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THE INFLUENCE OF CALCIUM IONS AND CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE ON LYMPHOCYTE TRANSFORMATION IN VITRO

by

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Department of Biophysics

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
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ABSTRACT

Lymphocytes, which are normally non-dividing cells, can be stimulated into an active cell cycle both in vivo and in vitro by antigens, allogeneic cells and a variety of plant derivatives including phytohemagglutinin (PHA). This transformation phenomenon is extremely important physiologically since it is an essential step in most immunological processes. However, the mechanism of the process is poorly understood.

The initial purpose of the present studies was to ascertain the possible role(s) of calcium ions (Ca++) and cyclic adenosine 3',5'-monophosphate (cAMP) in the mediation of stimulation of lymphocytes by PHA and allogeneic lymphocytes. Further studies were aimed at elucidating the mechanism of calcium transport by lymphocytes and how PHA stimulated it.

Measurements of RNA and DNA synthesis, morphological transformation, and calcium and amino acid transport were used to assess the response. The specific Ca++ requirements of stimulated lymphocytes were studied using EGTA, a chelator with an affinity for Ca++ approximately 10^5 times greater than its affinity for magnesium ions (Mg++).

It was found that transformation by either PHA or allogeneic lymphocytes did not occur in the absence of Ca++. Other divalent cations including Mg++ could not
substitute for Ca$^{++}$ in the PHA response. Further they were only required for the initiation of the response since removing them after DNA synthesis was initiated had no effect on the subsequent response. Ca$^{++}$ were also required for the early initiation of RNA synthesis as well as amino acid transport.

The uptake of Ca$^{++}$ in resting lymphocytes was found to be a saturable process which was competitively inhibited by manganese ions indicating that uptake was mediated by a membrane carrier. However, it was also characterized by a low temperature coefficient and little or no sensitivity to metabolic inhibitors indicating that uptake was an energy-independent process.

PHA caused a large increase in Ca$^{++}$ uptake which was detectable within one minute after exposure. The increased uptake was a result of an increase in the affinity of the membrane sites for Ca$^{++}$ rather than to an increase in the number of sites or an increased rate of transport by the available sites. Exchange diffusion did not occur in either resting or stimulated lymphocytes, and PHA did not affect Ca$^{++}$ efflux.

Cyclic AMP and agents which influence intracellular cAMP metabolism had no effect on either resting or PHA-stimulated lymphocytes at physiological concentrations. However, they inhibited the PHA response at higher concentrations. This inhibition may be due to an inhibition of essential membrane transport since all these agents
inhibited both calcium and amino acid uptake in PHA-
stimulated cells.
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I would like to express my gratitude to my advisor, Dr. Robert Sutherland, for his continued inspiration and guidance during the course of this work. Thanks are also due Dr. E.L. Medzon and Dr. I.G. Walker who acted as my advisory committee and Dr. J. MacDonald who did the cyclic AMP measurements.

I also would like to thank my parents for their unfailing encouragement and support and my wife, Mary, for typing this thesis in addition to her constant support.

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1. INTRODUCTION

1.1 The Physiological Significance of the Lymphocyte

During the course of evolution higher organisms have developed elaborate mechanisms for protecting their internal environment from invasion by foreign substances. These defense mechanisms are found in their most complex forms in man, and it is now clear that the majority of the functions of the immune system are mediated to a great extent by one cell type, the lymphocyte.

Until as recently as about fifteen years ago the cell identified morphologically as a small lymphocyte was generally believed to be an end-stage cell with no significant biological function (1). However, since then numerous immunological phenomena such as allograft rejection (2, 3), antibody formation (4, 5), delayed hypersensitivity (6, 7), and graft-versus-host reactions (8, 9) have been shown to be mediated mainly by this cell type. In conjunction with these roles it has been unequivocally demonstrated that the key immunological properties of antigen recognition (10, 11, 12) and memory (13, 14) reside in the lymphocyte. It has also become clear that this population of cells is functionally heterogeneous, and two major subpopulations, which are themselves heterogeneous, have been identified. One consists of cells which develop in the thymus gland (15)
and the other is comprised of cells of indeterminate source (16) but which are the cells that manufacture and secrete immunoglobulins (17).

1.2 Characteristics of the Resting Lymphocyte and Its Inherent Proliferative Potential

The small lymphocyte is normally a non-proliferating cell exhibiting minimal metabolic activity (18). Its morphological appearance is characteristic of a non-dividing cell with its small size (7-9 µ in diameter), condensed nuclear chromatin and cytoplasm containing only a few mitochondria and ribosomes and no endoplasmic reticulum (19). However, it is now realized that the lymphocyte can be forced out of its resting state both in vivo and in vitro by a large number of stimuli, and that this shift to an active state is a required prelude to many of its final immunological functions.

This phenomenon of lymphocyte transformation was first observed in vitro by Nowell in 1960 (20). He found that when lymphocytes were exposed to phytohemagglutinin (PHA), a mucoprotein extract of the red kidney bean (Phaseolus vulgaris), a significant proportion of the original small lymphocytes transformed to large immature cell types after several days in culture. It was subsequently demonstrated that this process also occurred in vivo mainly in the lymph nodes and spleen in response
to antigens (21, 22). These observations coupled with the findings that lymphocytes continuously recirculate between the blood and lymphatic system (23), and that some of these cells have a long life span (24), firmly established that they are not functionless, end-stage cells and provided the impetus for the subsequent discoveries of their many biological functions mentioned previously.

1.3 Research Interest in the Transformation Process

The process of *in vitro* lymphocyte transformation has been studied extensively during the past decade for a number of reasons. The *in vitro* system provides an excellent simplified model of the *in vivo* transformation response greatly facilitating investigations of the transformation mechanism. Also the fact that the lymphocyte is a differentiated resting cell which still retains its ability to proliferate under the appropriate circumstances offers the possibility that transformation studies may provide insight into the mechanism by which normally non-cycling cells are stimulated into a rapidly proliferating state such as occurs in wound healing, tissue regeneration and in certain types of malignancy. Further the observation that transformation is depressed in certain diseases associated with the lymphatic system (25, 26) has stimulated clinical studies aimed
at developing the in vitro reaction as a diagnostic or prognostic tool. Finally, studies of human chromosomes have been advanced by the ready availability of large numbers of cells undergoing mitosis (27).

1.4 Classification of Lymphocyte Stimulants

A wide variety of stimulants have been used to activate lymphocytes in vitro. They are generally divided into two main groups. One group consists of agents referred to as specific stimulants and includes protein antigens from protozoa, fungi, viruses and bacteria (28) of which tuberculin purified protein derivative (PPD) is the common example. Other proteins such as purified histocompatibility antigens (29) and synthetic polypeptides of strictly determined structure (30) have also been employed. The other group is comprised of agents which are termed non-specific stimulants for reasons to be described shortly. The most commonly used of these substances is PHA but other plant extracts such as concanavalin A (31), pokeweed mitogen (32) and lens culinaris mitogen (33) are also effective stimulants. Other diverse factors which affect cell membranes can induce varying degrees of non-specific transformation, and they include antigen-antibody complexes (34), proteolytic enzymes (35), mercury (36), zinc (37) and ultrasonic treatment (38). Stimulation also occurs in cultures
containing lymphocytes from two or more genetically unrelated donors, the mixed lymphocyte culture reaction (MLR) (39, 40). The degree of transformation induced by the various agents is normally quantitated by measurement of the extent of incorporation of radioactive precursors into nucleic acids particularly DNA and/or by counting the percentage of morphologically transformed cells in an appropriate smear.

Although the biochemical and morphological changes associated with activation appear to be essentially similar in cells exposed to agents from both groups, several important distinctions must be recognized. Stimulation by specific agents occurs only when the lymphocyte donor has been pre-sensitized to that molecule in vivo while stimulation by non-specific agents requires no such prior exposure (28). Additionally, the response to the non-specific plant derivatives occurs much more rapidly than does the corresponding specific response with DNA synthesis reaching a maximum by about three days after treatment (41, 42, 43) in contrast to the six days normally required to obtain maximal rates of DNA synthesis in specific antigen cultures (28). Also the absolute response observed is much greater in the former case with about 40-90% of the cells responding depending on the criteria used in declaring a cell transformed (44, 45), compared to the calculated 2% involved in antigenic stimulation (28). The mixed lymphocyte reaction
is considered to be a specific immunological response to foreign histocompatibility antigens, but prior exposure to the stimulating cellular antigens is not necessary (46, 47). The advantages of the non-specific stimulants is that the response develops more quickly and to a far greater extent make them ideal for studying early events in the activation process which are not yet detectable by present methods in the other weaker responses. PHA is the most commonly used stimulant, and further discussion will refer to data obtained from studies with this mitogen.

1.5 PHA Interactions with the Cell Surface and Membrane Receptors

Despite the recent voluminous research on lymphocyte transformation the actual mechanism of activation is still poorly understood. However, it is now generally accepted that the initial event involves an interaction of the stimulant with membrane receptors. Although some early reports claimed that PHA was taken up by lymphocytes (48, 49), no correlation between uptake and stimulation could be shown (48). More recent work has demonstrated that stimulation is achieved under conditions which block endocytosis, and also that mitogenically active PHA remains on cell surfaces for days (50). Furthermore, the PHA response can be inhibited by fetuin,
a fetal calf serum derivative, which removes PHA from the cell surface (51) so it is most likely that transformation is initiated by a surface rather than an intracellular reaction.

It is known that antigens bind to specific immunoglobulin receptors on the lymphocyte surface (52), but the nature of the PHA binding sites is not clearly defined. It has been shown that the PHA receptors are sensitive to trypsin and neuraminidase (53) suggesting that surface proteins and sialic acid are involved in forming the receptor site. The erythrocyte receptor for PHA has been isolated and was found to be a glycopeptide (54). The binding specificity was related to the oligosaccharide portion of the molecule with galactose and mannose residues being the critical moieties (54). It seems reasonable to expect the lymphocyte receptor to be similar.

There is evidence that the sugar portion of the PHA molecule is not involved in binding (53), and since PHA has no sulfur-containing amino acids (55), sulfhydryl groups are also not involved. However, the binding reaction is certainly more than a simple adsorption since the residual binding of PHA to washed lymphocytes is temperature, time and concentration dependent (53). It must be noted here that the erythro- and leuco-agglutinating properties of PHA associated with surface binding are not essential to the induction of transformation
since removal of the agglutinating factors does not alter the mitogenic properties of the molecule (56).

1.6 Biochemical and Cellular Alterations Induced by PHA

Many biochemical and cellular alterations occur after the initial membrane interaction with PHA. However, it is not yet known which ones are crucial to the induction of the response and which ones are just secondary effects possibly involved in the maintenance of the response once it is underway. The earliest event reported to date which was discovered during the course of the present work is an activation of the membrane-associated enzyme, adenylate cyclase (57, 58), with consequent increased production of cyclic adenosine 3',5'-monophosphate (cAMP) from adenosine 5'-triphosphate (ATP) which reportedly occurs within 1 to 2 minutes (59). Possibly associated with this observation is the recent finding that there is an immediate increase in the size and specific activity of the intracellular inorganic phosphate pool following PHA treatment (60). The intracellular level of cAMP then rises to a maximum at 1 hour before declining to below control levels by 6 hours (59). Killander and Rigler found that PHA caused a 3-fold increase in the binding of acridine orange to phosphate groups of deoxyribonucleoprotein within 10 minutes (61), and it was also reported very recently that there is an
increased incorporation of long chain fatty acids into lecithin within 10 minutes (62). Increased phosphorylation of nuclear protein (63) and increased acetylation of arginine-rich histones (64) are observed within 15 minutes after PHA treatment.

The rate of RNA synthesis is also increased very early in the response perhaps by as early as 15 minutes (64, 65, 66). The early RNA is predominantly a low molecular weight species which is probably a precursor to transfer RNA since it appears in the cytoplasm rapidly (67). This increase could be due, at least in part, to an alteration of the cellular uridine monophosphate (UMP) pool resulting from an increased uridine kinase activity, but more recent studies indicate that uridine incorporation into RNA precedes any rise in uridine kinase activity (68, 69, 70). Later dramatic increases in RNA synthesis mainly involve precursors of ribosomal RNA (71). The RNA polymerase content of nuclei does not increase until 6 to 12 hours after PHA addition (72) so a sufficient quantity must be available to permit the observed stimulation of RNA production.

An increased turnover of membrane phospholipid with enhanced incorporation of phosphate into phosphatidyl inositol is readily observed after 30 minutes (73) as is an increased rate of uptake of the non-utilizable amino acid, α-aminoisobutyric acid (51). Another interesting finding is that the acid hydrolases, β-glucuronidase and
acid phosphatase, are released from lysosome-like granules from 30 to 120 minutes after PHA treatment (74, 75). Potassium accumulation is also stimulated by PHA although significant increases only occur after 1 hour (76). Protein synthesis is also increased fairly early in the response at about 2 hours (77). It is important to note that cycloheximide, an inhibitor of protein synthesis, does not inhibit early RNA synthesis or any of the other early events indicating the existence of certain preformed precursors necessary to initiate the response (71). Carbohydrate metabolism by several metabolic pathways also increases after PHA stimulation (78, 79). No stimulation of DNA synthesis occurs until about 18 to 24 hours after stimulation (41, 42, 43) at which time a corresponding increase in DNA polymerase activity occurs (50, 80). However, DNA synthesis will not begin unless ribosome accumulation has proceeded unimpeded for at least 18 hours (71). DNA synthesis then increases progressively until it reaches a maximum level at about 72 hours (41, 42, 43). RNA and protein synthesis increase progressively from the time they begin to maximum levels at about 48 hours (66, 81). The final event of transformation, mitosis, does not normally occur before 72 hours (82).

Certain characteristic morphological changes accompany these biochemical and cellular alterations although they do not become apparent immediately. The
nucleolus enlarges and the chromatin becomes less condensed (83). The cytoplasm increases in size, and intense ribosome proliferation occurs (84) with subsequent aggregation to form polysomes (85). Additionally, the mitochondria enlarge and the number of lysosome-like bodies increases (85). Some cells may reach a size of 30 μ in diameter (84). Few fully-developed blast cells are observed until the latter part of the second day in culture (82).

1.7 Lymphocyte Membrane Properties

The preceding discussion revealed that the lymphocyte membrane has more than a passive role in the induction of transformation, and that extensive modifications of membrane structure and function occur rapidly after the initial binding reaction. This fits in well with the growing interest in the cell membrane as a regulator of cell division (86). It is now known that membrane properties including transport phenomena change throughout the cell cycle (87, 88), and also that the surfaces of virally-transformed malignant cells are markedly different from their normal counterparts (89, 90).

The molecular composition of the lymphocyte membrane is not yet well-known due to former difficulties in separating them completely from other blood cells.
However, the presence of adenylate cyclase is certain (59), and since ouabain inhibits transformation, a Na\(^+\)-K\(^+\)-ATPase is very likely (91). Common membrane lipids are also found (73) so in all probability their membrane molecular composition is similar to that of other cells.

