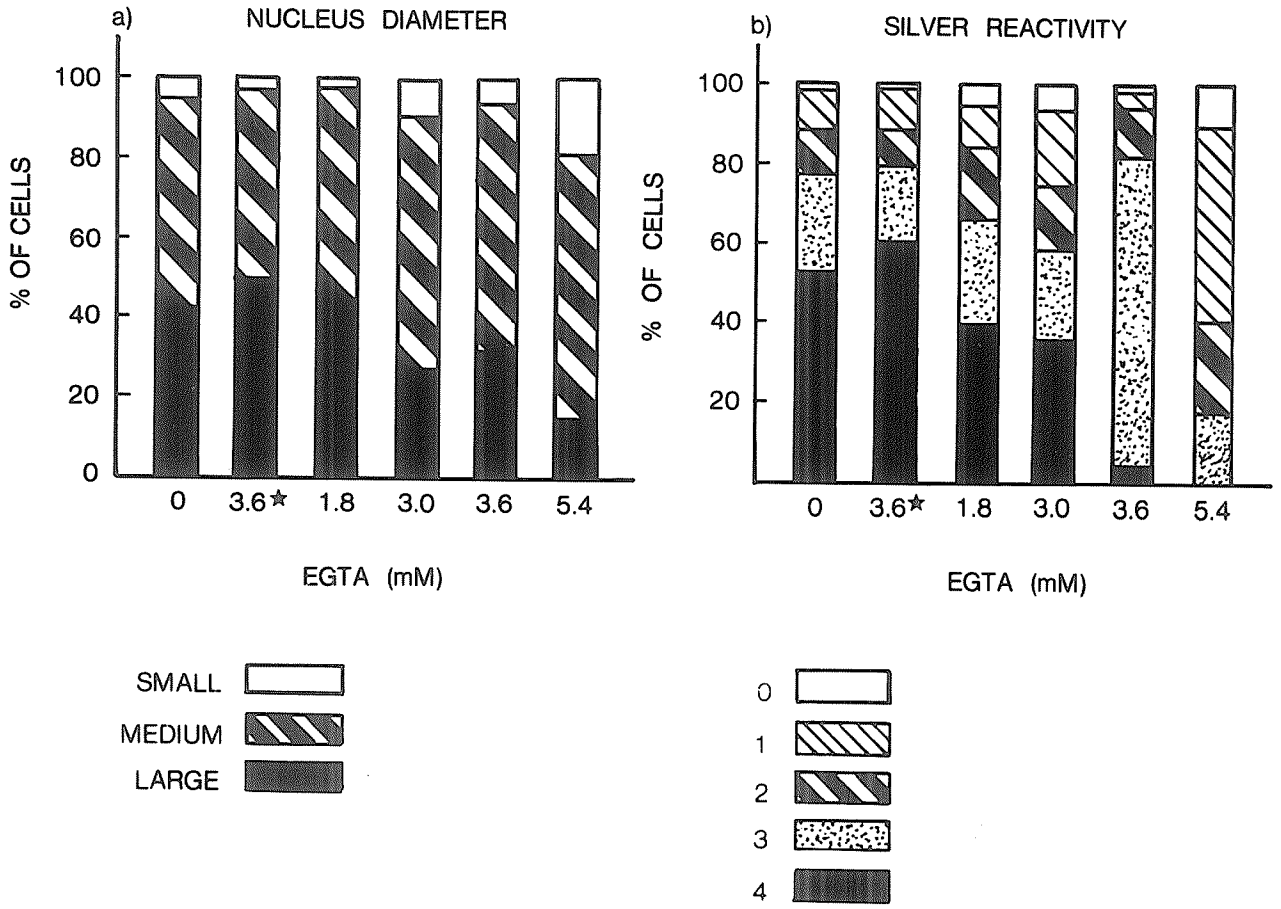
TABLE I

Mean number of silver reactive metaphase chromosomes (AgNORs) after various treatments.

Treatment	Hours of Treatment/ Hours of culturing with PHA	Total Number of Metaphase Cells	Mean Number of AgNORs
Controls	72/72	526	7.7
0.7 mM K^{+} (14% of normal)	72/72	20	8.0
0.14 mM Mg^{++} (14% of normal)	72/72	20	7.6
1.8 mM EGTA	Last 24/72	20	7.5
4.0 mM EGTA	72/72	No Mitosises	

Fig. 8: Histograms showing changes in lymphocyte a) nuclear diameter and b) silver reactivity after 24 hours of treatment with EGTA. After 48 hours of culturing, various concentrations of EGTA were added to PHA stimulated lymphocytes and $CaCl_2$ was added to one treatment group. All were harvested at 72 hours.

FIG. 8



☆ 2.6 mM $CaCl_2$ WAS ADDED AFTER 48 HOURS OF CULTURING.

EGTA, the concentration at which all ionized calcium was expected to be chelated. When some $CaCl_2$ was restored to treated cultures, the silver reactivity and nuclear diameter was comparible to control cells, indicating the decrease was not a toxic effect of EGTA.

The mean number of AgNORs in lymphocytes treated with 1.8 mM EGTA for the last 24 hours of a 72 hour culturing period was 7.5 per cell (Table I). This did not vary significantly from the control level of 7.7 AgNORs per cell.

Cells from one treatment group which was treated with 1.8 mM EGTA, for the last 24 hours of a 72 hour culturing period had silver reactive areas dissociating from the nucleolus of interphase cells and NOR of metaphase cells (Figs.9 & 10). Dissociated silver reactive areas formed uniform spheres with a ring of satellite bodies composed of RNP-like material. Due to this phenomenon further investigations into the role of Ca^{++} in lymphocyte transformation were undertaken. Repetition of the same experiment failed to produce a similar dissociation pattern.

Ca^{++} was chelated with 4.0 mM EGTA for varying periods of time during a 72 hour period of culturing PHA stimulated lymphocytes (Fig. 11). The nuclear diameter and silver reactivity was relatively constant, if EGTA (4.0 mM) was added 48 hours post PHA or later. When EGTA was added 0 to 24 hours post PHA, there was a dramatic reduction in nuclear diameter and silver reactivity.

Figs. 9 and 10: Lymphocytes with silver reactive areas dissociated from the nucleolus of interphase cells (Figs. 9 and 10a) and the NORs of metaphase cells (Fig. 10b).

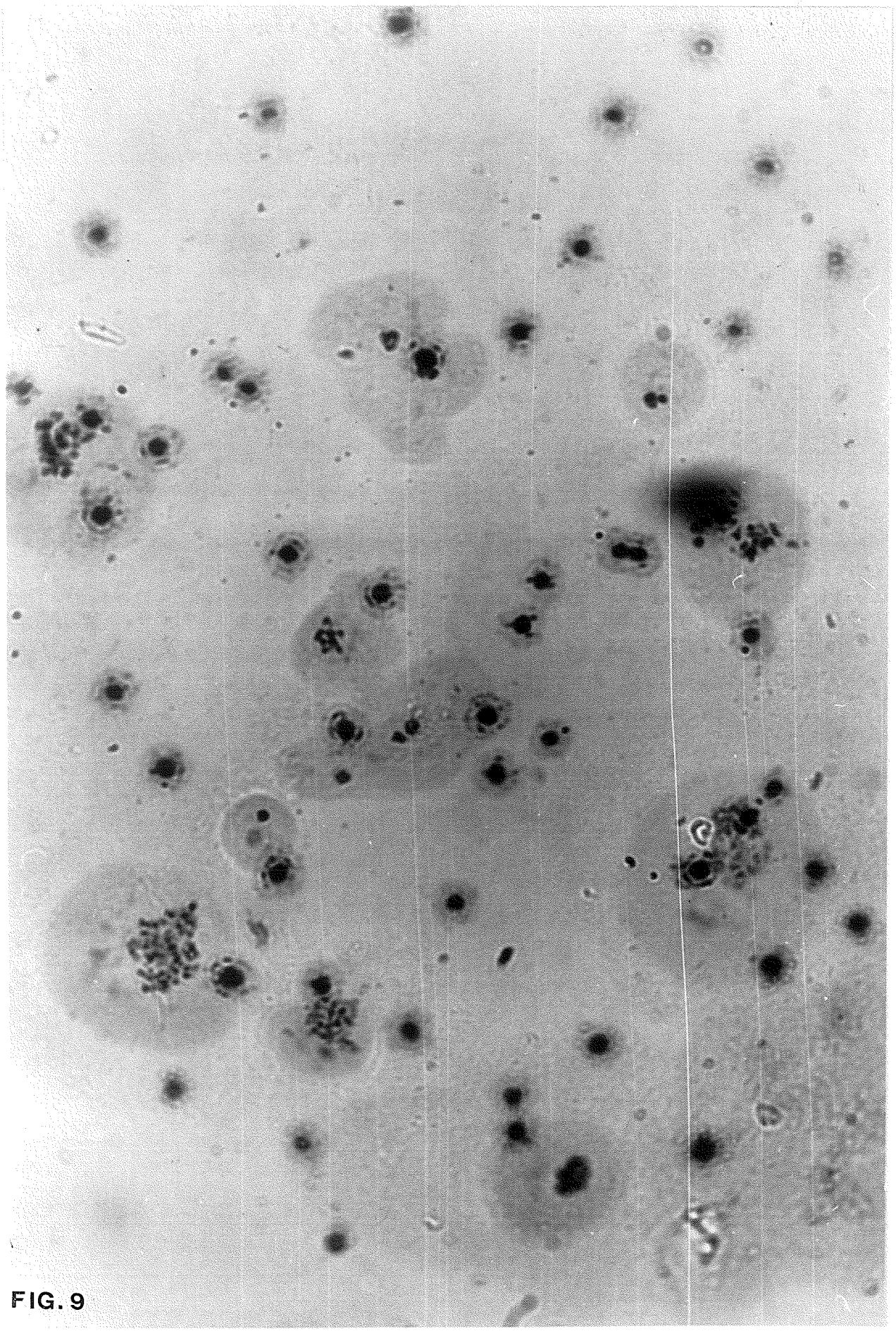


FIG. 9

A)