Considerably more is known about the external surface of the lymphocyte membrane. Like other mammalian cells, they have a carbohydrate-rich cell-coat containing acidic-residues (92) giving the cell a net negative surface charge. The major part of the negativity is due to the ionized carboxyl groups of N-acetyl neuraminic acid (93), but phosphate groups of membrane-bound RNA (94) and carboxyl groups of surface proteins (95) also contribute. Positively charged sulfhydryl and amino groups are also present (96, 97). A unique feature of the lymphocyte surface is the presence of immunoglobulin molecules (52) which are often grouped on a large projection of the cell termed a uropod (98). All these surface moieties contribute in some unknown manner to the formation of a mosaic of surface receptors for various exogenous agents which can stimulate the cell into an active, proliferative state.

1.8 Physiological Functions of Calcium

One factor which is intimately involved in membrane structure and function, and which also has many diverse
intracellular functions is calcium. Calcium ions (Ca\(^{++}\)) bind to mucopolysaccharides (99) and lipids (100) which are integral components of the membrane. They also form a stable chelate with ATP, and this complex may also have structural and functional significance in the membrane (101). Further it has been reported that as much as 87% of cellular calcium may be associated with the cell coat (102). The well-established importance of Ca\(^{++}\) in cellular adhesion (103) and cell-cell interactions (104) are undoubtedly functions of this extracellular calcium. Alterations in membrane binding of Ca\(^{++}\) can influence permeability to water (105), cations (106) and proteins (107) possibly by inducing conformational changes in membrane proteins (108). Calcium ions can also alter the membrane potential (109) as well as membrane deformability (110).

Calcium's various intracellular roles are not clearly defined, but it can affect the activities of a wide variety of enzymes (111). They also bind to nucleo-proteins (112) and mediate endoplasmic viscosity changes which occur prior to mitosis (113).

The importance of Ca\(^{++}\) in regulating major biological processes in which the above specific known functions are undoubtedly involved is clearly recognized. However, their exact mode(s) of influence is still poorly understood. The most widely accepted role of Ca\(^{++}\) is as a coupling factor between excitation and contrac-
tion in muscle and excitation and secretion at nerve endings (114). They also are crucial to the complex process of blood clotting (115). Further there is a great deal of evidence now available which indicates that calcium is a cofactor in the mechanism of many cAMP-mediated hormones (116).

Possibly relevant to the process of lymphocyte transformation is the work of Whitfield, Rixon and associates regarding the role of Ca^{++} in regulating cell proliferation. They have clearly demonstrated that Ca^{++} can stimulate mitotic activity in rat thymocyte and bone marrow populations which are already proliferating both in vitro and in vivo (117, 118, 119, 120). Also they have shown that Ca^{++} can enhance recovery of lethally irradiated animals (121, 122), and that the mitogenic actions of some hormones and other agents such as bradykinin and detergents all act through a similar Ca^{++}-mediated mechanism (123, 124).

1.9 Summary of Motivation for the Present Investigations

The lymphocyte is the most ubiquitous cell in the body. It is a multipotential cell, and its normal functioning is essential to the maintenance of the integrity of the immune system. The phenomenon of transformation is the common feature linking most of the diverse final events mediated by the lymphocyte. Thus, understanding
the mechanism of transformation is of top priority to the field of immunology. Also insight may be gained as to how normally resting cells of different types may be induced to enter an active cell cycle.

In view of the importance of the cell membrane in lymphocyte activation, the intimate relation of $\text{Ca}^{++}$ to membrane function, and the critical role of $\text{Ca}^{++}$ in regulating cell division in bone marrow and thymus cells the present studies were begun with the aim of determining the influence of $\text{Ca}^{++}$ on transformation.
2. MATERIALS AND METHODS

2.1 Introduction

The techniques for culturing lymphocytes are relatively simple and are similar to those used for other cell types although lymphocytes are not generally grown on surfaces since they do not attach to glass or plastics. However, there are many culture variables which must be standardized if reproducible results are to be obtained in transformation studies. A good review of many of the critical factors has been presented by Ling (125). Several of the most important ones are type of serum, cell concentration, shape of culture chamber, gas phase and method of cell separation. The methods which were used in these studies were a consolidation of what were thought to be the best points of previously described procedures with appropriate adaptations where necessary.

2.2 Separation and Culturing of Lymphocytes for Studies Involving Determination of Nucleic Acid Synthesis or Morphological Transformation

Venous blood from healthy volunteers was collected in 15 ml heparinized (15 IU/ml) "Vacutainer" tubes which were centrifuged at 160xg for 10 min. The plasma and buffy coat were removed with a Pasteur pipette and were placed in a
disposable plastic tissue culture tube (17 x 100 mm, Falcon). The tube was placed at room temperature at a 45\(^{\circ}\) angle for 45 min followed by 10 min in an upright position to remove most of the erythrocytes by simple sedimentation. The leukocyte-rich supernatant was pipetted into a 20 ml glass syringe packed loosely with cotton wool. The syringe was then incubated for 40 min at 37\(^{\circ}\). At the end of this period the non-adherent cells which were mostly lymphocytes were eluted from the column with saline into a 17 x 100 mm tube. The eluted cells were washed three times by centrifugation with saline of pH 7.1.

The final white cell and erythrocyte counts were performed with a hemocytometer, and a differential white cell count was done on a smear stained with Wright's stain. Normally 0.8 to 1.2 x 10\(^6\) lymphocytes were obtained from each ml of whole blood, and they comprised from 98-100% of the final leucocyte population. Erythrocytes were generally present at an erythrocyte to lymphocyte ratio of from 2 to 6:1.

The washed cells were diluted with Basal Medium Eagle (BME) or Ca\(^{++}\) and Mg\(^{++}\)-free BME supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and 20% homologous AB serum except where indicated to the contrary since autologous plasma was previously found to produce variable results (126). Magnesium ions were added to the medium where indicated. The final concentration was 5 x 10\(^5\) lymphocytes/ml. The cells were then dispensed in
0.7 ml volumes (3.5 x 10^5 lymphocytes) to loosely capped, round-bottomed disposable plastic culture tubes (12 x 75 mm, Falcon) which were subsequently incubated at 37° in a humidified atmosphere of 95% air-5% CO_2. For 2-way mixed lymphocyte reaction (MLR) experiments, lymphocytes from 2 donors were isolated and diluted as described above. Each mixed culture tube (12 x 75 mm) received 0.35 ml (0.175 x 10^6 lymphocytes) of the cell suspension from each donor. The cultures were then incubated at 37° as above.

Phytohemagglutinin was rehydrated with 5 ml of sterile distilled water. This was the stock solution from which further dilutions were made with saline. PHA was added to cultures at a maximally effective (Fig. 1) concentration of 0.75 μg/ml. Each vial of PHA was used for up to 2 months since the mitogenic potential did not decrease during this length of time as determined by dose-response experiments on the same individual.

2.3 Determination of Nucleic Acid Synthesis

Synthesis of nucleic acids by cultured lymphocytes was determined by measurement of the incorporation of tritium-labeled precursors into acid-insoluble material. Methyl-3H-thymidine (3HT, 6.0 Ci/m mole) for RNA studies were diluted with sterile, isotonic saline to an activity of 15 μCi/ml. At appropriate times 0.025 ml of the desired isotope was added to each culture to give a final
activity of 0.5 μCi/ml. Incubation of the cells with the isotope was continued at 37° for various times depending on the individual experiment. The exact length of time will be indicated in each case. Mixed lymphocyte cultures were incubated for 6 days with \(^3\text{H}\)-thymidine present for the final 4 hours.

At the end of the incubation period the reactions were terminated by adding 2 ml of ice-cold saline to the cultures and centrifuging them for 7 min at 1000xg at 4°. The supernatant was removed and the washing procedure repeated. After the second wash the cell button was resuspended in exactly 1 ml of saline, and 0.2 ml of the well-mixed suspension was placed on each of two filter paper discs of 22 mm diameter (#3 Whatman Qualitative). The papers were then dried thoroughly with a heat lamp and washed twice with ice-cold 5% trichloroacetic acid, twice with 95% ethanol and once with acetone for 5 min per wash. After the final acetone wash the papers were dried completely for 10 min with a heat lamp to remove any final traces of water. Finally they were placed in glass liquid scintillation vials with 5 ml of toluene-based scintillation fluid, \([3 \text{ liters toluene, 12.0 gm 2,5-diphenyl-oxazole (PPO) and 0.15 gm 1,4-Bis-2-(5-phenyloxazolyi)benzene (POPOP)}]\) which were then counted in a Nuclear Chicago Mark II liquid scintillation counter with counting errors 2%. Isotope incorporation was expressed as counts
per minute (CPM) per culture tube.

2.4 Determination of Morphological Transformation

The percentage of morphologically transformed cells was normally determined after 72 hours in culture. The cells were first concentrated by low speed centrifugation (50xg). Most of the supernatant was removed with a Pasteur pipette, and the cells were gently aspirated. A smear was then made which was stained with Wright's stain. A minimum of 500 cells per slide was counted. The criteria used for judging a cell transformed were significantly increased size, increased cytoplasmic basophilia and the presence of a nucleolus.

2.5 Determination of Lymphocyte Viabilities

Assessment of cell viability is critical in studies involving exogenous agents which inhibit the response being measured since these effects may be due to toxicity. There is, however, no single method of determining cell viabilities which is completely reliable. Therefore, three viability methods were used in this work, each of which ostensibly measured a somewhat different parameter of viability.

Dye exclusion which measures individual cell death by loss of membrane integrity was one technique used. At
appropriate times after initiation of cultures the cells were concentrated by low speed centrifugation (50xg) for 5 min. Most of the supernatant was removed with a Pasteur pipette. Several drops of trypan blue (0.2% w/v) in phosphate-buffered saline was added to the tube, and the cells were dispersed by gentle aspiration. They were then incubated at 4°C for 15 min. Finally the cells were placed in a hemocytometer, and a minimum of 500 cells was counted. The percentage of stained cells gave an estimate of the proportion of dead cells.

The second method used isotope release from pre-labeled cells as a viability estimate. This technique also uses loss of membrane integrity as the measure of cell death. It has the advantage of being less subjective than the dye-exclusion method. Lymphocytes (10-20 x 10^6) were suspended in 1 ml of BME without serum. Approximately 100 μCi of 51 chromium (sodium chromate, 0.13 Ci/mg) was added. The cells were incubated at 37°C for 30 min after which they were washed 3 times with saline at 4°C to remove extracellular isotope. They were then diluted to 5 x 10^5 cells/ml and were dispensed in 0.7 ml volumes to 12 x 75 mm plastic tubes which were incubated at 37°C. At desired times the cells were centrifuged at 1000xg for 5 min. The isotope in the supernatant and the cell pellet was determined with a Nuclear Chicago scaler coupled with a well-type sodium iodide crystal. The percentage of isotope in the supernatant provided a qualitative estimate of the degree of cell
death. The maximum amount of $^{51}$Cr which could be released was determined by 3 alternate freeze-thaw cycles after suspension in 3 ml of distilled H$_2$O. This correction was applied to the data.

The final technique involved measuring the ability of the lymphocytes to respond to PHA, and, thus, gave an estimate of the functional capacity of the cells. In this method the cells were treated with a potentially toxic agent with no PHA present for various lengths of time (commonly 48 hr). They were then washed 2 times with saline and resuspended in 0.7 ml of BME with 20% AB serum. PHA was added, and RNA synthesis was determined 48 hr later. The degree of synthesis in cultures previously exposed to the agent was compared with that in untreated cultures and used as a qualitative estimate of relative cell survival. When any agent did not increase the degree of cell death as determined by all three methods, it could safely be concluded that it was non-toxic.

2.6 Preparation of Lymphocytes for Transport Studies

The separation procedure described previously provided lymphocytes free of contamination by other leukocytes although a significant number of erythrocytes and platelets were still present. The presence of these factors is considered acceptable in most transformation studies which involve determination of nucleic acid
synthesis and morphological transformation, since neither platelets nor red cells transform or synthesize any significant quantities of nucleic acid. However, for studies on membrane transport a further purification was necessary.

The procedure used was similar to the one described by Mendelsohn et al. (51). About 200 ml of venous blood was collected in heparin or in some cases defibrinated to remove platelets by stirring with a glass rod. The blood was then centrifuged at 500xg for 10 min in 17 x 100 mm tubes. The plasma and buffy coat were aspirated and placed in a 17 x 100 mm tube followed by sedimentation at a 45° angle and then in an upright position as described earlier. The leukocyte-rich supernatant was removed and mixed with 2 volumes of saline. Thirty ml of this mixture was then carefully layered in 50 ml conical centrifuge tubes over 9 ml of a solution containing the following ingredients: hypanque (Sodium diatrizoate), 1.8 ml; Ficoll (9% w/v), 6.35 ml; distilled water, 0.85 ml. The tubes were centrifuged at 1500xg for 10 min at room temperature. This isopycnic centrifugation procedure produced an almost complete separation of white cells from red cells. The latter were deposited in a solid button at the bottom of the tube while the former were found in a layer at the interface. The leukocyte layer was aspirated with a Pasteur pipette and was placed in disposable plastic tubes. The cells were washed twice with saline and resuspended in
AB serum. They were pipetted into a syringe containing cotton wool as described previously and then incubated for 15 min at 37\degree. The cells were eluted with saline and were washed three times with saline to remove the serum. They were then resuspended in BME, and total white cell and differential counts were performed. The final leukocyte population normally consisted of 99-100% lymphocytes. The ratio of red cells to lymphocytes was often as low as 1:100 and was never more than 1:10. Platelets were not present in defibrinated blood and were generally found at a proportion of about 1:2 to lymphocytes in heparinized blood.

2.7 Calcium Transport Measurements

Lymphocytes prepared as described in the previous section were suspended in BME or Ca\textsuperscript{++} and Mg\textsuperscript{++}-free BME supplemented with antibiotics and 2% AB serum to provide a low initial Ca\textsuperscript{++} concentration of approximately 0.025 mM calculated assuming the level of Ca\textsuperscript{++} contamination of the Ca\textsuperscript{++}-free medium to be less than 10\textsuperscript{-5} M, as indicated by the suppliers, and the serum calcium level to be 2.6 mM (53% of which is normally ionized). The cell concentration was 3.33 x 10\textsuperscript{6} lymphocytes/ml. The cultures were maintained in the CO\textsubscript{2} incubator until used.

For 2-way mixed lymphocyte cultures the cells were diluted to a concentration of 10\textsuperscript{6} lymphocytes/ml, and
0.5 ml of each donor's cells were added to the same tube. Mouse spleen cells were diluted to a concentration of 10^7 nucleated cells/ml and 0.8 ml was dispensed to each tube.

PHA was added at a concentration of 1.5 μg/ml which was found to produce the maximum level of Ca^{++} uptake in the concentration range normally used when measuring nucleic acid synthesis. ^{45}\text{Ca} (^{45}\text{Ca}) as CaCl_2 (10-25 mCi/mg) was diluted with saline to the desired concentration at the time of each experiment.

If the cells were to be treated with some agent, the exposure to the agent prior to ^{45}\text{Ca} addition was carried out in the CO_2 incubator. Untreated control cultures were adjusted to the same total volume with saline instead of the agent. To begin the ^{45}\text{Ca} uptake determination the tubes were transferred to a water bath at the desired temperature, and ^{45}\text{Ca} was immediately added to each tube, and the incubation was continued for the desired length of time.