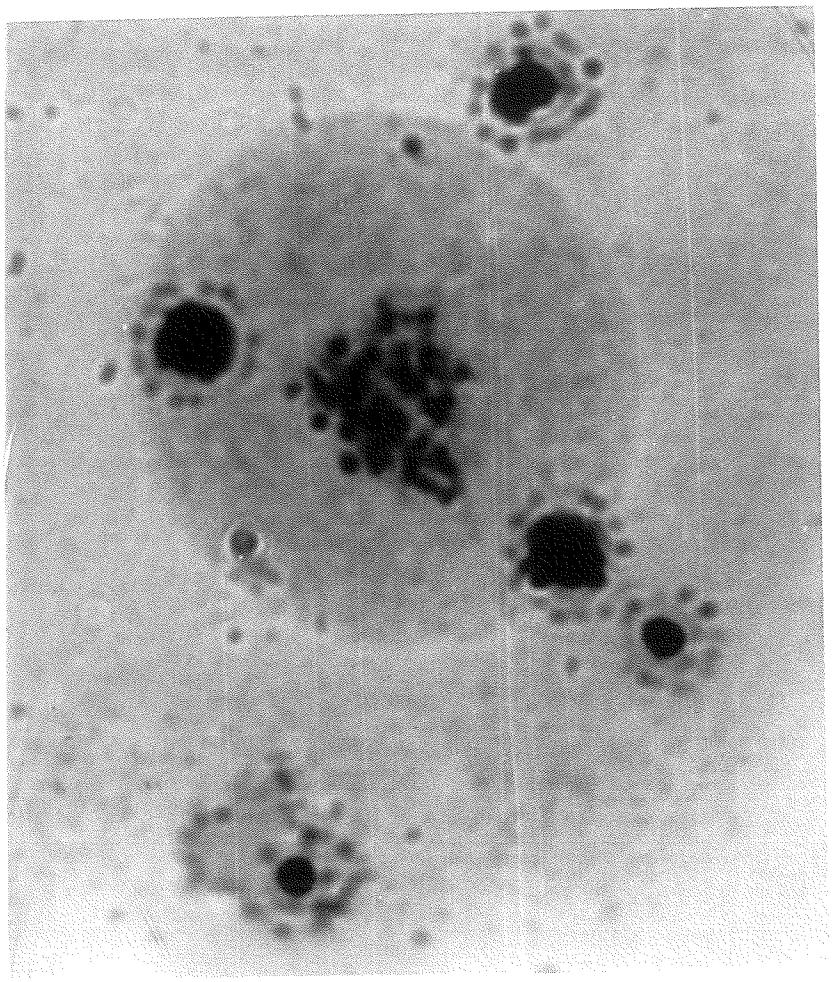


FIG. 10

B)

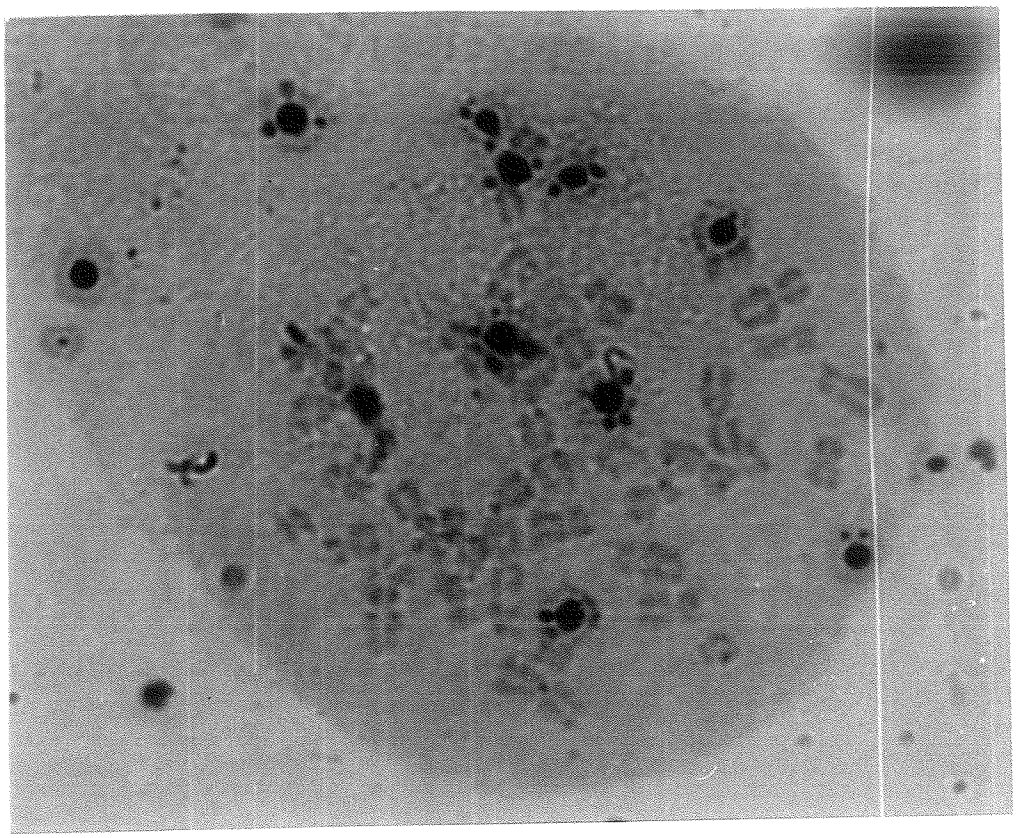
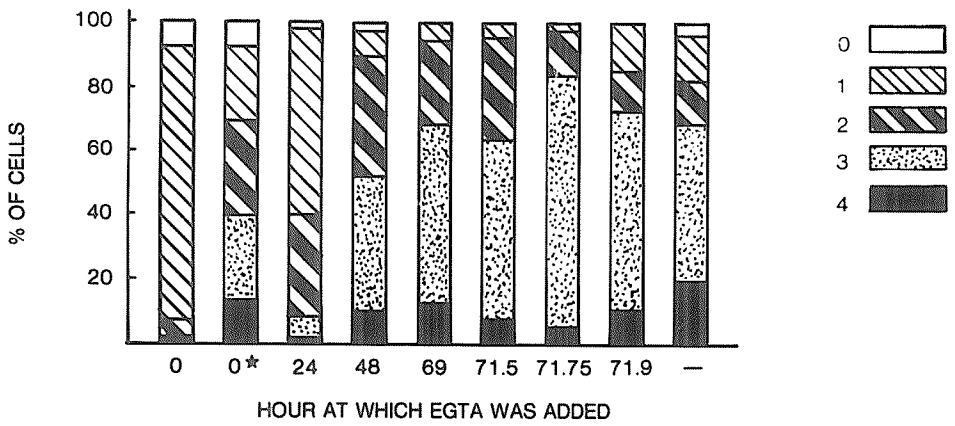


Fig. 11: Histograms showing changes in lymphocyte a) silver reactivity and b) nuclear diameter after various periods of treatment with 4.0 mM EGTA. All treatments were stimulated with PHA and harvested after 72 hours of culturing.

FIG. 11

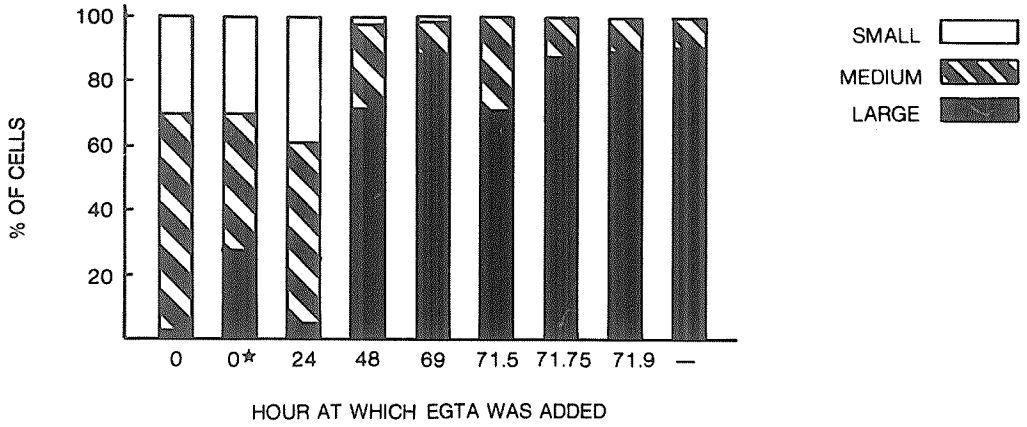
a) SILVER REACTIVITY

EGTA (4.0mM)



b) NUCLEUS DIAMETER

EGTA (4.0mM)



☆ 6.0 mM $CaCl_2$ WAS ADDED AFTER 24 HOURS FOR 48 HOURS .