At the end of the incubation period 10 ml of ice-cold saline was added to each tube. The tubes were centrifuged at 1500xg for 5 min at 4\(^\circ\) and the supernatant was then completely removed. The washing process was repeated twice. Preliminary experiments established that the reaction was halted by the first addition of the large volume of cold saline so incubation times expressed in the text refer to the time between ^{45}\text{Ca} addition and the first saline addition. It was also found that three
washes were sufficient to remove residual extracellular isotope. After the last wash the cell button was resus-
pended in 1 ml of saline, and 0.2 ml aliquots were placed on 0.22 mm filter paper discs (#3 Whatman Qualitative),
dried under a heat lamp and counted as described for nucleic acid synthesis determinations. The samples were
counted in a Nuclear Chicago liquid scintillation counter. The counts in the added isotope were also determined, and
results were expressed as nanomoles (10^{-9} moles) per 10^6 lymphocytes with n moles/10^6 cells equal to medium Ca^{++}
concentration times the ratio of counts in the cells to the total counts added. Measurements of total protein were
done using the Lowry method as modified by Eagle and Oyama (127). Cell size determinations were done in a
hemocytometer using a micrometer eyepiece. These methods indicated that mean cell protein and cell volume did not
increase in stimulated cells during the first three hours after PHA treatment.

For Ca^{++} efflux determinations 10^6 lymphocytes were incubated with 5 \mu C of ^{45}Ca at a Ca^{++} concentration of
0.025 mM for 2 hours to load the cells. They were then washed 3 times with saline as described for uptake. After
the final wash they were resuspended in 1.0 ml of Ca^{++} and Mg^{++}-free medium without the phenol red indicator sup-
plemented with 2% AB serum. At various times thereafter the percentage of isotope in both the cells and super-
natant was assayed as described for uptake.
2.8 Measurements of $\alpha$-Aminoisobutyric Acid Transport

Amino acid transport is commonly studied using $\alpha$-aminoisobutyric acid (AIB). It is a non-metabolizable amino acid analogue thereby eliminating possible alterations in transport properties due to subsequent utilization. Since it shares a common transport mechanism with glycine, alanine, serine, methionine and valine, it is a valid substitute for these naturally occurring amino acids (128).

The incubation procedure was essentially as described previously (51). The lymphocytes were prepared exactly as for the $^{45}$Ca transport studies. PHA was again added at a concentration of 1.5 $\mu$g/ml. After the pre-incubation period in the CO$_2$ incubator, the cultures were transferred to a 37$^\circ$ water bath. One microcurie of $^3$H-AIB (0.2–0.4 Ci/mrnole) was added to each tube, and the incubation was continued for 15 minutes. At the end of this period 6 ml of saline containing 1 mM of non-radioactive AIB was added to each tube. The tubes were centrifuged for 5 min at 4$^\circ$ at 1000xg. The supernatant was removed and the washing procedure repeated twice again. After the last wash the cells were resuspended in 1 ml of saline and then prepared and counted as described previously for calcium transport.
2.9 Sources of Materials

All medium and supplements except serum (Red Cross) were obtained from Grand Island Biological Company, Grand Island, New York. Phytohemagglutinin (PHA-P) came from Difco Laboratories, Detroit, Michigan. The source of $5^3$H-uridine and $^3$H-thymidine was Schwarz Bioresearch, Inc., Orangeburg, New York. $^{51}$Cr, $^{45}$Ca and $^3$H-AIB were obtained respectively from Charles E. Frosst and Co., Montreal, Quebec; Amersham-Searle, Arlington Heights, Illinois; New England Nuclear, Boston, Massachusetts. All other chemicals used were of the top grade available and were obtained from Sigma Chemical Company, St. Louis, Missouri, with a few exceptions. Sodium diatrizoate (Hypaque) was purchased from Winthrop Laboratories, Aurora, Ontario. Calcium, magnesium and manganese chlorides, ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis ($\beta$-aminoethyl ether)-N,$N'$-tetraacetic acid (EGTA), imidazole and toluene were obtained from Fisher Scientific Co., Montreal, Quebec. The scintillation fluors, POPOP and PPO, came from Kent Laboratories, Vancouver, British Columbia.

2.10 Statistical Methods and Other Calculations

Each point in all experiments was the mean value of from 3 to 5 replicate cultures. The error bars represent
standard errors of the mean (SEM). In any case where no error bar was shown, the SEM was smaller than the size of the point. Statistical significance was determined using Student's t-test. Correlation coefficients were calculated for all potential linear relationships, and the significance of the correlation was estimated by the t-test. The best fit for linear data was determined by the method of least squares. When results were expressed as a percentage of the normal or maximum response, the mean value of replicate cultures for that point was taken as the normal or maximum response respectively. The SEM of that mean value was shown in the figure proper.
3. RESULTS

3.1 Characteristics of the Lymphocyte Response to PHA

3.1.1 Introduction

The experimental technique employed for lymphocyte culturing was a composite of many that have been described previously. In view of the many factors which can produce variability in results it was necessary to establish the dose-response and time-course relationships for nucleic acid synthesis for this composite technique. The time-course of early amino acid transport (AIB) by stimulated cells was also determined. These parameters also had to be established for the first time for Ca\(^{++}\) uptake which was found to be stimulated by PHA.

Since many studies in this thesis involved treating the cells with potentially toxic agents, an assessment of cell viabilities was critical. Accordingly the precision of each of three viability methods was determined.

3.1.2 Effect of PHA Concentration

Little or no stimulation of RNA or DNA synthesis or Ca\(^{++}\) uptake occurred using PHA concentrations less
than about 0.05 µg/ml (Fig. 1a, b, c). Increasing the PHA concentration above this threshold level progressively increased each of the three responses until a plateau was reached which extended over the concentration range from about 0.2 to 3.0 µg/ml. When the concentration was raised further, RNA and DNA synthesis began to decrease until these responses were reduced to the control level at 48 µg/ml. Ca\(^{++}\) uptake was also depressed at PHA concentrations above 3.0 µg/ml, but it always remained above control levels. An unexpected observation was the dramatic increase in Ca\(^{++}\) accumulation which occurred at the highest PHA concentration tested (48 µg/ml). The uptake in this case was two times the plateau value. It should be noted that Ca\(^{++}\) uptake was a very early event in the PHA response and was determined in this experiment during the first hour following PHA treatment. In contrast RNA synthesis was determined at 48 hours and DNA at 64 hours the times of maximum levels of synthesis for each parameter. The effect of PHA concentration on AIB uptake was not determined since it did not constitute a major area of investigation, and since clear stimulation occurred at the same concentration used in Ca\(^{++}\) transport studies. A dose-response curve for AIB transport was reported by Mendelsohn et al. (51).
Dose-response effects of PHA on several parameters of lymphocyte transformation.
a. RNA synthesis was determined at 48 hours with $^3$H-uridine present for the final 6 hours. Each point was the mean CPM ± SEM of 3 or 4 replicate cultures expressed as a percentage of the maximum response observed (16,200 CPM).
b. DNA synthesis was determined at 64 hours with $^3$H-thymidine present for the final 6 hours. Each point was the mean CPM ± SEM of 3 or 4 replicate cultures expressed as a percentage of the maximum response observed (15,300 CPM).  
c. Calcium uptake was determined at a Ca$^{++}$ concentration of 1.25 mM with a 60 minute incubation with $^{45}$Ca immediately after PHA addition. Each point was the mean uptake in n moles/10$^6$ lymphocytes ± SEM of 3 or 4 replicate cultures expressed as a percentage of the maximum response observed (135 n moles/10$^6$ lymphocytes).
3.1.3 Time-Course of Increased Nucleic Acid Synthesis and Morphological Transformation

The time-courses of RNA and DNA synthesis and morphological transformation were followed for 4 or 5 days after PHA treatment using the following procedure. The appropriate isotope was added to several PHA or control cultures immediately after adding PHA or saline. The cultures were incubated for 8 hours and then harvested as described in the methods. At that time isotope was added to another set of cultures which were incubated for the next 8 hours i.e. during the interval of 8 to 16 hours after stimulation. This procedure was carried on for 4 days providing a continuous description of the time-course of nucleic acid synthesis. Blast transformation was determined only at each 24 hour interval up to day 5.

RNA synthesis in PHA-treated cultures was about 4 times the control level (1 to 2% of the maximum stimulated synthesis) over the first 8 hour interval (Fig. 2a). A rapid linear increase in isotope incorporation then occurred with each successive interval until the maximum value was reached at 48 hours. The level of synthesis declined gradually thereafter.

No stimulation of DNA synthesis was observed until 24 hours (Fig. 2b) by which time RNA synthesis had reached about 50% of its maximum level. Isotope incorporation
then increased to a maximum at 64 hours. Similar to RNA
the level of synthesis declined continuously after reach-
ing its peak value. DNA synthesis in unstimulated cells
remained at about 1 to 2% of the maximum level observed
in stimulated cultures throughout the experiment.

Few true lymphoblasts appeared in PHA-treated cul-
tures before 48 hours (Fig. 2c). The percentage of these
transformed cells then increased to a maximum of 82% at
96 hours before declining to 38% at 120 hours. Lympho-
blasts were not commonly observed in unstimulated cul-
tures during the first 4 or 5 days, but an increased
degree of spontaneous transformation (up to 4%) occa-
sionally occurred in long-term cultures.

The various stages of lymphocyte transformation
in vitro in response to PHA are depicted in Fig. 3. The
normal lymphocyte population before stimulation consist-
et of morphologically homogeneous small lymphocytes of
7 to 9 μ in diameter, approximately the size of red
cells (Fig. 3a). After exposure to PHA for about 24
hours some intermediate stages commonly referred to as
"immature cells" were found (Fig. 3b), and after about
48 hours fully-developed lymphoblasts were common
(Fig. 3c). These were often as large as 30 μ in di-
ameter with a greatly increased amount of basophilic
cytoplasm and a large nucleus with one or more nucleoli.
The lymphoblasts may divide as shown in Fig. 3d.
Figure 2.

Time-course of increased nucleic acid synthesis and morphological transformation in PHA-stimulated lymphocytes. a. RNA synthesis was determined by incubation with $^3$H-uridine during each 8 hour interval for 4 days. Each point was the mean CPM ± SEM of 3 or 4 replicate cultures expressed as a percentage of the maximum response observed (22,200 CPM). b. DNA synthesis was determined by incubation with $^3$H-thymidine during each 8 hour interval for 4 days. Each point was the mean CPM ± SEM of 3 replicate cultures expressed as a percentage of the maximum response observed (26,100 CPM). c. The percentage of transformed cells was determined at the end of each 24 hour period for 5 days. Each point was the mean of duplicate cultures.
Figure 3.

Photomicrograph of the various morphological stages of lymphocyte transformation. a. Normal small lymphocytes before PHA exposure. b. Intermediate cell types observed about 24 hours after PHA treatment. c. Fully-developed blast cells generally found in large numbers 48 hours after stimulation. d. Mitosis (telophase) which is the fate of many of the blast cells by 72 hours and later. The magnification = 1700x.
3.1.4 Time-Course of Increased Calcium and Amino Acid Uptake

Stimulation of Ca\textsuperscript{++} uptake by PHA was first demonstrated in these studies. In the experiment shown in Fig. 4a uptake was determined with a 10 minute incubation with isotope at the indicated times after PHA treatment. PHA significantly (p < 0.001) enhanced the rate of Ca\textsuperscript{++} accumulation during the interval from 0 to 10 minutes after exposure. The rate of uptake in PHA-treated cultures then increased linearly for 3 hours and subsequently became constant. The rate of uptake by unstimulated cells rose only slightly during the five hour experiment.

PHA also stimulated the initial rate of AIB uptake although not quite as early (Fig. 4b). A significant increase (p < 0.05) occurred only after a 30 minute exposure to the mitogen which agreed with a previous study (51). The rate of stimulated uptake increased progressively after 1 hour while the control uptake remained constant for 5 hours.

3.1.5 Viability Determinations

The ability of viable cells to exclude vital dyes has been the most commonly used means of estimating cell survival. However, it was felt that more than one technique should be used in these studies so that decisions
Figure 4.

Time-course of increased calcium and amino acid uptake in PHA-stimulated lymphocytes. •—•, PHA; ○—○, unstimulated. a. The initial rate of calcium uptake was determined at 37° at a Ca^{++} concentration of 1.25 mM with a 10 minute exposure to ^{45}Ca at the indicated times. Each point was the mean uptake in n moles/10^6 lymphocytes/min ± SEM of 3 replicate cultures expressed as a percentage of the maximum rate of uptake observed (8.7 n moles/min). b. The initial rate of AIB uptake was determined at 37° with a 15 minute exposure to ^{3}H-AIB at the indicated times. Each point was the mean uptake in CPM/10^6 lymphocytes/15 min ± SEM of 3 replicate cultures expressed as a percentage of the maximum uptake observed (1190 CPM).
regarding the toxicity of an agent could be made with complete confidence. Consequently, the viability of lymphocytes cultured with or without PHA was followed for 8 days using the three techniques described in the methods.

In unstimulated cultures the percentage of dye-excluding, i.e. viable, cells decreased at a rate of 1 to 1.5% per day (Fig. 5a). However, the $^{51}$Cr release method indicated a much greater degree of cell death with the amount of isotope released increasing by an average of about 9% per day to day 5 (Fig. 5a). The data was scattered for the final 3 days, but the rate of release seemed to be decreasing.

The dye-exclusion method indicated that in PHA-treated cultures the proportion of viable cells was significantly less ($p < 0.05$) than in unstimulated cultures at 24 hours, and that the viable fraction then declined by an average of about 9% per day (Fig. 5a). The stimulated cells released $^{51}$Cr at a rate of 13% per day until day 5 (Fig. 5b). This was also greater than the corresponding release from unstimulated cells. No further release occurred over the final 3 days possibly indicating that a steady state between cell loss by death and cell gain by mitosis had been achieved or else that there was a long-lived subpopulation.

The above two methods provided estimates of viability based on the structural integrity of the cell
Figure 5.

Viability of unstimulated and PHA-stimulated lymphocytes with time in culture. ●—●, PHA; O—O, controls. a. The percentage of dye-excluding (viable cells) was determined at the end of each 24 hour interval. Results are expressed as the mean percentage ± SEM of 3 replicate cultures. b. The percentage of $^{51}$Cr retained in the cells (viable cells) was determined at the end of each 24 hour interval also. Results are expressed as the mean percentage ± SEM of 3 replicate cultures.
membrane. However, they probably measure different parameters of membrane breakdown. The final method gave an indication of the functional integrity of the cell population as a whole. PHA was added to a set of previously unstimulated cultures every 24 hours, and $^3$H-uridine incorporation was determined 48 hours after PHA addition. There was no marked change in isotope incorporation in cells stimulated as late as 5 days after initiation of the culture (Table 1). However, incorporation decreased by about 50% over the final 3 days. Comparison of these results with the other two methods indicates that they agreed with the dye exclusion method which showed little death of unstimulated cells during the experiment (Fig. 5a).

3.1.6 Summary

The effects of different concentrations of PHA on RNA and DNA synthesis were characterized by a plateau range with smaller response at both higher and lower concentrations outside this range, as observed by others previously (129, 130) using somewhat different methods. The effect of PHA concentration on Ca$^{++}$ uptake was similar except for a sharp increase at the highest concentration of PHA tested. This dramatically increased uptake may have been due to early membrane damage since this high PHA concentration increased the degree of cell
TABLE 1

The Ability of PHA-Stimulated Lymphocytes to Incorporate \(^3\)H-Uridine as a Viability Test\(^1\)

<table>
<thead>
<tr>
<th>Time PHA Added (Hrs)</th>
<th>(^3)H-Uridine Incorporation (^2) (CPM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15,600 ± 952</td>
</tr>
<tr>
<td>24</td>
<td>18,600 ± 838</td>
</tr>
<tr>
<td>48</td>
<td>17,400 ± 767</td>
</tr>
<tr>
<td>72</td>
<td>13,900 ± 1980</td>
</tr>
<tr>
<td>96</td>
<td>19,900 ± 789</td>
</tr>
<tr>
<td>120</td>
<td>16,000 ± 469</td>
</tr>
<tr>
<td>144</td>
<td>12,700 ± 352</td>
</tr>
<tr>
<td>168</td>
<td>9,590 ± 271</td>
</tr>
<tr>
<td>192</td>
<td>7,980 ± 433</td>
</tr>
</tbody>
</table>

\(^1\)Unstimulated lymphocytes were cultured for the indicated times. PHA was then added, and the cultures were incubated for 48 additional hours with \(^3\)H-uridine present for the final 4 hours.

\(^2\)Each value is the mean CPM ± SEM of 4 replicate cultures.
death in long-term cultures. However, it should be noted that other early events such as cAMP production (59) and early RNA synthesis (64) were observed only at very high PHA doses.

The time-course of nucleic acid synthesis has also been reported by other workers (41, 42, 43, 81), and the present studies showing maximum RNA and DNA synthesis occurring at 48 and 64 hours respectively were in close agreement. The maximum percentage of transformed cells was observed at 96 hours. Also increased DNA synthesis did not begin until between 16 to 24 hours.

PHA increased the rate of Ca$$^{++}$$ accumulation within 10 minutes of treatment, and the rate then increased linearly until 3 hours before leveling off. Enhanced uptake of AIB was not observed until 30 minutes after exposure to PHA, but the rate of uptake increased rapidly thereafter agreeing with a previous study (51).

The dye-exclusion and PHA stimulation methods for measuring viability indicated that very little cell death occurred in unstimulated cultures. The $^{51}$Cr method seemed to disagree in that there was a high degree of release by resting cells. However, other studies on the cytolysis potential of lymphocytes have shown that there is a large amount of spontaneous release from cells which are likely still viable (131, 132). Since the purpose of the viability tests was to assess the toxic effects of various chemical agents which involves only
relative differences, it was not necessary to have
strict quantitative agreement among the methods. The
precision among replicates for each method was excellent.
3.2 Exogenous Calcium Requirements of PHA-Stimulated Lymphocytes

3.2.1 Introduction

The initial event in transformation is an interaction of the stimulant with the cell membrane, and alterations in membrane properties become apparent soon after exposure to PHA. Since Ca$^{++}$ are important in maintaining membrane structure and function (116), it seemed conceivable that Ca$^{++}$ could exert a critical influence on the activation process. In addition Whitfield et al. had previously reported that Ca$^{++}$ had a profound influence on the proliferation of rat bone marrow and thymus cells (118, 119, 120).

The experiments reported in this chapter were initiated first of all to determine if medium Ca$^{++}$ were required for transformation. Further studies were aimed at elucidating possible loci of action both by direct measurements of several aspects of the response and by selective removal of Ca$^{++}$ at various times throughout the response.

The roles of Ca$^{++}$ in many biological phenomena have been successfully elaborated using a variety of agents which can selectively complex the ion (133). The most commonly employed chelating compound has been ethylenediaminetetraacetic acid (EDTA). This agent has an
affinity for Ca\textsuperscript{++} which is about 10\textsuperscript{2} times greater than its affinity for Mg\textsuperscript{++} (133). In more recent studies EDTA has been largely replaced by EGTA. The affinity of EGTA for Ca\textsuperscript{++} is more than 10\textsuperscript{5} times greater than its affinity for Mg\textsuperscript{++} (133) so the use of this agent provides much greater assurance that observed effects are indeed due to a reduction of the level of available Ca\textsuperscript{++}. However, these chelators do bind other divalent cations of biological importance such as zinc and manganese (134) so a possible influence of the chelators associated with binding of these ions has to be considered.

3.2.2 Effects of EDTA on Transformation

PHA-induced activation of lymphocytes as assessed by nucleic acid synthesis and morphological transformation was inhibited by EDTA at concentrations above 10\textsuperscript{-4}\text{M} (Fig. 6). Complete inhibition of DNA synthesis occurred at about 10\textsuperscript{-3}\text{M} while RNA synthesis and blast transformation were slightly less sensitive. The highest concentration of EDTA used was non-toxic to the cells as judged by all three viability methods, agreeing with a previous study showing that it was non-toxic to mouse spleen cells up to 10\textsuperscript{-2}\text{M} (135).

The previous experiments indicated that divalent cations were required to permit transformation to proceed. However, no conclusions could be drawn regarding
Effect of EDTA concentration on RNA and DNA synthesis and morphological transformation in PHA-stimulated lymphocyte cultures. O--O, DNA; ••, RNA; X--X, blast transformation. EDTA was added to cultures at the indicated concentrations 15 minutes before addition of PHA. RNA synthesis was assessed at 40 hours and DNA at 64 hours after PHA addition with the appropriate isotope present for the final 16 hours. Blast transformation was determined at 72 hours. Each point is the mean CPM ± SEM of 3 to 5 replicate cultures for RNA and DNA and duplicates for blast transformation expressed as a percentage of the maximum response observed for each parameter (DNA, 33,630 CPM; RNA, 18,510 CPM; blast cells, 74.2%).
Ca^{++} specifically. Addition of excess Ca^{++} to PHA cultures treated with EDTA at any time up to 3 hours after PHA addition resulted in only a partial reversal of the inhibition of DNA synthesis even with as high a concentration as 7.5 mM (Fig. 7). Magnesium ions were completely ineffective in reversing the EDTA inhibition. Independent studies by Alford (136) agreed with the above findings and also showed that various combinations of divalent cations could not completely restore the normal response. Therefore, it was concluded that the EDTA inhibitory effects were not due solely to Ca^{++} binding so it was not a satisfactory agent for studying lymphocyte Ca^{++} requirements.

3.2.3 Effects of EGTA on Transformation

EGTA was employed to achieve low, stable concentrations of free Ca^{++}. A system containing EGTA and Ca^{++} constitutes a Ca^{++} buffer identical in principle to a hydrogen ion buffer system except that the Ca^{++} concentration is controlled. The reaction depends on the dissociation constant of the reaction 2H^{+} + EGTA-Ca \rightleftharpoons EGTA-H_{2} + Ca^{++} so that the free Ca^{++} level can be altered by changing the EGTA, Ca^{++} or H^{+} concentration. Thus, by using different EGTA concentrations the free Ca^{++} level could be adjusted at the same pH and total calcium concentrations (137, 138).
Figure 7.

Ability of Ca++ and Mg++ to reverse EDTA-induced inhibition of PHA-stimulated DNA synthesis. □, PHA only; □, unstimulated lymphocytes; □, PHA + 1.5 mM EDTA; □, PHA + 1.5 mM EDTA + Ca++; □, PHA + 1.5 mM EDTA + Mg++. EDTA was added to the cultures 15 minutes before PHA. Ca++ and Mg++ were added 15 minutes after PHA. DNA synthesis was determined at 64 hours with 3H-thymidine present for the final 16 hours. Each point is the mean CPM ± SEM of 3 replicate cultures expressed as a percentage of the response obtained with PHA alone (28,300 CPM).
EGTA also inhibited the stimulatory effects of PHA beginning at concentrations slightly greater than the medium Ca\(^{++}\) concentration of 1.26 mM (Fig. 8). Complete inhibition of nucleic acid synthesis occurred at an EGTA concentration of 1.39 mM. Morphological transformation was significantly less sensitive indicating that some morphological changes could occur in the absence of nucleic acid synthesis as has been proposed before (38). Since the chelator effectively inhibited all three parameters, it could be concluded that the inhibition was not due to isolated effects on thymidine and/or uridine metabolic pathways. EGTA was definitely non-toxic although a slightly increased degree of cell death (10-20%) was observed at concentrations above 1.45 mM.

DNA synthesis in unstimulated lymphocytes was not affected by EGTA at concentrations up to 1.39 mM which completely inhibited the PHA-stimulated DNA synthesis (Table 2). However, concentrations of 1.42 mM and higher caused almost a 50% inhibition of the basal level of DNA synthesis.

The inhibitory effect of EGTA was due specifically to its binding of Ca\(^{++}\) and not other divalent cations since addition of excess Ca\(^{++}\) at as low a concentration as 0.35 mM reversed most of the inhibition of DNA synthesis (Fig. 9). Calcium ions at concentrations above 0.70 mM completely restored the normal response. Magnesium ions were completely ineffective at reversing
Figure 8.

Effect of EGTA concentration on RNA and DNA synthesis and morphological transformation in PHA-stimulated lymphocytes.

O—O, DNA; •—•, RNA; X—X, blast transformation. EGTA was added to the cultures at the indicated concentrations 15 minutes before addition of PHA. RNA and DNA synthesis were assessed at 40 and 64 hours respectively after PHA addition with the appropriate isotope present for the final 16 hours. Blast transformation was determined at 72 hours. Each point is the mean CPM ± SEM of 3 to 5 replicate cultures for RNA and DNA and duplicates for blast transformation expressed as a percentage of the maximum response observed for each parameter (DNA, 25,540 CPM; RNA, 14,690 CPM; blast cells, 62.5%).
TABLE 2

Effect of EGTA on $^3$H-Thymidine Incorporation by Unstimulated Lymphocytes$^1$

<table>
<thead>
<tr>
<th>EGTA Concentration (mM)</th>
<th>$^3$H-Thymidine Incorporation$^2$ (CPM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>385 ± 14</td>
</tr>
<tr>
<td>1.28</td>
<td>387 ± 16</td>
</tr>
<tr>
<td>1.30</td>
<td>378 ± 16</td>
</tr>
<tr>
<td>1.32</td>
<td>408 ± 10</td>
</tr>
<tr>
<td>1.34</td>
<td>372 ± 6</td>
</tr>
<tr>
<td>1.36</td>
<td>371 ± 30</td>
</tr>
<tr>
<td>1.39</td>
<td>374 ± 19</td>
</tr>
<tr>
<td>1.42</td>
<td>233 ± 15</td>
</tr>
<tr>
<td>1.48</td>
<td>206 ± 29</td>
</tr>
</tbody>
</table>

$^1$Unstimulated lymphocytes were cultured with various concentrations of EGTA for 64 hours with $^3$H-thymidine present for the final 16 hours.

$^2$Each value is the mean CPM ± SEM of 4 replicate cultures.
Figure 9.

Reversibility of the inhibitory effect of EGTA on PHA-stimulated lymphocytes, ■, PHA only; □, control (no PHA or EGTA); ■■, PHA + 1.4 mM EGTA; :●: , PHA + 1.4 mM EGTA + Ca++; ■■■, PHA + 1.4 mM EGTA + Mg++. EGTA was added 15 minutes prior to PHA; Ca++ or Mg++ were added with the PHA. DNA synthesis was determined at 48 hours with ³H-thymidine present for the final 2 hours. Each bar is the mean CPM ± SEM of 3 to 5 replicate cultures expressed as a percentage of the maximum level of synthesis observed (2180 CPM).
$^{3}\text{H-THYMIDINE INCORPORATION}$

(% OF MAXIMUM)

CONCENTRATION OF DIVALENT CATION ADDED (mM)

0  0.35  0.70  1.4  2.8
EGTA inhibition even at concentrations higher than are shown in Fig. 9. Manganese ions were similarly ineffective and were toxic at concentrations above 1.0 mM.

The observed inhibition of the PHA response was conceivably due to a slowing down of some step(s) in the transformation process rather than to a complete inhibition. This possibility was tested by following the time-course of DNA synthesis for 4 days in PHA cultures containing 0, 1.30 or 1.39 mM EGTA. DNA synthesis in cultures without EGTA followed the normal time-course beginning at about 24 hours and then increasing to a maximum at 72 hours before declining (Fig. 10). The shape of the curve for cultures with 1.30 mM EGTA was the same, but the absolute response was lower at each point. DNA synthesis did not rise above control levels in cultures with 1.39 mM EGTA at any time.

The requirement of Ca\(^{++}\) in the medium for transformation was tested further by culturing lymphocytes in commercial calcium-free medium supplemented with different concentrations of homologous AB serum in order to vary the initial calcium ion concentration. The human serum used had a total calcium concentration of 2.7 mM. In normal serum about 53\% of the total calcium is ionized so a Ca\(^{++}\) concentration of about 1.4 mM was assumed for this serum. The cells responded very poorly in the absence of serum, but a reasonable amount of RNA synthesis was obtained with as little as 0.5% serum.
Figure 10.

Effect of EGTA on the time-course of DNA synthesis in PHA-stimulated lymphocytes. ●—●, PHA only; ○—○, PHA + 1.30 mM EGTA; ×—×, PHA + 1.39 mM EGTA. PHA was added at 0 time, and EGTA was added 15 minutes prior to PHA. DNA synthesis was determined at the indicated times with $^3$H-thymidine present for the final 2 hours. Each point is the mean CPM ± SEM of 4 replicate cultures expressed as a percentage of the maximum level of synthesis observed (6820 CPM).
Maximum responses were observed in cultures with 20% serum. Additional Ca\textsuperscript{++} were then added to the cultures to provide a range of Ca\textsuperscript{++} concentrations for each serum concentration.

Cultures containing 0.5% or 1% serum with no added Ca\textsuperscript{++} gave responses not significantly different from those of control cultures not treated with PHA. Their Ca\textsuperscript{++} levels were near $7.0 \times 10^{-6}$M and $1.4 \times 10^{-5}$M respectively (calculated assuming 53% of the 2.7 mM serum calcium was ionized). There was then a progressive increase in isotope incorporation with increasing Ca\textsuperscript{++} concentration until maximum responses were obtained at concentrations in the range $6.0 \times 10^{-4}$ to $1.6 \times 10^{-3}$M (Fig. 11). A similar increase was observed for cultures containing 10% and 20% serum. In these cases values of RNA synthesis amounting to 60 to 75% of the maximum response were obtained with no added calcium. However, these cultures had initial Ca\textsuperscript{++} concentrations of about $1.4 \times 10^{-4}$ and $2.8 \times 10^{-4}$M respectively due to their serum levels. Thus, the results with 10% and 20% serum actually were in close agreement with the results from equivalent cultures which had the same total Ca\textsuperscript{++} concentration after additions to the medium containing 0.5% and 1% serum. Raising the Ca\textsuperscript{++} concentration above the physiological level (1.3 mM) resulted in slightly decreased isotope incorporation although increased variability among samples made the differences not significant
Figure 11.