Restoration of Ca^{++} in the form of $CaCl_2$, partially reversed the inhibitory effects of EGTA.

The addition of EGTA (4.0 mM) at the beginning of culturing completely inhibited PHA stimulation of nucleolar silver reactivity (Fig. 12a) and nuclear diameter (Fig. 13a). This inhibition was completely reversed when Ca^{++} was restored to the medium during the first 24 hours of culturing (Figs. 12b and 13b). After this critical period the effects of EGTA could not be reversed by the addition of $CaCl_2$.

Treatment of lymphocytes with EGTA, prior to the last 24 hours of culturing, prohibited mitosis, and therefore AgNORs were not observed (Table I).

Greatly increasing extracellular $CaCl_2$, from 1.8 mM to 7.8 mM, did not stimulate lymphocyte activity during a 72 hour culture period without PHA (Fig. 14).

The addition of calmodulin to cultured lymphocytes produced some inconsistent results (Figs.15, 16 and 17). Fig. 15 illustrates that the addition of 0.75 and 3.0 mg/ml calmodulin slightly stimulated nucleolar silver reactivity when compared with untreated, unstimulated controls (Fig. 15a). There was no parallel increase in nuclear diameter (Fig. 15b). Upon repetition, it was found that treatment of lymphocytes with calmodulin (3.0 µg/ml) for varying periods of culturing time, neither stimulated silver reactivity (Fig. 16) nor nuclear

Fig. 12: Histograms comparing changes in lymphocyte silver reactivity

a) at various times after culturing PHA stimulated cells in EGTA treated medium.

b) after culturing PHA stimulated cells in EGTA treated medium for 72 hours and adding $CaCl_2$ at various intervals.

c) at various times after culturing cells not stimulated with PHA.

d) at various times after culturing cells stimulated with PHA.

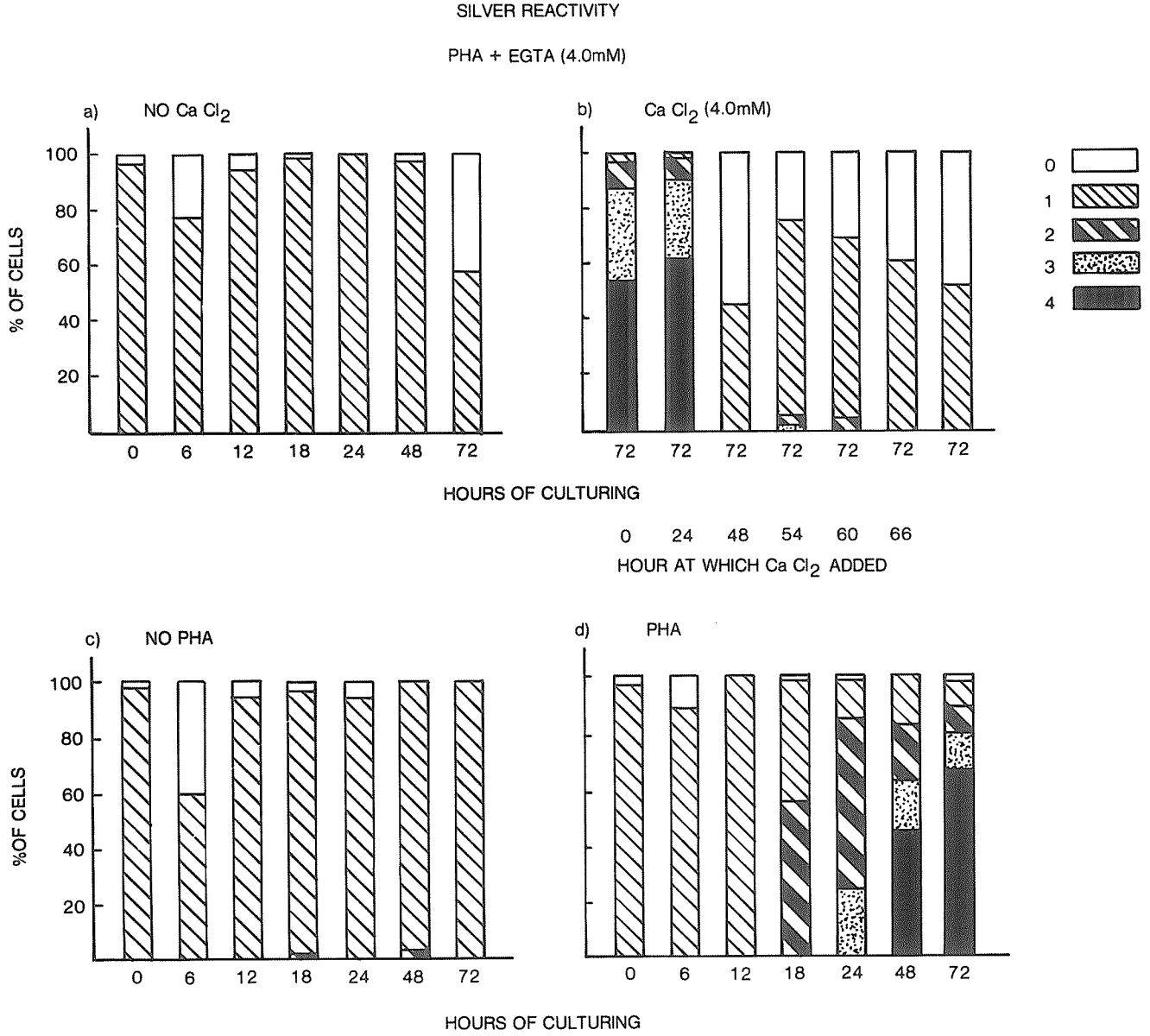

SILVER REACTIVITY
PHA + EGTA (4.0mM)
a) NO Ca Cl2
b) Ca Cl2 (4.0mM)
% OF CELLS
100
80
60
40
20
0 6 12 18 24 48 72
72 72 72 72 72 72 72
0
1
2
3
4
HOURS OF CULTURING
0 24 48 54 60 66
HOUR AT WHICH Ca Cl2 ADDED
c) NO PHA
d) PHA
%OF CELLS
100
80
60
40
20
0 6 12 18 24 48 72
0 6 12 18 24 48 72
HOURS OF CULTURING

Fig. 13: Histograms comparing changes in lymphocyte nuclear diameter

a) at various times after culturing PHA stimulated cells in EGTA treated medium.

b) after culturing PHA stimulated cells in EGTA treated medium for 72 hours and adding $CaCl_2$ at various intervals.

c) at various times after culturing cells not stimulated with PHA.