Influence on PHA-induced RNA synthesis of adding Ca\textsuperscript{++} at various concentrations to cultures grown in 4 different concentrations of AB serum. □—□ , 20% serum; ○—○ , 10% serum; ●—● , 1% serum; ×—× , 0.5% serum. Ca\textsuperscript{++} were added to commercial calcium-free medium plus serum 15 minutes before addition of PHA. RNA synthesis was determined at 48 hours with \textsuperscript{3}H-uridine present for the final 16 hours. Each point is the mean CPM ± SEM of 3 or 4 replicate cultures expressed as a percentage of the maximum response for that serum concentration (21,200 CPM, 20%; 14,650 CPM, 10%; 3,870 CPM, 1%; 2,230 CPM, 0.5%).
statistically. Precipitation due to serum phosphate and/or toxic effects probably account for the variation in these cases. Magnesium ions could not substitute for Ca\(^{++}\) in this experiment.

The previous data established that PHA-induced transformation, as measured by nucleic acid synthesis during the late synthetic phase of the response, could not develop without a minimum level of exogenous Ca\(^{++}\). However, the data did not distinguish whether Ca\(^{++}\) were specifically required for early events occurring during the induction phase of transformation i.e. the period preceding initiation of DNA synthesis or whether they simply functioned as a normal growth requirement once the response was underway. If Ca\(^{++}\) only had a non-specific growth function in lymphocyte transformation, one would expect that their removal at any time during the response would result in an inhibition of growth, i.e. nucleic acid synthesis.

This possibility was tested by treating lymphocytes with PHA and allowing the response to develop for 48 hours by which time the lymphocytes had become a population of proliferating cells. EGTA or EDTA, both at 1.42 mM, were then added to determine whether DNA synthesis, measured subsequently, would be inhibited. Ouabain, an inhibitor of Na\(^{+}\) and K\(^{+}\) transport, was also tested. The results shown in Fig. 12 clearly demonstrated that EGTA had no effect on PHA-stimulated DNA
synthesis even as late as 72 hours after addition of the chelator. Even higher concentrations of EGTA did not inhibit this late DNA synthesis although some toxicity occurred above 1.45 mM. In contrast EDTA had begun to inhibit DNA synthesis by 8 hours after it was added, and the response was totally inhibited within 24 hours. When the cells were treated with ouabain (3 x 10^{-7}M), a time-course of inhibition similar to that for EDTA was observed. Each agent produced similar effects on RNA synthesis measured at the same times.

The data in Fig. 12 indicated that a reduction of Ca^{++} concentration using EGTA did not inhibit lymphocyte DNA (or RNA) synthesis directly. Therefore, its inhibitory effect must be on some event(s) involved in the initiation of nucleic acid synthesis which occur early in the response i.e. during the first 16 hours after PHA treatment before DNA synthesis is initiated.

In order to determine the critical time(s) when Ca^{++} must be present in the medium to permit development of the response, EGTA (1.4 mM) was added to PHA-treated cultures at various times relative to the time of PHA addition (Table 3). The amount of ^3H-thymidine incorporation was determined at 48 hours with the isotope present for the final 2 hours. Addition of EGTA at any time prior to adding PHA or up to 2 hours after completely inhibited the eventual response with 92% or greater inhibition considered to be complete since this was not
Figure 12.

Effect of EGTA on DNA synthesis in previously stimulated lymphocytes. ●—●, PHA only; ○—○, PHA + 1.42 mM EGTA; ■—■, PHA + 1.42 mM EDTA; ×—×, PHA + 3 x 10⁻⁷M ouabain. All lymphocyte cultures were treated with PHA alone for 48 hours. Then EGTA, EDTA or ouabain was added at the indicated concentrations, and the incorporation of $^3$H-thymidine was determined at various times thereafter with a 2 hour pulse. Each point is the mean CPM ± SEM of 3 to 5 replicate cultures expressed as a percentage of the maximum level of synthesis observed (3300 CPM).
### TABLE 3

Effect of EGTA Added at Various Times Relative to PHA Exposure on Incorporation of \(^{3}\)H-Thymidine

<table>
<thead>
<tr>
<th>Time of EGTA Addition Relative to PHA (Hrs)</th>
<th>(^{3})H-Thymidine Incorporation (^{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM ± SEM</td>
</tr>
<tr>
<td>PHA Only</td>
<td>1740 ± 120</td>
</tr>
<tr>
<td>-1</td>
<td>231 ± 34</td>
</tr>
<tr>
<td>-1/4</td>
<td>240 ± 32</td>
</tr>
<tr>
<td>0</td>
<td>351 ± 65</td>
</tr>
<tr>
<td>+1</td>
<td>382 ± 20</td>
</tr>
<tr>
<td>+2</td>
<td>320 ± 17</td>
</tr>
<tr>
<td>+4</td>
<td>490 ± 85</td>
</tr>
<tr>
<td>+8</td>
<td>722 ± 168</td>
</tr>
<tr>
<td>+12</td>
<td>1123 ± 143</td>
</tr>
<tr>
<td>+16</td>
<td>1631 ± 97</td>
</tr>
<tr>
<td>+24</td>
<td>1852 ± 97</td>
</tr>
<tr>
<td>+46</td>
<td>1807 ± 172</td>
</tr>
</tbody>
</table>

\(^{1}\)EGTA at a concentration of 1.4 mM was added to cultures at the indicated times with 0 being the time when PHA was added. The cultures were incubated for 48 hours with \(^{3}\)H-thymidine present for the final 2 hours.

\(^{2}\)Each value represents the mean CPM ± SEM of 3 replicate cultures expressed as % inhibition of the PHA-stimulated increment in isotope incorporation.
significantly different from unstimulated levels of synthesis. Addition of EGTA at progressively later times after PHA treatment resulted in a progressively decreasing degree of inhibition up to 12 hours. Later additions produced no inhibition.

A possible explanation for the previous results showing that EGTA was progressively less effective at inhibiting transformation when added at increasing times after PHA could be that EGTA was removing essential bound PHA since it is known that PHA must remain on the membrane for long periods to achieve complete transformation (51).

This possibility was investigated by treating lymphocytes with PHA for 1 hour. Then either saline as a control or 1.42 mM EGTA was added to the cultures. After exposures to EGTA or saline of 10 minutes or 1 or 3 hours the cultures were washed two times with BME without serum and were resuspended in the original volume of BME with serum. No additional PHA or EGTA was added. It was found that washing reduced the normal (no washing) PHA response (48 hour sample in Table 4) to a progressively lesser extent with increasing time after PHA treatment as expected. Cells treated with EGTA but not washed exhibited a response equivalent to unstimulated cells. However, PHA-stimulated lymphocytes treated with EGTA, from which the chelator was removed by washing, gave responses not significantly different from cells
exposed only to saline (Table 4). Even exposure to EGTA for as long as 3 hours did not reduce the response compared with the saline control. A similar experiment in which EGTA was added only 10 minutes after PHA yielded identical results. Since the final response of washed cells was the same regardless of whether or not they were exposed to EGTA, it was unlikely that EGTA removed bound PHA.

The preceding results established that Ca\(^{++}\) have an essential role in the initiation of PHA-induced transformation. There are many early events which could be influenced by Ca\(^{++}\) each of which could be the limiting step in the process. One of the earliest events is initiation of RNA synthesis, predominantly of a low molecular weight species. Therefore, the effect of EGTA on the development of this early RNA synthesis was determined in order to see if Ca\(^{++}\) were required for the response to proceed to that stage. In PHA-treated cultures in the absence of EGTA \(^3\)H-uridine incorporation was found to be slightly above control levels at 2 hours (Fig. 13). It then increased rapidly to 2.3 times the control value at 6 hours. When EGTA was added at a concentration which completely inhibited the usual final response (1.39 mM), RNA synthesis only increased slightly above control levels by 2 hours where it remained through 6 hours. EGTA had no effect on control RNA synthesis.
<table>
<thead>
<tr>
<th>Length of Exposure to EGTA (Hrs)</th>
<th>EGTA Concentration</th>
<th>$^3$H-Uridine Incorporation (CPM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1940 ± 180 2320 ± 205</td>
</tr>
<tr>
<td></td>
<td>1.42 mM</td>
<td>2460 ± 174 2240 ± 320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3480 ± 236 4005 ± 362</td>
</tr>
<tr>
<td>48 (not washed)</td>
<td></td>
<td>5280 ± 326 150 ± 3</td>
</tr>
</tbody>
</table>

$^1$Lymphocytes were exposed to PHA alone for 1 hour. Either 1.42 mM EGTA or saline was then added.

$^2$The cultures were washed 2 times with BME 10 minutes or 1 or 3 hours after adding EGTA and were resuspended in BME plus serum without additional PHA or EGTA. The 48 hour samples were not washed.

$^3$RNA synthesis was determined at 48 hours with $^3$H-uridine present for the final 4 hours.

The results were the mean CPM ± SEM of 3 replicate cultures.
Figure 13.

Effect of EGTA on the initiation of RNA synthesis by PHA. O—O, unstimulated controls; ■■■, unstimulated controls + 1.39 mM EGTA; ●●●, PHA; X—X, PHA + 1.39 mM EGTA. EGTA was added 15 minutes prior to adding PHA, and the incorporation of $^3$H-uridine was determined at the indicated times with a 1 hour pulse. Each point is the mean CPM ± SEM of 3 or 4 replicate cultures expressed as a percentage of the control response in the absence of EGTA (165 CPM).
% of Control Level

$\text{H-Uridine Incorporation}$
Another early event in PHA-induced transformation is an enhancement of amino acid uptake which could be a critical preliminary preparation for the dramatic increases in protein synthesis observed later. As mentioned previously Ca\textsuperscript{++} can influence membrane permeability to other materials so the effect of lowering the Ca\textsuperscript{++} concentration on \textsuperscript{3}H-AIB uptake was studied in both resting and PHA-treated lymphocytes. The uptake of AIB by both unstimulated and PHA-treated lymphocytes was influenced by the Ca\textsuperscript{++} level in the medium (Table 5). It was clear that PHA enhanced AIB uptake after 3 hours by almost a factor of 6 at 1.25 mM Ca\textsuperscript{++}. If EGTA (1.4 mM) was added to the cultures, AIB uptake was inhibited by about 62% in both PHA and control cultures. The inhibitory effect of EGTA could be reversed by adding more Ca\textsuperscript{++}. Addition of 3 mM Ca\textsuperscript{++} to the EGTA-treated cells resulted in a reversal of most of the inhibition of AIB uptake (Table 5). Culturing the cells in medium containing 0.025 mM Ca\textsuperscript{++} resulted in a slight inhibition of uptake by unstimulated cells (20%) and a considerable inhibition of the PHA-enhanced uptake (59%).

These results did not show whether the inhibition of the PHA-stimulated AIB uptake at low Ca\textsuperscript{++} levels was due to a direct requirement of Ca\textsuperscript{++} for AIB accumulation or an indirect effect due to a general inhibition of the response. In order to clarify this point EGTA was added to cultures at various times relative to PHA and AIB
**TABLE 5**

Influence of Medium Calcium on the Uptake of AIB$^1$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AIB Uptake$^2$ (CPM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>1.25 mM Ca$^{++}$</td>
<td>694 ± 7</td>
</tr>
<tr>
<td>0.025 mM Ca$^{++}$</td>
<td>284 ± 42</td>
</tr>
<tr>
<td>1.40 mM EGTA</td>
<td>123 ± 6</td>
</tr>
<tr>
<td>1.40 mM EGTA + 3.00 mM Ca$^{++}$</td>
<td>635 ± 27</td>
</tr>
</tbody>
</table>

$^1$Lymphocytes were cultured with or without PHA for 3 hours in the presence of 1.25 or 0.025 mM Ca$^{++}$ or the even lower concentration available when 1.4 mM EGTA was added. $^3$H-AIB uptake was determined with a 15 minute pulse.

$^2$Each value is the mean CPM/10$^6$ lymphocytes/15 min ± SEM of 3 replicate cultures.
addition. Addition of EGTA \(\frac{1}{2}\) hour before or \(\frac{1}{2}\) hour after PHA reduced uptake to control levels (100% inhibition) (Table 6). When it was added 2 or 4 hours after PHA, the latter of which was immediately prior to the take determination, a marked degree of inhibition was still observed (80%). From this experiment it was concluded that most of the inhibition could be attributed to a direct requirement of Ca\(^{++}\) for AIB transport.

3.2.4 Summary

The results demonstrated that both EDTA and EGTA inhibited the PHA-induced transformation response as assessed by nucleic acid synthesis and morphological transformation. The effect of EGTA was entirely due to its ability to reduce the concentration of free Ca\(^{++}\) since only Ca\(^{++}\) could reverse the inhibition. From experiments in which Ca\(^{++}\) were added to Ca\(^{++}\)-free medium it could be estimated that transformation was a function of Ca\(^{++}\) concentration between about \(10^{-5}\) and \(6 \times 10^{-4}\)M. The latter was about one-half the normal physiological level. Normal responses occurred at higher concentrations. EGTA affected early events in transformation since it had no effect on nucleic acid synthesis in cells which had been stimulated for 48 hours before addition of the chelator. Also EGTA treatment was progressively less effective at causing inhibition with pro-
TABLE 6

Effect on AIB Uptake of Adding EGTA at Different Times Relative to PHA Addition

<table>
<thead>
<tr>
<th>Time of EGTA Addition Relative to PHA (Hrs)</th>
<th>AIB Uptake&lt;sup&gt;2&lt;/sup&gt;</th>
<th>CPM ± SEM</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA only</td>
<td></td>
<td>757 ± 17</td>
<td>0</td>
</tr>
<tr>
<td>-1/2</td>
<td></td>
<td>123 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>+1/2</td>
<td></td>
<td>140 ± 1</td>
<td>100</td>
</tr>
<tr>
<td>+2</td>
<td></td>
<td>260 ± 10</td>
<td>81</td>
</tr>
<tr>
<td>+4</td>
<td></td>
<td>256 ± 31</td>
<td>80</td>
</tr>
<tr>
<td>no PHA</td>
<td></td>
<td>136 ± 11</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>1</sup>Lymphocytes were cultured with PHA for 4 hours.

<sup>2</sup><sup>3</sup>H-AIB uptake was then determined with a 15 minute pulse.

<sup>2</sup>Each value is the mean uptake in CPM/10<sup>6</sup> lymphocytes/15 min ± SEM of 3 replicate cultures. They were then expressed as % inhibition of the PHA-stimulated uptake. Hence, 100% inhibition refers to a reduction in uptake to control levels.
gressively later additions relative to the time of PHA addition. This effect was not due to removal of essential PHA bound to the membrane. Adding it later than 12 hours after PHA had no effect on the subsequent response. Amino acid transport and early RNA synthesis were also inhibited at low exogenous Ca^{++} levels.
3.3 Calcium Transport in Lymphocytes and Alterations Induced by PHA

3.3.1 Introduction

The mechanism(s) of Ca\(^{++}\) transport across the plasma membrane of mammalian cells is as yet poorly understood. It has been established that Ca\(^{++}\) accumulation by subcellular organelles such as mitochondria (139, 140) and sarcoplasmic reticulum (141) is an energy-dependent active transport process. However, it is highly unlikely that a similar mechanism exists in the plasma membrane. There is a large inwardly directed Ca\(^{++}\) concentration gradient since the concentration ratio of extracellular to intracellular Ca\(^{++}\) is on the order of \(10^2\) to \(10^5\) (116). Nevertheless, Ca\(^{++}\) exchange takes place across the membrane which would tend to indicate that uptake would be a passive process with ions moving down their concentration gradient. Efflux in contrast would probably require expenditure of energy. These speculations have received some experimental support (116, 146, 147).

The influx of Ca\(^{++}\) in a variety of cells has been found to be energy-independent although it was not clear whether the process was more complex than simple diffusion (142, 143, 144). However, one recent report claimed that Ca\(^{++}\) uptake in chick embryo fibroblasts was
dependent on exogenous adenosine 5'-triphosphate (ATP) and Mg\(^{++}\) which was similar to the subcellular transport systems (145). Efflux of Ca\(^{++}\) from HeLa cells (146) and from human red cells (147) is energy-dependent considering its great sensitivity to reduced temperature and metabolic inhibitors. Also convincing evidence for the existence of a Ca\(^{++}\)-ATPase in red cell membranes has been presented (147, 148). Further it has been suggested that in nerve axons at least part of the Ca\(^{++}\) efflux is related to Na\(^+\) influx (149).

The previous chapter provided considerable evidence that Ca\(^{++}\) have an essential role in initiating lymphocyte transformation. Therefore, experiments were performed with the initial object of determining whether PHA could increase the intracellular concentration of Ca\(^{++}\) by stimulating its uptake. Further studies were aimed at elucidating the mechanism of Ca\(^{++}\) transport across the normal human lymphocyte plasma membrane since this had not been studied previously. It was important to consider this in order to see if the cells had a means of regulating Ca\(^{++}\) flux other than through equilibration by diffusion. The kinetics of PHA-induced alterations in the process of Ca\(^{++}\) translocation were also investigated in order to obtain information about the mechanism of stimulation of transport.
3.3.2 Effect of PHA on Calcium Uptake

Stimulation of lymphocytes with PHA caused an immediate increase in Ca\(^{++}\) uptake (Fig. 14a). A significant (p < 0.05) enhancement was detectable after only 1 minute. Equilibrium was reached after about 90 minutes, and the PHA-treated cells had accumulated three times as much Ca\(^{++}\) as the unstimulated cells by that time. More information can be gained if these data are transformed so as to give a straight line on a semilog plot (143, 144, 150). \(P_\infty\) refers to the uptake after 90 minutes, and \(P(t)\) refers to the uptake at any time "t". The semilog plot showed that uptake by both unstimulated and stimulated cells could be described by single although different rate constants i.e. only one straight line was obtained (Fig. 14b). PHA increased the rate constant for influx from 0.029 to 0.042/minute and correspondingly decreased the \(t_{1/2}\) from 21.5 to 15.5 minutes. PHA also rapidly stimulated Ca\(^{++}\) accumulation in cultures of mouse spleen cells (Table 7).

In order to test the possibility that Ca\(^{++}\) accumulation by lymphocytes might involve synthesis of new transport protein particularly after PHA stimulation the cells were treated with cycloheximide or actinomycin D. These inhibitors of protein and RNA synthesis respectively had no effect on Ca\(^{++}\) uptake by either resting or stimulated lymphocytes even 12 hours after PHA treatment.
Calcium uptake with time in unstimulated and PHA-treated lymphocytes. ●—●, PHA; O—O, unstimulated. a. PHA was added to cultures at 0 time, and $^{45}$Ca was immediately added. The cultures were incubated at 37° at a medium Ca$^{++}$ concentration of 1.25 mM for various lengths of time before assessing the uptake. Uptake was expressed as n moles/10$^6$ lymphocytes $\pm$ SEM of 3 or 4 replicate cultures. b. Semilog plot of the data in a. P$_{\infty}$ equalled uptake at 90 minutes, and P(t) equalled uptake at time t. The slope of the line equaled k, the rate constant, while $t_{1/2}$ was the time required to obtain $1/2$ the maximal uptake. The regression lines were fitted by the method of least squares. They were ln y = -0.042x + 4.58 and ln y = -0.029x + 4.54 for PHA-stimulated and unstimulated cells respectively.
TABLE 7

Enhanced Calcium Uptake by Mouse Spleen Cells Treated with PHA¹

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PHA</th>
<th>Control</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64.2 ± 2.0</td>
<td>41.6 ± 1.0</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>2</td>
<td>53.5 ± 1.9</td>
<td>38.9 ± 2.7</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

¹Mouse spleen cells were cultured with or without PHA for 1 hour at a Ca²⁺ concentration of 1.25 mM with ⁴⁵Ca present.

²Results are expressed as the mean uptake in n moles/10⁶ nucleated spleen cells ± SEM of 4 replicate cultures.
by which time both protein and RNA synthesis has increased considerably (Fig. 15).

The pH of the incubation medium had no effect on Ca\textsuperscript{++} uptake by PHA-treated cells within the range 6.5 to 7.5 (Fig. 16). Raising the pH to 8.0 resulted in a slight increase in accumulation while lowering it to 6.0 caused a highly significant decrease in uptake. A similar pattern was observed in unstimulated cultures except that uptake was significantly lower at pH 6.5 than it was at pH 7. These observations probably indicate a competition of H\textsuperscript{+} with Ca\textsuperscript{++} for binding sites or possibly other effects of pH on membrane structure and function.

3.3.3 Effect of Temperature and Metabolic Inhibitors on Calcium Uptake

The effects of temperature and various agents which inhibit cellular metabolism were tested in order to determine if Ca\textsuperscript{++} uptake by lymphocytes required expenditure of metabolic energy.

The temperature coefficient (Q\textsubscript{10}) for Ca\textsuperscript{++} uptake was determined by measuring the rate of accumulation at 7\degree, 17\degree, 27\degree and 37\degree. Calcium accumulation increased linearly with increasing temperature in both unstimulated and PHA-treated cultures (Fig. 17a). The average Q\textsubscript{10} values calculated from the regression lines were 1.82
Figure 15.

Calcium uptake in unstimulated and PHA-treated lymphocytes in the presence of cycloheximide and actinomycin D. [ ], PHA-treated (uptake determined from 0 to 1 hour after exposure); [ ], unstimulated; [ ], PHA-treated (uptake determined from 11 to 12 hours after exposure). Results are expressed as the mean uptake in n moles/10^6 lymphocytes ± SEM of 3 replicate cultures.
Figure 16.

Variation in lymphocyte calcium transport with pH of the incubation medium. ●—●, PHA; ○—○, unstimulated. Lymphocytes were suspended in media of different pH. PHA was added to the appropriate cultures, and calcium uptake was determined immediately at 37°C with a 30 minute incubation with $^{45}$Ca. Uptake was expressed as n moles/10$^6$ lymphocytes $\pm$ SEM of 3 replicate cultures.
and 1.58 respectively. Fig. 17b is an Arrhenius plot of the data in Fig. 17a. He showed that the activation energy (E) could be determined experimentally. The rate of a reaction depends on the number of molecules having the required activation energy. By plotting the natural log of the rates of the reaction at different temperatures against the reciprocal of the absolute temperature, E can be calculated from: 

$$E = 2.303 \left( \log k_2 - \log k_1 \right) \frac{R}{T_1 - T_2},$$

with R the gas constant, $k_1$ and $k_2$ the reaction rates at the absolute temperatures $T_1$ and $T_2$, 2.303 the factor for converting the natural log to log$_{10}$ (151). The E values were found to be 7990 and 6530 cal/mole for the control and PHA case respectively. The observed values for the temperature effect parameters are far below those observed in metabolically-dependent processes (151).

The effects of metabolic inhibitors on Ca$^{++}$ uptake fell into three groups. Azide and ouabain had no effect on Ca$^{++}$ accumulation by either stimulated or control cells (Table 8). Iodoacetamide and N-ethylmaleimide inhibited both normal and stimulated uptake with the latter being more effective. In contrast sodium fluoride at 5 mM enhanced Ca$^{++}$ uptake in both cases by a factor of more than 2.

3.3.4 Kinetics of Calcium Uptake by Unstimulated Lymphocytes

The previous observations that Ca$^{++}$ uptake has a
Figure 17.

Influence of temperature on calcium uptake by unstimulated and PHA-treated lymphocytes. •---, PHA; ○—○, unstimulated. a. The initial rate of calcium uptake was determined with a 15 minute incubation with $^{45}$Ca at a Ca$^{++}$ concentration of 0.025 mM. The cultures were pre-cooled to the respective temperature before the isotope was added. Uptake was determined immediately after PHA addition. The rate of uptake was expressed as n moles/10$^6$ lymphocytes/min + SEM of 3 replicate cultures. b. An Arrhenius plot of the data in a. The regression lines were fitted by the method of least squares. For a. they were $y = 0.0046x + 0.0102$ and $y = 0.0080x + 0.0579$ for unstimulated and PHA-stimulated cells respectively. For b. they were $\ln y = -4.02x + 15.9$ and $\ln y = 3.29x + 14.2$ respectively.
TABLE 8

<table>
<thead>
<tr>
<th>Agent</th>
<th>Calcium Uptake $^3$ (n moles ± SEM)</th>
<th>PHA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>12.5 ± 0.49</td>
<td>8.22 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>Azide</td>
<td>12.3 ± 0.17</td>
<td>8.09 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>12.8 ± 0.45</td>
<td>7.97 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>9.03 ± 0.29</td>
<td>7.32 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>7.16 ± 0.52</td>
<td>5.38 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Fluoride</td>
<td>26.8 ± 1.2</td>
<td>18.1 ± 0.87</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Lymphocytes were incubated with or without PHA for 30 minutes at a Ca$^{++}$ concentration of 0.05 mM.

$^2$ Each agent was added at a concentration of 1 mM except for sodium fluoride (5 mM) for 1 hour before PHA addition. Calcium uptake was determined for 30 minutes after PHA addition.

$^3$ The results were expressed as the mean uptake in n moles/10$^6$ lymphocytes ± SEM of 3 replicate cultures.
low $Q_{10}$ and is relatively insensitive to metabolic inhibitors provided evidence that Ca$^{++}$ accumulation by lymphocytes is an energy-independent process. However, it was likely that uptake was a more complex process than simple diffusion possibly involving participation of a carrier molecule.

A typical characteristic of a carrier-mediated transport system is that the system becomes saturated at high substrate concentrations so that further increases in concentration do not increase the rate of uptake (152, 153). The kinetics of transport of the substance are then analogous to enzyme kinetics and can be expressed by the standard Michaelis-Menten formulation which describes a rectangular hyperbola (154).

$$v = \frac{S \ \text{V}_{\text{max}}}{S + K_m}$$

In this equation for Ca$^{++}$ transport $v$ = the initial rate of uptake, $V_{\text{max}}$ = the maximum rate of uptake which can be attained under the specified conditions, $S$ = substrate concentration (Ca$^{++}$) and $K_m$ = the apparent Michaelis constant which is numerically equivalent to the concentration at which $\frac{1}{2} V_{\text{max}}$ occurs. It offers an estimate of the affinity of the carrier molecule for the substrate (Ca$^{++}$ in this case).

The rate of Ca$^{++}$ uptake by lymphocytes was found to
be constant for at least 16 minutes at both high and low Ca$^{++}$ concentrations (Table 9). Therefore, the initial rate of uptake, or the slope of the uptake curve would be the same when determined at any time up to 16 minutes. In all further experiments the rate of uptake was assessed with a 10 minute incubation with isotope.

Calcium uptake by unstimulated lymphocytes exhibited saturation kinetics with the maximum rate of uptake occurring at about 0.8 mM when the initial rate of uptake was plotted against Ca$^{++}$ concentration on a linear scale (Fig. 18a). It is rather difficult to determine $V_{\text{max}}$ and $K_m$ values from the hyperbolic relationship of the Michaelis-Menten equation. However, the equation can be rearranged in several simple ways (154) to provide linear relationships two of which are given below.

$$\frac{1}{v} = \frac{K_m}{V_{\text{max}}} \cdot \frac{1}{S} + \frac{1}{V_{\text{max}}} \quad \text{(Lineweaver-Burk)}$$

$$v = -K_m \cdot \frac{v}{S} + V_{\text{max}} \quad \text{(van Hofstee)}$$

Transforming the data in Fig. 18a by the van Hofstee method gave a straight line ($r = -0.980$, $p < 0.001$) from which the value of $K_m = 0.73$ mM and $V_{\text{max}} = 13.0$ nmoles/minute were calculated (Fig. 18b).

Inhibition of a transport process by structurally
### TABLE 9

**Demonstration of the Constant Rate of Calcium Uptake**

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Calcium Uptake (nmoles ± SEM)</th>
<th>0.025 mM Ca(^{++})</th>
<th>1.25 mM Ca(^{++})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
<td>Control</td>
<td>PHA</td>
</tr>
<tr>
<td>4</td>
<td>2.46 ± .19</td>
<td>0.98 ± .05</td>
<td>26.6 ± 1.6</td>
</tr>
<tr>
<td>8</td>
<td>3.07 ± .10</td>
<td>1.46 ± .04</td>
<td>39.8 ± 2.2</td>
</tr>
<tr>
<td>12</td>
<td>3.87 ± .32</td>
<td>1.62 ± .07</td>
<td>47.5 ± .81</td>
</tr>
<tr>
<td>16</td>
<td>4.32 ± .22</td>
<td>1.90 ± .09</td>
<td>64.2 ± 1.0</td>
</tr>
</tbody>
</table>

**correlation**

- 0.995
- 0.978
- 0.991
- 0.987

**t-test**

- p < 0.01
- p < 0.02
- p < 0.01
- p < 0.02

\(^1\)PHA or saline and Ca\(^{45}\) was added to cultures. They were then assayed at the indicated times.

\(^2\)Each value is the mean uptake in nmoles/10\(^6\) lymphocytes ± SEM of 3 replicate cultures.

\(^3\)The constant rate of uptake is indicated by the respective correlation coefficients.
Figure 18.

Effect of medium Ca$^{++}$ concentration on calcium uptake by unstimulated lymphocytes.  a.  The initial rate of calcium uptake (v) was determined at 37$^\circ$ with a 10 minute incubation with $^{45}$Ca at different Ca$^{++}$ concentrations (S in mM). The rate of uptake was expressed as n moles/10$^6$ lymphocytes/min $\pm$ SEM of 3 or 4 replicate cultures.  b.  A van Hofstee plot (v versus v/S) of the data in a.  for the determination of the maximal velocity ($V_{max}$) and the apparent Michaelis constant ($K_m$). The regression line was fitted by least squares analysis and was found to be $y = -0.728x + 13.0.$
similar molecules, other divalent cations in this case, can also be taken as evidence for the mediation of a carrier. The effect of manganese (Mn\(^{++}\)) was tested since it has been found to inhibit Ca\(^{++}\) accumulation by chick embryo fibroblasts (145), and since it seems to be accumulated by the same mechanism as Ca\(^{++}\) in mitochondria (140). Manganese at a concentration of 5 mM inhibited Ca\(^{++}\) accumulation by unstimulated lymphocytes (Fig. 19a). Replotting this data by the method of Lineweaver and Burk yielded a straight line for uptake in the absence \(r = 0.999, p < 0.001\) and presence \(r = 0.993, p < 0.01\) of Mn\(^{++}\) (Fig. 19b). From the calculated regression equations it was apparent that Mn\(^{++}\) did not alter the \(V_{\text{max}}\) of Ca\(^{++}\) uptake \(2.77 \text{nmoles/minute in the uninhibited case compared to 2.84 with Mn}^{++}\) present) which is consistent with a competitive type of inhibition (154). The Lineweaver-Burk equation can be modified to allow calculation of an affinity constant for the inhibitor \(K_I\) as follows (154) with I equal to the inhibitor concentration.

\[
\frac{1}{v} = \frac{K_m}{V_{\text{max}}} \left(1 + \frac{I}{K_I}\right) \cdot \frac{1}{S} + \frac{1}{V_{\text{max}}}
\]
Thus, the intercept on the 1/S axis for the inhibited case would be given by the following expression.

\[
-\frac{1}{K_m (1 + I/K_I)}
\]

The apparent Michaelis constant \(K_m\) for the uninhibited case was 0.48 mM, I was 5 mM and the intercept was -0.362. From these values \(K_I\) was calculated to be 1.1 mM. Thus, the affinity of the carrier for Ca\(^{++}\) was greater than its affinity for Mn\(^{++}\) by about a factor of 2.3.