FIG. 13

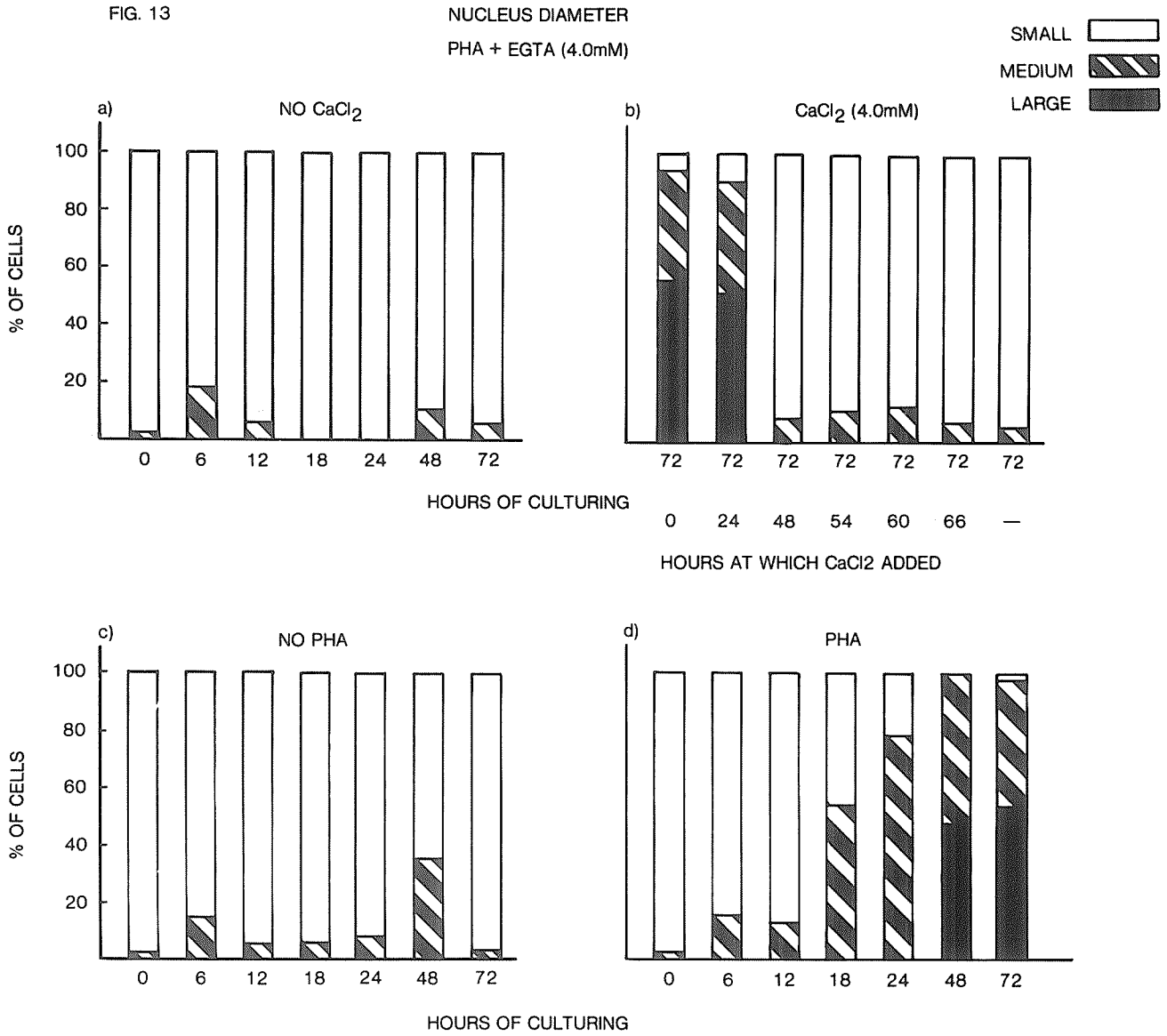

NUCLEUS DIAMETER
PHA + EGTA (4.0mM)
SMALL
MEDIUM
LARGE
a)
NO CaCl2
b)
CaCl2 (4.0mM)
% OF CELLS
100
80
60
40
20
0 6 12 18 24 48 72
72 72 72 72 72 72 72
HOURS OF CULTURING
0 24 48 54 60 66 —
HOURS AT WHICH CaCl2 ADDED
c)
NO PHA
d)
PHA
% OF CELLS
100
80
60
40
20
0 6 12 18 24 48 72
0 6 12 18 24 48 72
HOURS OF CULTURING

Fig. 14: Histograms showing lymphocyte silver reactivity and nuclear diameter with and without adding extra $CaCl_2$ to the culture medium. Untreated McCoy's medium contained 1.8 mM $CaCl_2$ and treated medium 7.8 mM $CaCl_2$. PHA was not added to the cultures. Cells were harvested after 72 hours of culturing.

FIG. 14

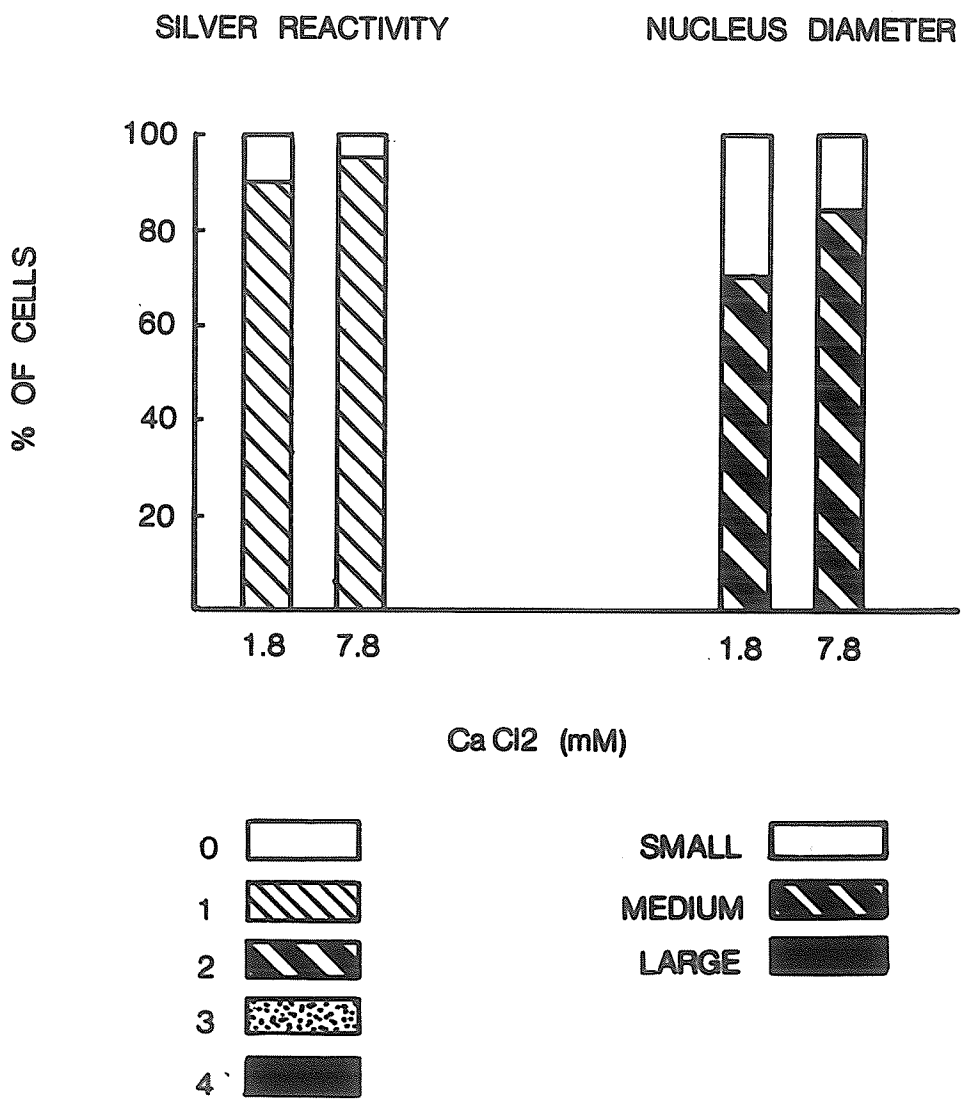

SILVER REACTIVITY
NUCLEUS DIAMETER
100
80
60
40
20
% OF CELLS
1.8
7.8
1.8
7.8
Ca Cl2 (mM)
0
1
2
3
4
SMALL
MEDIUM
LARGE

Fig. 15: Histograms showing lymphocyte
a) silver reactivity and b) nuclear diameter
after culturing cells for various periods of
time with and without calmodulin.