Another phenomenon often associated with the presence and the reversibility of carrier-mediated transport systems is exchange diffusion (128). The phenomenon refers to an exchange of intracellular molecules or ions with extracellular ones with no net flux. The rate of radioactive tracer influx would be greater if exchange diffusion were operating than the maximum obtainable net rate of influx in the absence of exchange diffusion. Two different experiments were performed to test for an exchange diffusion mechanism for Ca\(^{++}\) uptake in unstimulated lymphocytes.

In one experiment some lymphocytes were pre-loaded at 2 mM Ca\(^{++}\) for 2 hours while others were not pre-loaded. All samples were then washed and resuspended
Inhibition of calcium uptake in unstimulated lymphocytes by manganese. ●—●, no Mn\(^{++}\); ○—○, 5 mM Mn\(^{++}\). a. The initial rate of calcium uptake (v) was determined at 37° with a 10 minute incubation with \(^{45}\)Ca at different Ca\(^{++}\) concentrations (S in mM). The rate of uptake was expressed as n moles/10^6 lymphocytes/min ± SEM of 3 replicate cultures. b. A Lineweaver-Burk plot (1/v versus 1/S) of the data in a. from which \(V_{\text{max}}\) and \(K_m\) could be determined. The regression lines were fitted by the method of least squares and were found to be \(y = 0.174x + 0.360\) and \(y = 0.973x + 0.350\) in the absence and presence of manganese.
in medium containing 0.025 mM Ca\textsuperscript{++} plus \textsuperscript{45}Ca. No significant differences in uptake were observed in the pre-loaded cells (Fig. 20a).

In the other experiment the rate of Ca\textsuperscript{++} uptake under steady state conditions was compared to the net rate of uptake at the same exogenous Ca\textsuperscript{++} level; that is, one set of cultures was incubated at normal Ca\textsuperscript{++} levels (1.25 mM) for several hours to pre-load the cells. Calcium uptake at 1.25 mM exogenous Ca\textsuperscript{++} by these pre-loaded cells was then compared to the uptake in cells which were not pre-loaded. Again there was no difference in uptake in the two culture conditions (Fig. 20b).

### 3.3.5 Alterations in the Kinetics of Calcium Transport Induced by PHA

The PHA-stimulated Ca\textsuperscript{++} uptake also exhibited saturation kinetics when uptake was determined immediately after PHA treatment (Fig. 21a). There was a significant increase in the initial rate of Ca\textsuperscript{++} accumulation at all external Ca\textsuperscript{++} concentrations. When the data was re-plotted by the van Hofstee method, linear relationships were obtained for both unstimulated ($r = -0.927, p < 0.001$) and PHA-enhanced ($r = -0.954, p < 0.001$) uptake (Fig. 21b). From the regression lines the values for $V_{\text{max}}$ were calculated to be 2.6 and 2.5 nmoles/minute
Figure 20.

Test for an exchange diffusion mechanism for calcium transport in unstimulated lymphocytes. ●—●, pre-loaded cells; ○—○, non-loaded cells. a. Uptake was determined at 37° at a Ca++ concentration of 0.025 mM. b. Uptake was determined at 37° at a Ca++ concentration of 1.25 mM. In both cases uptake was expressed as n moles/10⁶ lymphocytes ± SEM of 3 replicate cultures.
Effect of medium Ca$^{++}$ concentration on calcium uptake in unstimulated lymphocytes and PHA-stimulated lymphocytes immediately after PHA addition. ●●, PHA; ○○, unstimulated. a. The initial rate of uptake (v) was determined at 37° with a 10 minute incubation with $^{45}$Ca at different Ca$^{++}$ concentrations (S) initiated immediately after PHA addition. The rate of uptake was expressed as n moles/10$^6$ lymphocytes/min$^+$ SEM of 3 or 4 replicate cultures. b. A van Hofstee plot of the data in a. (v versus v/S) for the determination of $V_{max}$ and $K_m$. The regression lines were fitted by the method of least squares. They were $y = -0.469x + 2.6$ and $y = -0.231x + 2.5$ for unstimulated and PHA-stimulated cells respectively.
respectively which were not significantly different. However, the \( K_m \) was markedly reduced by PHA treatment from 0.47 to 0.23 mM.

When the kinetics of uptake were followed 3 hours after PHA stimulation, by which time many other processes in the response were in progress, the rate of Ca\(^{++}\) uptake again was saturable (Fig. 22a). The van Hofstee plot yielded linear relationships for control (\( r = -0.813, p<0.02 \)) and stimulated uptake (\( r = -0.975, p<0.001 \)) (Fig. 22b). In this case PHA reduced the \( K_m \) value from 0.74 to 0.30 mM but also increased the \( V_{max} \) from 4.4 to 5.3 nmoles/minute.

The effect of Mg\(^{++}\) on Ca\(^{++}\) uptake was tested next. Magnesium ions usually do not compete with Ca\(^{++}\) for transport, and they may potentiate Ca\(^{++}\) uptake so it was conceivable that Mg\(^{++}\) could have had an important effect on the PHA-stimulated uptake. However, it was observed that Mg\(^{++}\) at 1 mM, which is close to physiological, had no effect whatsoever on either unstimulated or stimulated uptake (Fig. 23).

Tests for exchange diffusion in PHA-stimulated lymphocytes were also made essentially as described previously for unstimulated cells. In one type of experiment lymphocytes were pre-incubated with 0.025 or 1.25 mM Ca\(^{++}\) or with 1.4 mM EGTA to remove most of the residual medium Ca\(^{++}\). They were then washed and resuspended in 0.025 mM Ca\(^{++}\), and uptake was determined for 20
Effect of medium Ca\textsuperscript{++} concentration on calcium uptake in unstimulated lymphocytes and lymphocytes exposed to PHA for 3 hours. ●●, PHA; ○○, unstimulated.

a. The initial rate of uptake (v) was determined at 37° with a 10 minute exposure to \(^{45}\)Ca at different Ca\textsuperscript{++} concentrations (S in mM) 3 hours after PHA treatment. The rate of uptake was expressed as n moles/10\(^6\) lymphocytes/min ± SEM of 3 or 4 replicate cultures. b. A van Hofstee plot (v versus v/S) of the data in a. for the determination of \(V_{\text{max}}\) and \(K_m\). The regression lines were fitted by the method of least squares. They were \(y = -0.741x + 4.4\) and \(y = -0.295x + 5.3\) for unstimulated and PHA-stimulated cells respectively.
Figure 23.

Effect of Mg$^{++}$ on calcium uptake in unstimulated and PHA-stimulated lymphocytes. 
●—●, PHA (no Mg$^{++}$); ■—■, PHA (1 mM Mg$^{++}$); ○—○, unstimulated (no Mg$^{++}$); 
□—□, unstimulated (1 mM Mg$^{++}$). The initial rate of calcium uptake ($v$) was determined at 37$^\circ$ with a 10 minute incubation with $^{45}$Ca at different Ca$^{++}$ concentrations (S in mM). The rate of uptake was expressed as n moles/10$^6$ lymphocytes/min $\pm$ SEM of 3 replicate cultures.
minutes. There were no significant differences in either stimulated or unstimulated uptake regardless of the pre-incubation conditions (Table 10).

In the other experiment the cells were pre-incubated with 0.025 or 1.25 mM Ca++, and uptake was determined after 10 and 30 minutes at an extracellular Ca++ concentration of 1.25 mM. Again there were no significant differences in uptake by either stimulated or control cells regardless of the pre-incubation conditions (Table 11).

It was possible that PHA might also increase the net Ca++ accumulation by lymphocytes by inhibiting Ca++ efflux. In order to test this possibility unstimulated lymphocytes were pre-incubated with $^{45}$Ca and then were washed with saline and resuspended in medium. PHA was then added, and the fraction of activity in the supernatant was determined at various times thereafter. However, it was found that PHA had no effect on Ca++ efflux from pre-loaded cells even after 90 minutes exposure (Table 12).

3.3.6 Summary

PHA caused a marked increase in Ca++ accumulation within 1 minute after exposure. The maximum uptake was about 3 times greater in the PHA-stimulated cells than in the unstimulated controls. PHA also enhanced uptake
TABLE 10

Test for Exchange Diffusion Transport of Calcium by Lymphocytes at a Medium Calcium Ion Concentration of 0.025 mM

<table>
<thead>
<tr>
<th>Pre-incubation Conditions</th>
<th>Calcium Uptake(^2) (n moles ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>1.25 mM Ca(^{++})</td>
<td>0.867 ± 0.06</td>
</tr>
<tr>
<td>0.025 mM Ca(^{++})</td>
<td>0.888 ± 0.07</td>
</tr>
<tr>
<td>1.40 mM EGTA</td>
<td>0.895 ± 0.04</td>
</tr>
</tbody>
</table>

\(^1\) Unstimulated lymphocytes were incubated for 2 hours in medium containing 1.25 or 0.025 mM Ca\(^{++}\) or at the even lower concentration afforded by adding 1.4 mM EGTA. The cells were then washed and resuspended in medium with 0.025 mM Ca\(^{++}\). \(^{45}\)Ca and PHA were added, and the uptake was determined after a 20 minute incubation period.

\(^2\) The results were expressed as the mean uptake in n moles/10\(^6\) lymphocytes ± SEM of triplicate cultures.
<table>
<thead>
<tr>
<th>Pre-incubation Calcium Uptake</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca++</td>
<td></td>
</tr>
<tr>
<td>Concentration (mM)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>0.025</td>
<td>54.8 ± 2.3</td>
</tr>
<tr>
<td>1.25</td>
<td>52.0 ± 4.6</td>
</tr>
</tbody>
</table>

1Unstimulated lymphocytes were incubated for 2 hours with either 0.025 or 1.25 mM Ca++. At the end of this period PHA and Ca++ were added to the low Ca++ cultures to bring their Ca++ level to 1.25 mM. $^{45}$Ca was added, and the uptake was determined after 10 and 30 minutes of incubation.

2The results were expressed as the mean uptake in n moles/10^6 lymphocytes ± SEM of 3 replicate cultures.
TABLE 12

Effect of PHA on Calcium Efflux from Pre-labeled Lymphocytes

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>$^{45}$Ca in supernatant $^2$ (%)</th>
<th>PHA</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>$62.3 \pm 1.4$</td>
<td>64.7 $\pm$ 2.3</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>$77.4 \pm 2.0$</td>
<td>79.0 $\pm$ 2.5</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>$82.7 \pm 1.8$</td>
<td>84.3 $\pm$ 1.4</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>$84.3 \pm 2.0$</td>
<td>85.3 $\pm$ 2.6</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Unstimulated lymphocytes were incubated for 2 hours with 5 $\mu$Ci of $^{45}$Ca. They were then washed 3 times at 4°C and resuspended in 1.0 ml of medium having a Ca$^{++}$ concentration of 1.25 mM. PHA was then added to some of the cultures which were incubated at 37°C. At the indicated times the cells were spun down, and the percentage of the activity in the supernatant was determined.

$^2$The results are expressed as the mean % $\pm$ SEM of 3 replicate cultures.
by mouse spleen cells. Neither cycloheximide nor actinomycin D had any effect on PHA-stimulated or control Ca\(^{++}\) accumulation. Uptake was increased at high pH while it was decreased at low pH.

The Ca\(^{++}\) transport process had a low \(Q_{10}\) and activation energy and was not affected by the metabolic inhibitors, azide and ouabain, indicating that the process was mainly energy-independent. However, uptake was stimulated by fluoride and was inhibited by sulphydryl reagents.

Kinetic studies showed that Ca\(^{++}\) uptake in unstimulated cells was a saturable process. Manganese competed with Ca\(^{++}\) for transport by resting cells, but Mg\(^{++}\) did not, and exchange diffusion was not observed. PHA altered the kinetics of uptake by immediately increasing the affinity of the membrane binding sites for Ca\(^{++}\) (decrease in \(K_m\)). As the response progressed (3 hours), a small increase in \(V_{max}\) occurred in addition to the decreased \(K_m\). Exchange diffusion was also not observed for stimulated cells, and Mg\(^{++}\) again had no effect on either unstimulated or PHA-stimulated uptake. PHA did not affect Ca\(^{++}\) efflux from pre-loaded cells.
3.4 Effects of Cyclic AMP and Related Agents on Resting and Activated Lymphocytes

3.4.1 Introduction

Cyclic AMP has for some time been recognized as being the main mediator of the action of many hormones on their target cells (116). However, in the past few years it has been realized that cAMP can exert a regulatory influence on the transcription of certain genes (156) and on the translation of mRNA (156) which suggests that the nucleotide may be an important regulator of cell proliferation. Indeed Whitfield et al. have provided strong evidence that cAMP can regulate proliferation of rat thymocytes (157, 158, 159, 160, 161) and it has been shown that contact inhibition of growth is restored to virally transformed fibroblasts by treating them with cAMP (162, 163).

Therefore, the effects of exogenous cAMP and related agents on unstimulated and PHA-stimulated lymphocytes were tested in order to provide information on the possible role of cAMP in the mediation of transformation. These experiments were particularly relevant since the previous results had established that exogenous Ca$^{++}$ are essential to transformation, and that PHA stimulated Ca$^{++}$ uptake. One or both of these features are observed in cAMP-mediated hormone action (116) and
in the cAMP-mediated enhancement of rat thymocyte proliferation (157, 158, 159, 160, 161).

3.4.2 Effects on Unstimulated and PHA-Stimulated Nucleic Acid Synthesis

Exogenous cAMP and a dibutyril derivative, N^6, O^2'-dibutyril adenosine 3',5'-cyclic monophosphate (DB-cAMP), which penetrates the membrane more readily than cAMP (164) and which is less susceptible to enzymatic degradation (165), had no effect on PHA-stimulated DNA synthesis in the physiological concentration range of 10^{-8} to 10^{-6}M (Fig. 24). However, with increasing concentration above 10^{-5}M for DB-cAMP and 10^{-4}M for cAMP each agent progressively inhibited isotope incorporation. Complete inhibition was observed at concentrations of 3 \times 10^{-3} and 8 \times 10^{-3}M for DB-cAMP and cAMP respectively. The non-cyclic nucleotide, ATP, which is the substrate for cAMP production also progressively inhibited DNA synthesis in the concentration range from 10^{-5} to 8 \times 10^{-3}M (Fig. 24).