FIG. 15

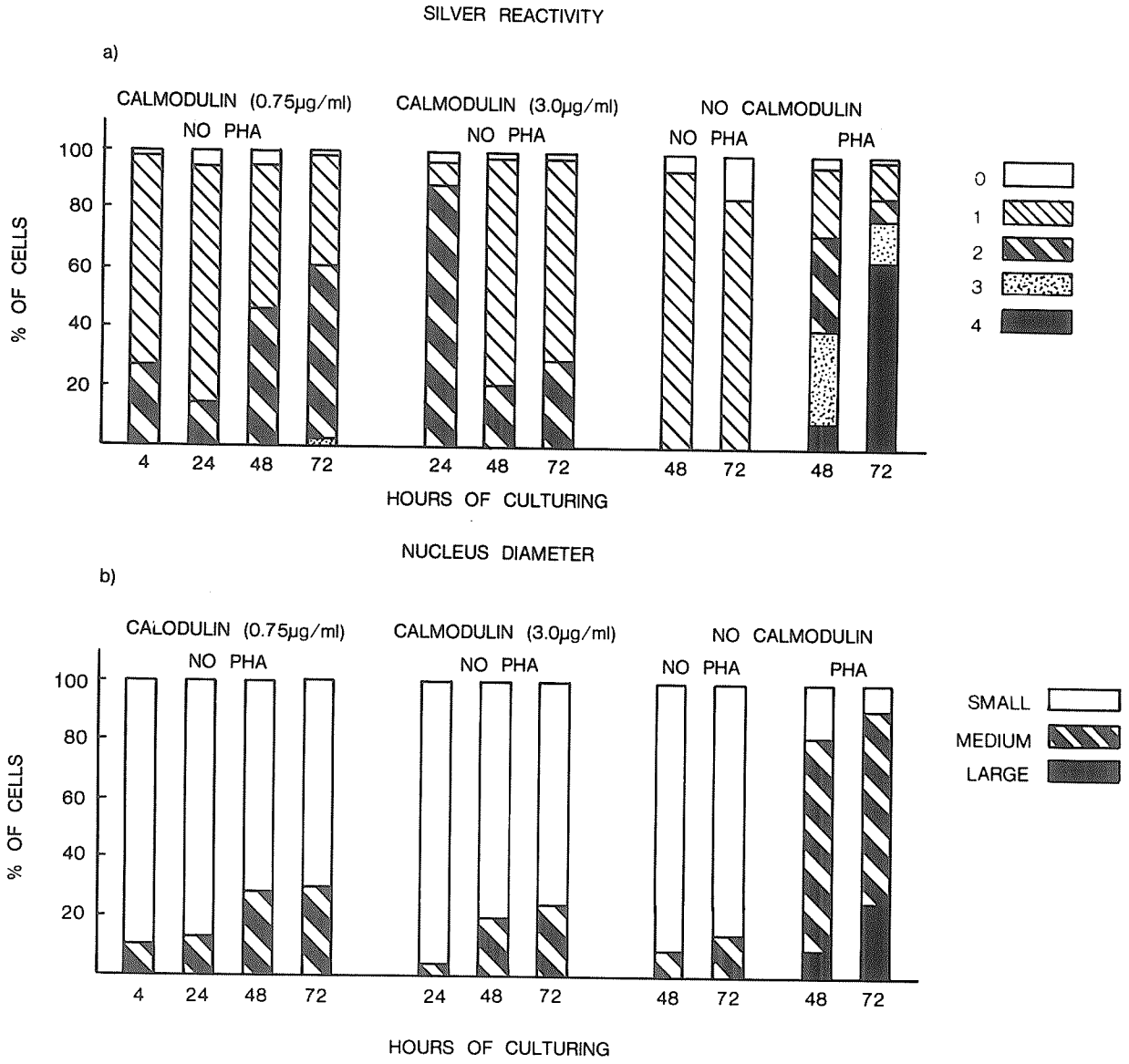
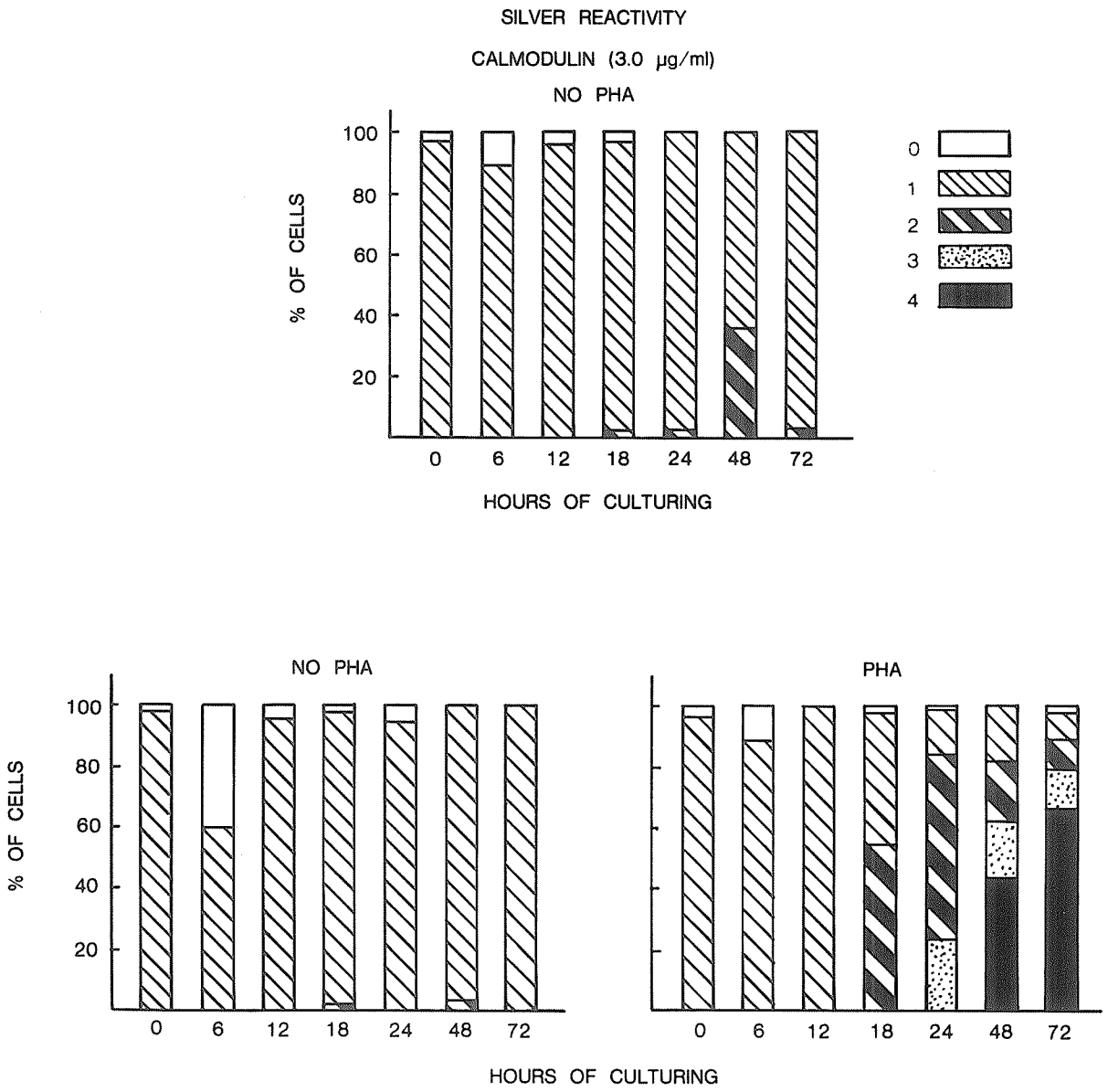

SILVER REACTIVITY
a)
CALMODULIN (0.75µg/ml)
NO PHA
CALMODULIN (3.0µg/ml)
NO PHA
NO CALMODULIN
NO PHA
PHA
% OF CELLS
100
80
60
40
20
4
24
48
72
24
48
72
48
72
48
72
HOURS OF CULTURING
0
1
2
3
4
NUCLEUS DIAMETER
b)
CALODULIN (0.75µg/ml)
NO PHA
CALMODULIN (3.0µg/ml)
NO PHA
NO CALMODULIN
NO PHA
PHA
% OF CELLS
100
80
60
40
20
4
24
48
72
24
48
72
48
72
48
72
HOURS OF CULTURING
SMALL
MEDIUM
LARGE

Fig. 16: Histograms showing lymphocyte silver reactivity after culturing cells for various periods of time with and without calmodulin.

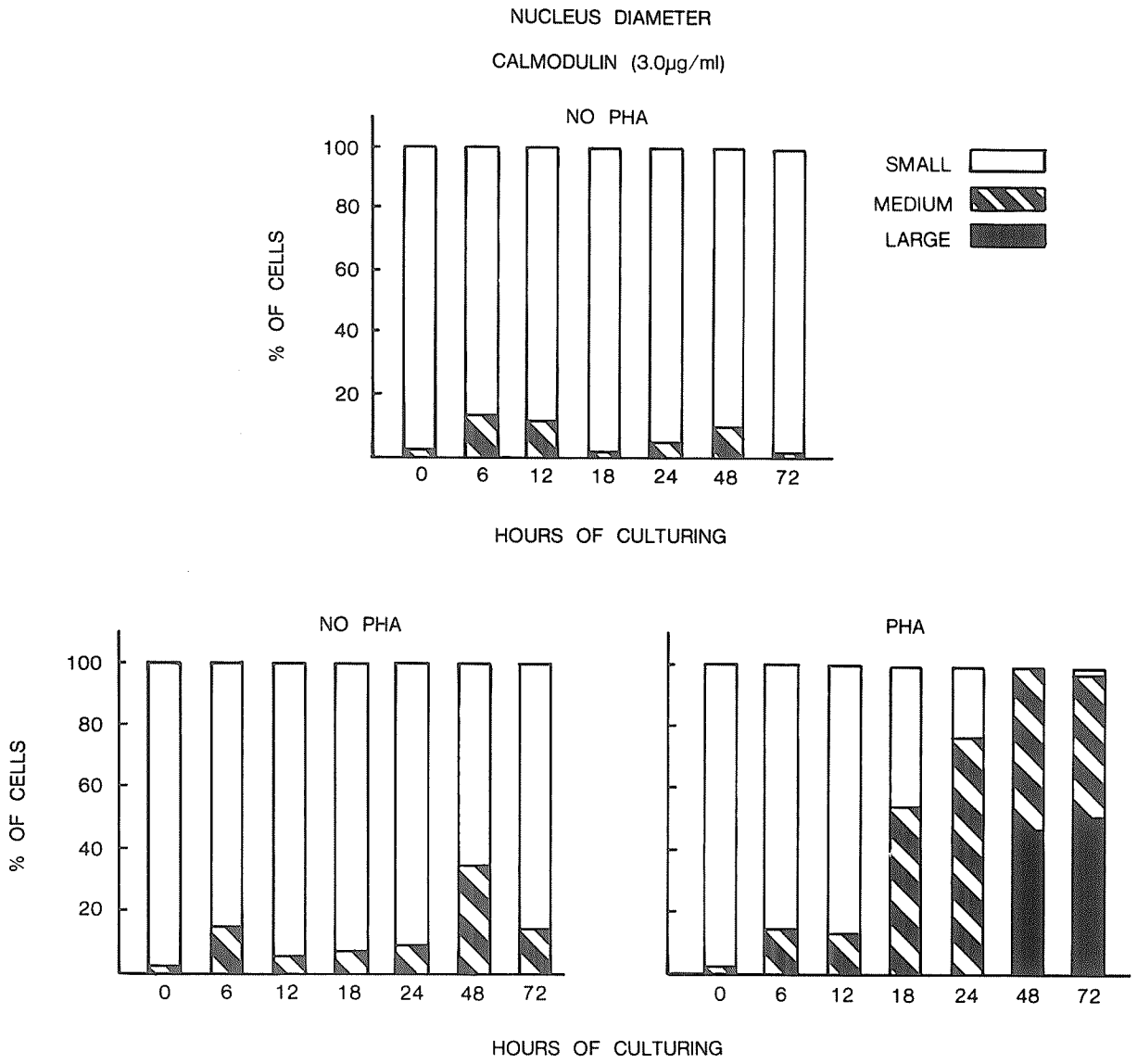
FIG. 16


SILVER REACTIVITY
CALMODULIN (3.0 µg/ml)
NO PHA
% OF CELLS
100
80
60
40
20
0
6
12
18
24
48
72
HOURS OF CULTURING
0
1
2
3
4
NO PHA
PHA
% OF CELLS
100
80
60
40
20
0
6
12
18
24
48
72
HOURS OF CULTURING

diameter (Fig. 17). Further experiments were prohibited by the limited quantity of calmodulin.

Fig. 17: Histograms showing lymphocyte nuclear diameter after culturing cells for various periods of time with and without calmodulin.

FIG. 17


NUCLEUS DIAMETER
CALMODULIN (3.0μg/ml)
NO PHA
% OF CELLS
100
80
60
40
20
0
6
12
18
24
48
72
SMALL
MEDIUM
LARGE
HOURS OF CULTURING
NO PHA
PHA
% OF CELLS
100
80
60
40
20
0
6
12
18
24
48
72
HOURS OF CULTURING

DISCUSSION

I. PARAMETERS OF LYMPHOCYTE ACTIVATION

Quantification of data was difficult. The silver nitrate method could not be completely standardized. Even within limited areas of a preparation, there were differences in intensity of the silver reaction. Metallic silver granules were deposited randomly over treated slides. Over treatment led to non-specific reactions, first within the nucleus and later over the entire slide.

Despite these problems, the size of the reactive area is a good indication of rRNA synthesis. There was almost precise (0.96 and 0.99) correlation between the ratio of silver reactive area to nucleus area and the rate of uridine incorporation (Hofgärtner et al., 1979). This correlation was quantified by cytophotometrically measuring the silver reactive area and total nuclear area, during preferential inhibition of rRNA with Actinomycin D. There was also good correlation between nuclear area and the area of silver reactivity, but not between nuclear area and the number of silver reactive areas (Hofgärtner et al., 1979).

In this study, silver reactivity was linearly related to the log of the nuclear diameter (Fig. 6). As silver reactivity increased, nuclear diameter tended to increase by 10-fold, indicating that the ratio of silver reactivity to nuclear diameter decreased in larger cells. Since the determination of silver reactivity was

subjectively evaluated and not based on accurate measurement of the silver reactive area, the apparent decrease in ratio is likely an artifact of subjectively categorizing silver reactivity into a limited number of categories. The important point to be noted is the correlation between silver reactivity, as measured in this experiment and nuclear diameter.

II. TRANSFORMATION

Unstimulated cultured lymphocytes were characterized by a small nuclear diameter and a small silver reactive area, neither of which increased with time (Figs. 3 & 5). After PHA stimulation, lymphocytes reenter the cell cycle, enlarge and the amount of silver reactivity increased (Figs. 4 & 5). This is comparible to the findings of Schwarzacher *et al.*, (1978) and Arrighi *et al.*(1980).

Schwarzacher *et al.* (1978) reported small, non-activated lymphocytes, in cultures 48 hours post PHA stimulation. These lymphocytes did not have silver reactive nucleoli. In this study, small non-activated lymphocytes were observed even 72 hours post PHA stimulation, but most had a small, distinct silver reactive area. Even unstimulated, uncultured peripheral lymphocytes (as represented by 0 hours of culturing, Fig. 5) nearly always had distinct silver reactive areas (Fig. 3).

Transformation with PHA did not induce uniform changes in the lymphocyte population. After stimulation, variability in lymphocyte size and silver reactivity was observed, even within one culture. This must reflect a difference in the rate of response and/or intensity of response to the same stimulus. Several environmental factors influence *in vitro* transformation. For example, the number of cells, duration and surface area of cultures, pH and the concentration of

stimulant affect transformation (Alford, 1970). Presumably these factors are relatively constant within a single culture, and yet great heterogeneity exists.

Morimoto and Wolff (1980) attributed this heterogeneity to variability in the times at which lymphocytes began to synthesis DNA (S-phase) in response to PHA. Cells have about the same generation time after entering their first S-phase. In a 64 hour PHA stimulated culture, 10% of the metaphases were in their first division, 40% in their second and 50% in their third. The first generation metaphase began DNA synthesis after 40 hours, while most of the third generation metaphases began at 24 to 32 hours. As silver reactivity and nucleus size are dependent on the cell cycle, all hetergeneity observed may be dependent on the variation in rate of response to PHA.

This does not eliminate the possibility that lymphocytes within a given population, also vary in how they respond. Purtell and Anthony (1975) found guinea pig lymphocytes had at least two rRNA maturation pathways, only one of which was stimulated by PHA. This indicates either all lymphocytes have the potential for both pathways, or that there are subpopulations of cells which may be differentiated by their response to PHA.

The peak period of increase of silver reactivity and nuclear diameter was 18 to 48 hours after the addition of PHA (Fig. 5).

This is similar to the findings of Arrighi et al. (1980) and is correlated with the period of increased rRNA and DNA synthesis. Lymphocyte rRNA synthesis peaks at about 18 hours (Purtell and Anthony, 1975) to 20 hours (Cooper, 1972) post PHA. DNA synthesis begins at 24 to 32 hours post stimulation with nearly 90% of cells having entered S-phase before 40 hours (Morimoto and Wolff, 1980).

III. DECREASING EXTRACELLULAR POTASSIUM

Decreasing extracellular K^+ from normal levels (5.0 mM) to 14% of normal (0.7 mM) did not affect the silver reactivity or nuclear diameter of PHA treated lymphocytes (Fig. 7), nor did it affect the number of AgNORs in metaphase cells (Table 1). A mean of 8.0 AgNORs/cell was observed in cells cultured in medium containing 0.7 mM K^+. This is comparable with the results of Ray and Pearson (1979) who reported a mean of 7.7, and Goodpasture et al. (1976) who reported a mean of 8.1.

External concentrations of K^+ are in dynamic equilibrium with internal levels. As external K^+ rises from zero, internal K^+ levels of human lymphocytes rise steeply in a sigmoidal fashion (Negendank and Shaller, 1979). At 5mM external K^+, internal K^+ is saturated at about 129 mM. At 0.7mM external K^+, internal K^+ is approximately 85 mM. Thus the 86% reduction in extracellular K^+ in this experiment, resulted in only an approximate 34% decrease in intracellular K^+.

Ledbetter and Lubin (1977) found that when intracellular K^+ levels of human fibroblasts fell below 60 to 80% of control levels, protein synthesis decreased in proportion to further reduction of K^+. Low cellular K^+ limits the transfer of amino acids from amino-acyl soluable RNA to the polypeptide during protein synthesis (Lubin and Ennis, 1964). The degree of K^+ reduction required to

inhibit growth or protein synthesis varies with the cell type.

Pollack and Fisher (1976) decreased extracellular K^+ of mammalian cell lines from 6 mM to 0 mM. Less than 2 mM K^+ reduced protein synthesis without affecting RNA synthesis. Cell division occured at 1.2 mM K^+. According to Negendank and Shaller (1979), 2 mM external K^+ resulted in near saturation of internal lymphocyte K^+ and thus the protein inhibition observed by Pollock and Fisher (1976) would not be expected. This incongruity may be explained by cellular differences in response to extracellular K^+.

Fluctuations as great as those tested in this experiment are not known to occur in vivo. K^+ influx doubles within minutes after PHA addition (Quastel and Kaplan, 1970b), but cellular K^+ concentrations remain constant due to an increased efflux of the ions (Hamilton and Kaplan, 1977). The approximate 34% decrease in cellular K^+ in this experiment was not sufficient to limit cell growth or division. Potassium is universally required by all cells, but it is unlikely that it is important in regulation of rRNA synthesis.

IV. DECREASING EXTRACELLULAR MAGNESIUM

Decreasing extracellular Mg^{++} from normal levels (1.0 mM) to 14% of normal (0.14 mM) did not affect the silver reactivity or nuclear diameter of PHA treated lymphocytes (Fig. 7), nor did it affect the number of AgNORs in metaphase cells (Table 1). The mean of 7.6 AgNORs/cell observed in cells cultured in Mg^{++} reduced medium (Table 1) is comparable to the results of Ray and Pearson (1979) who reported a mean of 7.7, and Goodpasture et al. (1976) who reported a mean of 8.1.

As intracellular and extracellular Mg^{++} remains relatively constant at about 1 mM (Bygrave, 1978b), further reduction of Mg^{++} level (below 0.14 mM), should not be necessary to demonstrate any physiologically feasible regulatory role that Mg^{++} might have. The lack of hormones and other mechanisms controlling Mg^{++} levels, further emphasizes the unlikelihood that Mg^{++} is important in the regulation of gene activity.

V. CALCIUM

A. Decreasing extracellular Ca^{++}

Chelation of extracellular Ca^{++} with EGTA early during activation, completely inhibited the increases in silver reactivity and nuclear diameter associated with PHA stimulation (Figs. 11, 12a, & 13a). EGTA could be added as late as 24 hours post PHA with resultant inhibition. This study confirms those of Alford (1970) and Whitney and Sutherland (1972) who found Ca^{++} to be essential for mitogenetic effects of PHA.

The inhibitory effects of EGTA were totally reversible if $CaCl_2$ was added to the medium within 24 hours of PHA stimulation (Figs. 12b & 13b). By 48 hours the effects were irreversible. Alford (1970) and Whitney and Sutherland (1972) also found the inhibitory effects to be reversible.

A slight decline in silver reactivity and nuclear diameter was observed when Ca^{++} was chelated during the last 24 hours of a 72 hour culture period with high concentrations of EGTA (Fig. 8). Since partial restoration of Ca^{++} partially reversed the inhibitory effects, this was not a toxic effect of EGTA. Possibly the reduced Ca^{++} levels inhibited stimulation of lymphocytes which were late to enter their first metaphase.

A mean of 7.5 AgNORs per cell occurred in cells treated with

1.8 mM EGTA for the last 24 hours of culturing (Table 1). This was comparable with control values and with values reported by Goodpasture et al. (1976). As this treatment also did not affect nucleolar silver reactivity and nuclear diameter, it is not surprising that the number of AgNORs was not affected. Longer treatment periods with EGTA prohibited mitosis and therefore NORs were not observed.

Whitney and Sutherland (1972) found that inhibition of DNA synthesis was not possible if EGTA was added later than 12 hours post PHA. As S-phase begins at about 24 hours post PHA, these authors concluded that EGTA does not directly inhibit DNA synthesis. The present study showed EGTA inhibits lymphocyte activation if it was added as late as 24 hours post PHA. This coincides with the beginning of S-phase.

If Ca^{++} had a direct effect on DNA or RNA synthesis, its removal prior to these events would result in inhibition of growth. As the lymphocyte cell cycle was found to be constant at 16 to 18 hours (Morimoto and Wolff, 1980), all cycling cells in a 72 hour culture would have entered their last S-phase at 52 to 54 hours. The addition of EGTA, 4 to 6 hours prior to this last S-phase did not inhibit lymphocyte stimulation. Once the cells have been stimulated to enter the dividing population, they are insensitive to EGTA.

Sufficient PHA to stimulate a full response binds to the lymphocyte membrane within minutes in an irreversible manner

(Whitney and Sutherland, 1972). As inhibition can occur as late as 24 hours post PHA, the essential need for Ca^{++} must not only be involved in the binding of PHA to the cell membrane.

Within minutes after the addition of PHA a surge of Ca^{++} moves into the cells. Whitfield et al. (1979) discounted this surge as being the stimulating factor for the following two reasons:

(i) Firstly, studies of Rosentreich and Blumenthal (1977) and Gemsa et al. (1979) showed that Ionophore A23187 did not produce a Ca^{++} influx surge and yet did stimulate DNA synthesis. According to Claret-Berthon et al. (1977), this ionophore initiates a surge of calcium ions and "non-exchangeable" calcium salts from the mitochondria. Mobilization of Ca^{++} from intracellular reservoirs may have the same effect as the Ca^{++} influx surge.

(ii) Secondly, if the surge initiates DNA synthesis, then adding EGTA after the surge should not have any effect on DNA synthesis. The addition of EGTA as late as 24 hours post PHA is still able to inhibit DNA synthesis. This does not prove that the surge is not essential for transformations. It does demonstrate that the active role of Ca^{++} in transformation is not finished immediately after the surge.

The Ca^{++} surge results in a cellular cAMP surge. Within 1 to 2 minutes post PHA, up to a 300% increase in cAMP occurred, which returned to control levels by 6 hours (Smith et al., 1971). High concentrations of exogenous cAMP can stimulate lymphoblast

Fig. 18: Possible explanation of the role of Ca^{++} in PHA stimulation of lymphocytes. PHA stimulates a Ca^{++} influx surge. This activates calmodulin (CDR) to bind with adenylate cyclase and stimulate an increase in production of endogenous cAMP. cAMP binds to and activates various protein kinases, some of which are membrane bound and may also be dependent upon calmodulin for activation. Activated kinases stimulate transformation via a phosphorylated product.

Based upon this model the possible mechanisms of EGTA action are (the numbers below correspond with the numbered locations on the diagram):

1) EGTA chelates extracellular Ca^{++} preventing the post PHA Ca^{++} influx surge and thus stimulation of rRNA and DNA synthesis.

2) EGTA chelates membrane associated Ca^{++}, inhibiting enzyme reactions leading to stimulation of rRNA and DNA synthesis.

3) If the cell membrane is disturbed allowing EGTA to enter the cell, intracellular Ca^{++} would be chelated and Ca^{++} dependent enzyme reactions inhibited. Nucleolar rRNA synthesis may be regulated by a Ca^{++} dependent enzyme(s).

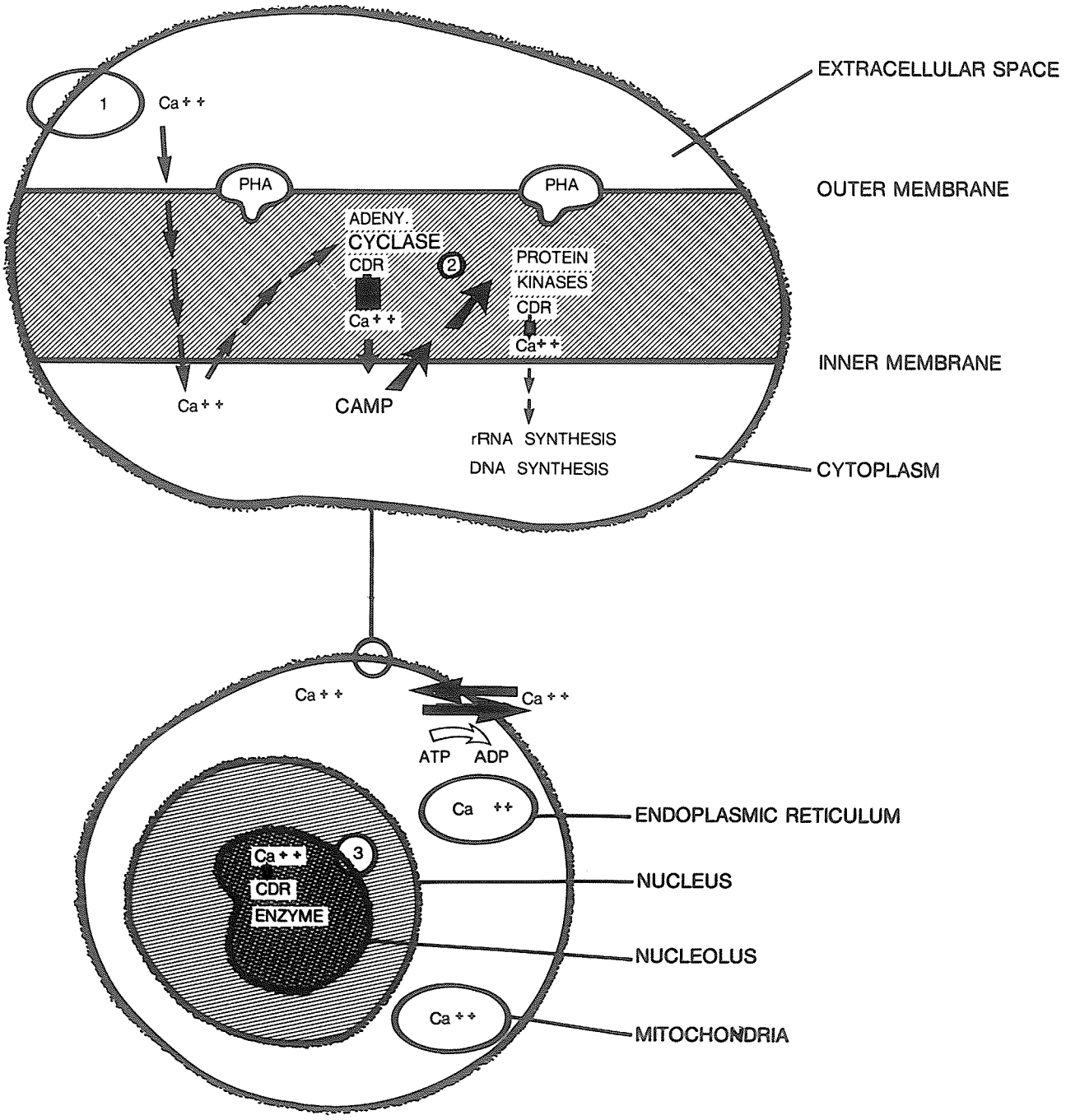

EXTRACELLULAR SPACE
1
Ca++
PHA
PHA
OUTER MEMBRANE
ADENY.
CYCLASE
CDR
2
PROTEIN
KINASES
CDR
Ca++
Ca++
Ca++
INNER MEMBRANE
CAMP
rRNA SYNTHESIS
DNA SYNTHESIS
CYTOPLASM
Ca++
Ca++
ATP
ADP
Ca ++
ENDOPLASMIC RETICULUM
Ca++
3
CDR
ENZYME
NUCLEUS
NUCLEOLUS
Ca++
MITOCHONDRIA

FIG. 18

proliferation (Whitfield et al., 1972).

EGTA displaces approximately 63% of total exchangeable Ca^{++} (Claret-Berthon et al., 1977). It does not penetrate the cell membrane, totally chelates the 1st compartment of rapidly exchangeable Ca^{++} (4 min.), and chelates 85% of the 3rd compartment of slowly exchangeable Ca^{++} (223 min.). This 3rd compartment is presumed to be Ca^{++} in more intimate contact with the membrane.

EGTA may inhibit lymphocyte transformation by chelating Ca^{++} bound to calmodulin associated with membrane bound adenylate cyclase and protein kinases (Fig. 18). The Ca^{++} surge activates calmodulin which binds with adenylate cyclase. Adenylate cyclase, when activated by calmodulin increases endogenous levels of cAMP. cAMP binds to and activates various protein kinases, some of which are membrane bound and may be also dependent upon calmodulin for activation. Whitfield et al. (1980) proposed that these activated kinases may stimulate transformation via a phosphorylated product.

According to this model the initial Ca^{++} surge is critical because it stimulates adenylate cyclase which in turn stimulates a chain of events leading to activation of proliferation genes. Ca^{++}, through its association with calmodulin, is also essential for one or more of the critical events leading to transformation. Therefore the addition of EGTA after the Ca^{++} surge would also inhibit transformation.

This hypothesis could be tested by adding lanthanum to PHA stimulated lymphocytes after the initial Ca^{++} surge. Lanthanum

specifically displaces the 1st compartment of rapidly exchangeable calcium, without disturbing other external pools (Claret-Berthon et al. 1977). Since it does not displace Ca^{++} which is in more intimate contact with the membrane, it would not be expected to inhibit transformation.

B. Increasing extracellular Ca^{++}

Increasing extracellular $CaCl_2$ from 1.8 mM to 7.8 mM did not stimulate lymphocyte activity during a 72 hour culture period without PHA (Fig. 14). Anghileri and Heidbreder (1978) found that increasing extracellular Ca^{++} to 10 to 50 mM stimulated DNA, RNA, and protein synthesis in liver cells. Yang and Morton (1971) found Ca^{++} and Mg^{++} stimulated mitotic activity and DNA synthesis in cells of various differentiated systems. Whitfield et al. (1969b) found that increasing Ca^{++} to 1.2 mM stimulated mitotic activity of rat thymocytes. Whitfield et al. (1979) found that suddenly raising extracellular Ca^{++} of fetal mouse cells from 1.8 mM to 5.0 mM, stimulated 20 to 60% of cells to resume cycling and initiate DNA synthesis. The authors considered this a meaningless artifact of a physiologically improbable Ca^{++} shock.

Rapidly responding thymic lymphocytes, which includes 10-15% of the thymic population, are proliferatively activated but require exogenous stimulation to initiate DNA synthesis (Whitfield et al. 1980). Raising extracellular Ca^{++} from 1.0 mM to 1.5 mM directly

initiated DNA synthesis. Circulating peripheral lymphocytes are not proliferatively activated and cannot be activated by increasing exogenous Ca^{++} alone. Additional membrane stimulation is required.

C. Increasing exogenous calmodulin

The addition of exogenous calmodulin did not significantly activate the proliferative genes of lymphocytes (Figs. 15, 16, & 17). A slight increase in nucleolar silver reactivity was observed in comparison to untreated, unstimulated controls (Fig. 15a), but this was not sufficient to induce mitosis. As synthesis of the protein would inhibit the speed of response to stimulation, it is unlikely that the quantity of calmodulin would be a rare limiting factor in regulating rRNA and DNA synthesis. It is more likely that an excess of calmodulin is activated by Ca^{++} surges to stimulate transformation.

VI. SIGNIFICANCE OF SILVER NITRATE REACTIVITY

Histochemical studies on the nature of silver reactivity have demonstrated that interactions of silver ions with the carboxyl group of acidic proteins, involved in rRNA transcription, are primarily responsible for the reaction (Olert et al., 1979). Esterification of carboxyl groups inhibits the reaction. Reactive protein is located in the fibrillar region of the nucleolus, not in the granular region. Thus it is not part of the RNP complex (Hubbell et al., 1979), but it is strongly correlated with rRNA synthesis (Hofgärtner, 1979).

Harper et al. (1980) reported labelling of the nucleus of rat liver and adrenal cortex cells with antibody for calmodulin in a pattern consistent with nucleolar organization. The amount of labelling increased with hormonal activity. Thus it appears that calmodulin localizes in the nucleolus, in response to appropriate stimulation.

Calmodulin is characterized by thermal stability and abundant acidic amino acids which furnish carboxylate groups for the reversible binding of Ca^{++} (Cheung, 1980). These characteristics match with the requirements for the silver reactive protein. The silver nitrate reaction was stable at temperatures in the range of 60-70 ^{0}C (personal observations). The carboxylate groups provide binding sites for silver ions.

Most regulatory roles previously attributed to calcium ions, require its association with calmodulin (Means and Dedman, 1980). When calmodulin binds Ca^{++} it becomes an active more helical molecule (Liu and Cheung, 1976; Dedman et al., 1977), which binds to an apoenzyme to form a holoenzyme. Such a holoenzyme may govern the rate of rRNA synthesis and be responsible for reducing colourless silver nitrate to black metallic silver.

VII. SIGNIFICANCE OF Ca^{++}

Ca^{++} is essential for activation of the genetic information necessary for proliferation of lymphocytes *in vitro*. Chelatin of Ca^{++} with EGTA during the first 24 hours post PHA addition inhibited transformation. The molecular basis of Ca^{++} regulation of proliferation is hypothetical, but it is likely that Ca^{++} activates calmodulin, which in turn activates enzymes stimulating cAMP, rRNA and DNA synthesis (Fig. 18). Circumstantial evidence supports the hypothesis that calmodulin may be the nucleolar protein which reacts with silver nitrate.

Ca^{++} is an important *in vivo* regulator of cell proliferation. Lowering Ca^{++} by removal of the parathyroid and low dietary intake, reversibly reduced cell proliferation in the bone marrow and thymus of rats (Whitfield *et al.*, 1973). Most cellular responses to stimuli are mediated by Ca^{++} fluxes. Cell proliferation is one of these. Cells from one culture group, treated with 1.8 mM EGTA for the last 24 hours of 72 hour culturing period, had silver reactive spheres, which were dissociated from the nucleolus or NOR of metaphase chromosomes (Figs. 9 & 10). The spheres were surrounded by a ring of satellite bodies composed of RNP-like material. This dissociation may be more than an artifact, even though the experimental conditions causing it were not defined.

Possibly intracellular Ca^{++} was disturbed by EGTA, causing

chelation of nuclear Ca^{++} and inactivation of nuclear calmodulin (Fig. 18). The inactivated calmodulin could then have dissociated from its active site, pulling RNP particles with it. This could implicate nuclear Ca^{++} levels in the regulation of RNP transportation, in addition to regulation of rRNA synthesis.

Unusual metabolic patterns and behaviour of aberrant cells may be related to altered distributions of Ca^{++} within the cells. Ca^{++} uptake of erythrocytes from cystic fibrosis patients was significantly reduced, due to decreased Mg^{++}-and Ca^{++}-ATPase activity (Ansah and Katz, 1980). Tumor cells invariably have low cytoplasmic Ca^{++}, and high levels in the mitochondria, indicating a lack of control of Ca^{++} cycling (Bygrave, 1978b). During transformation of a normal cell to a neoplastic cell there is often an increase in size and number of nucleoli (Busch and Smetana, 1970), which may be related to the altered Ca^{++} distribution. Control of rRNA synthesis is important in inhibition of tumor growth.

Because Ca^{++} responds to and induces a wide range of events, understanding the regulation of the ionic environment is fundamental to understanding the functioning of normal and abnormal cells.

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