The methyl xanthines, caffeine and theophylline, cause an increase in intracellular cAMP levels by inhibiting the cAMP-degrading enzyme, phosphodiesterase, while imidazole decreases the intracellular cAMP concentration by stimulating the phosphodiesterase (Fig. 25) (166). Both caffeine and theophylline progressively
Inhibition of PHA-stimulated lymphocyte transformation by exogenous nucleotides. 

●●, DB-cAMP; ○○, cAMP; □□, ATP. The agents were added 15 minutes before PHA. DNA synthesis was determined at 72 hours with $^3$H-thymidine present for the final 4 hours. Each point is the mean CPM $\pm$ SEM of 3 or 4 replicate cultures expressed as a percentage of the PHA response observed with no agent added (5230 CPM).
Figure 25.

Diagram of the intracellular metabolism of cyclic AMP. —— , inhibition; —— , potentiation.
inhibited PHA-stimulated DNA synthesis (Fig. 26). Theophylline was a more effective inhibitor, but complete inhibition was obtained with both at about $5 \times 10^{-3}$ to $7 \times 10^{-3}$M. Imidazole had no effect on DNA synthesis up to $10^{-3}$M (Fig. 26). However, concentrations from $2 \times 10^{-3}$ to $8 \times 10^{-3}$ produced about a 45% inhibition of isotope incorporation.

Cyclic AMP and DB-cAMP at concentrations ranging from $10^{-8}$ to $10^{-4}$M were both completely ineffective at stimulating DNA synthesis in resting lymphocytes in contrast to PHA which stimulated synthesis by a factor of about 70 over the control level (Table 13).

It was important to distinguish whether the cyclic nucleotides and related agents directly inhibited DNA synthesis or whether they blocked earlier initiating events as was found in the EGTA studies. Therefore, lymphocytes were treated with PHA alone for 48 hours. The various agents were then added, and DNA synthesis was followed for an additional 72 hours. Both imidazole and theophylline at 1 mM had no effect on subsequent isotope incorporation even up to 72 hours after their addition (Fig. 27a). However, ATP immediately reduced synthesis by about 55% at which level it remained for 72 hours except for a slight increase at 6 hours (Fig. 27a). Dibutyryl cAMP at either the inhibitory concentration of $10^{-4}$M or at $10^{-7}$M had no effect on the late synthetic phase of the response even after 72 hours.
PHA response in the presence of agents which influence intracellular cyclic AMP metabolism. ●—●, theophylline; ○—○, caffeine; □—□, imidazole. The agents were added 15 minutes before PHA. DNA synthesis was determined at 72 hours with $^3$H-thymidine present for the final 4 hours. Each point is the mean CPM ± SEM of 3 or 4 replicate cultures expressed as a percentage of the PHA response observed with no agent added (5230 CPM).


<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>$^3$H-Thymidine Incorporation (CPM ± SEM)</th>
<th>cAMP</th>
<th>DB-cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>409 ± 20</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>418 ± 13</td>
<td>407 ± 6</td>
<td></td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>431 ± 9</td>
<td>412 ± 12</td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>397 ± 6</td>
<td>438 ± 10</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>460 ± 15</td>
<td>407 ± 12</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>29,000 ± 1100</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

1Unstimulated lymphocytes were cultured with the cyclic nucleotides for 72 hours with $^3$H-thymidine present for the final 16 hours. DNA synthesis in the PHA-treated cells was determined over the same interval.

2Each value is the mean CPM ± SEM of 3 to 5 replicate cultures.
exposure (Fig. 27b). Although it was not tested in the particular experiment shown, cAMP also had no effect on subsequent DNA synthesis. EGTA and DB-cAMP in combination did not inhibit the fully-developed response either (Fig. 27b).

It was possible that the cyclic nucleotides were able to initiate RNA synthesis in unstimulated cells in spite of their ineffectiveness at stimulating DNA synthesis which is a late event in PHA-induced transformation. Also it was necessary to determine whether they could either potentiate or inhibit the early development of RNA synthesis in cells stimulated with PHA at both the optimal and sub-optimal concentration. The latter case was investigated because it was conceivable that stimulation with optimum doses caused the maximum possible level of activation such that any cAMP potentiating effect would be masked. Cyclic AMP at physiological concentrations ($10^{-8}$ or $10^{-7}$M) or at concentrations which inhibited DNA synthesis in PHA-stimulated cultures ($10^{-4}$ or $10^{-3}$M) had no effect on $^3$H-uridine incorporation by resting lymphocytes after 6 hours (Table 14). Similarly it had no effect on incorporation by lymphocytes treated with a very low dose of PHA (0.038 µg/ml) which does not stimulate DNA synthesis. However, at $10^{-3}$M cAMP produced a 35% inhibition of the development of early RNA synthesis in cells treated with an optimal concentration of PHA (0.75 µg/ml) while other concentra-
Figure 27.

Effect of cyclic nucleotides and related agents on DNA synthesis in previously stimulated lymphocytes. a. •• , PHA only; O—O , theophylline (1 mM); ■■ , imidazole (1 mM); X—X , ATP (5 mM). b. •• , PHA only; O—O , DB-cAMP (10^{-3}M); ■■ , DB-cAMP (10^{-7}M); X—X , DB-cAMP (10^{-7}M) + EGTA (1.42 mM). All cultures were treated with PHA alone for 48 hours. Then the respective agents were added at the indicated concentrations, and the incorporation of ^3H-thymidine was determined at various times thereafter with a 2 hour pulse. Each point is the mean CPM ± SEM of 3 or 4 replicate cultures expressed as a percentage of the maximum level of synthesis observed (4120 CPM).
### TABLE 14

Influence of Cyclic AMP on the Early Incorporation of $^3$H-Uridine by PHA-Stimulated and Unstimulated Lymphocytes

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>$^3$H-Uridine Incorporation $^2$ (CPM ± SEM)</th>
<th>PHA Concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.038 0.750</td>
</tr>
<tr>
<td>0</td>
<td>114 ± 9</td>
<td>137 ± 5 358 ± 8</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>110 ± 4</td>
<td>126 ± 11 328 ± 18</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>110 ± 4</td>
<td>127 ± 9 332 ± 21</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>99 ± 3</td>
<td>125 ± 6 322 ± 12</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>105 ± 8</td>
<td>119 ± 10 225 ± 17</td>
</tr>
</tbody>
</table>

$^1$Unstimulated lymphocytes or lymphocytes treated with either a sub-optimal or an optimal dose of PHA were cultured with various concentrations of cyclic AMP for 6 hours with $^3$H-uridine present for the final 1 hour.

$^2$Each value is the mean CPM ± SEM of 3 replicate cultures.
tions of cAMP had no effect. This concentration also produced a 50% inhibition of DNA synthesis.

3.4.3 Effects on Lymphocyte Transport Properties

Cyclic AMP, DB-cAMP, theophylline and the combination of DB-cAMP and theophylline all caused a small but statistically significant enhancement of Ca\(^{++}\) accumulation by unstimulated lymphocytes (Table 15). Lymphocytes treated with a sub-optimal concentration of PHA (0.19 \(\mu\)g/ml) were unaffected by DB-cAMP at either 2 \(\times\) 10\(^{-7}\) or 10\(^{-3}\)M or by cAMP at 10\(^{-3}\)M while addition of 2 \(\times\) 10\(^{-7}\)M cAMP, 5 \(\times\) 10\(^{-3}\)M theophylline or DB-cAMP plus theophylline, produced small increases in Ca\(^{++}\) uptake. However, all the agents inhibited Ca\(^{++}\) accumulation by cells treated with an optimal concentration of PHA (1.5 \(\mu\)g/ml).

Exogenous ATP is known to stimulate Ca\(^{++}\) accumulation by subcellular transport systems such as in mitochondria which are energy-dependent (139, 140) and also by cultured chick embryo fibroblasts (145). Exogenous ATP produced a small but significant stimulation of Ca\(^{++}\) uptake by unstimulated lymphocytes in the concentration range from 5 \(\times\) 10\(^{-4}\) to 4 \(\times\) 10\(^{-3}\)M although it inhibited uptake at 8 \(\times\) 10\(^{-3}\)M (Fig. 28). However, it progressively inhibited PHA-stimulated Ca\(^{++}\) accumulation with increasing concentration above 5 \(\times\) 10\(^{-4}\)M.
### Table 15

Alterations in Calcium Uptake by Lymphocytes in the Presence of Cyclic AMP, Dibutryl Cyclic AMP and Theophylline

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (M)</th>
<th>Calcium Uptake (nmoles ± SEM)</th>
<th>PHA Concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.19</td>
</tr>
<tr>
<td>none</td>
<td></td>
<td>17.0 ± .17</td>
<td>36.3 ± 1.3</td>
</tr>
<tr>
<td>cAMP</td>
<td>1 x 10^{-3}</td>
<td>18.9 ± .78</td>
<td>38.3 ± 2.4</td>
</tr>
<tr>
<td>DB-cAMP</td>
<td>1 x 10^{-3}</td>
<td>19.6 ± .61</td>
<td>37.0 ± .87</td>
</tr>
<tr>
<td>cAMP</td>
<td>2 x 10^{-7}</td>
<td>19.0 ± .61</td>
<td>40.0 ± .35</td>
</tr>
<tr>
<td>DB-cAMP</td>
<td>2 x 10^{-7}</td>
<td>20.3 ± .70</td>
<td>36.0 ± .78</td>
</tr>
<tr>
<td>theophylline</td>
<td>5 x 10^{-3}</td>
<td>19.7 ± .87</td>
<td>40.3 ± .43</td>
</tr>
<tr>
<td>DB-cAMP</td>
<td>2 x 10^{-7}</td>
<td>22.6 ± 1.0</td>
<td>42.1 ± 2.9</td>
</tr>
<tr>
<td>theophylline</td>
<td>5 x 10^{-3}</td>
<td>19.7 ± .87</td>
<td>40.3 ± .43</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Uptake of $^{45}$Ca was determined with a 1 hour isotope incubation initiated at the time of PHA addition.

2. The various agents were added to the cultures 15 minutes before the $^{45}$Ca addition. The Ca$^{++}$ concentration was 1.25 mM.

3. Each value is the mean uptake in nmoles/10^6 lymphocytes ± SEM of 3 replicate cultures.
Influence of exogenous ATP on calcium uptake in unstimulated and PHA-treated lymphocytes. ●●, PHA; ○○, unstimulated. ATP was added 15 minutes before the PHA, and uptake was determined with a 30 minute incubation with $^{45}\text{Ca}$ initiated immediately after PHA addition. Results are expressed as the mean uptake in n moles/10^6 lymphocytes ± SEM of 3 replicate cultures.
In order to better characterize the manner in which the cyclic nucleotides affected Ca\textsuperscript{++} uptake the effect of DB-cAMP on the kinetics of Ca\textsuperscript{++} accumulation was studied. Dibutyryl cyclic AMP at a concentration of 2 mM significantly inhibited Ca\textsuperscript{++} uptake by PHA-stimulated lymphocytes up to a medium Ca\textsuperscript{++} concentration of 0.6 mM although no inhibition was observed at 1.0 mM (Fig. 29a). Replotting this data by the method of Lineweaver and Burk produced a straight line for both the uninhibited case \((r = 0.998, p < 0.001)\) and the inhibited case \((r = 0.998, p < 0.001)\) (Fig. 29b). From these equations the values for \(V_{\text{max}}\) were calculated to be 5.7 and 4.5 nmoles/minute respectively. The corresponding \(K_m\) values were 0.53 and 0.47 mM. Hence, DB-cAMP decreased both \(V_{\text{max}}\) and \(K_m\) so the inhibition could not be classified as either competitive or non-competitive but may actually be a combination of the two. DB-cAMP significantly stimulated Ca\textsuperscript{++} uptake in controls at 0.05 and 1.0 mM Ca\textsuperscript{++}, but did not significantly alter it at 0.2 and 0.6 mM Ca\textsuperscript{++} (Fig. 29a). The Lineweaver-Burk plot (Fig. 29b) yielded straight lines for uptake in the absence of DB-cAMP \((r = 0.999, p < 0.001)\) and in its presence \((r = 0.956, p < 0.01)\). The values of \(V_{\text{max}}\) were 4.4 and 2.2 nmoles/minute, and the \(K_m\) values were 0.86 and 0.31 mM respectively. Thus, DB-cAMP alters both, but it increased the affinity for Ca\textsuperscript{++} (decreased \(K_m\)) to a greater extent. Comparing the values for normal
control uptake to PHA-stimulated uptake in the presence of DB-cAMP, it may be significant that $V_{\text{max}}$ values were identical (4.4 and 4.5) while $K_m$ was considerably decreased from 0.86 to 0.47 mM.

The effects of cAMP and related molecules on AIB uptake by lymphocytes were also tested. Cyclic AMP, DB-cAMP, ATP and theophylline all reduced PHA-stimulated AIB uptake to control levels when present at concentrations which were previously found to inhibit nucleic acid synthesis (Table 16). None of them had significant effects on unstimulated uptake.

3.4.4 Summary

Cyclic nucleotides, ATP and other agents including caffeine, theophylline and imidazole which alter cAMP metabolism were found to inhibit the PHA response as measured by nucleic acid synthesis when present from the beginning of the culture period and at higher than physiological concentrations. However, similar to EGTA they had no effect on DNA synthesis when added after the response had developed completely except for ATP which caused about a 50% inhibition. Physiological concentrations of cAMP (about $10^{-8}$ to $10^{-6}$M) had no effect on the PHA response. Cyclic AMP and DB-cAMP had no effect on RNA or DNA synthesis in unstimulated cells at any concentration.
Figure 29.

Effect of dibutyryl cyclic AMP on the kinetics of calcium uptake. \( \bullet-\bullet \), PHA (no DB-cAMP); \( \bigcirc-\bigcirc \), PHA + 2 mM DB-cAMP; \( \square-\square \), unstimulated (no DB-cAMP); \( \bigboxtimes-\bigboxtimes \), unstimulated + 2 mM DB-cAMP. a. The initial rate of calcium uptake (v) was determined at \( 37^\circ \) with a 10 minute incubation with \( ^{45}\text{Ca} \) at different \( \text{Ca}^{++} \) concentrations (S in mM). The rate of uptake was expressed as n moles/\( 10^6 \) lymphocytes/min \( \pm \) SEM of 3 replicate cultures. b. Lineweaver-Burk plot (1/v versus 1/S) of the data in a. The regression lines were fitted by the method of least squares. For the PHA case they were \( y = 0.093x + 0.18 \) and \( y = 0.103x + 0.22 \) in the absence and presence of DB-cAMP respectively. For the unstimulated case they were \( y = 0.194x + 0.23 \) and \( y = 0.142x + 0.46 \) respectively.
TABLE 16

Effect of Cyclic AMP, Dibutyryl Cyclic AMP, Adenosine Triphosphate and Theophylline on AIB Uptake by Lymphocytes

<table>
<thead>
<tr>
<th>Agents</th>
<th>Concentration (M)</th>
<th>AIB Uptake&lt;sup&gt;3&lt;/sup&gt; (CPM ± SEM) PHA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td>278 ± 18</td>
<td>153 ± 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>$5 \times 10^{-3}$</td>
<td>141 ± 11</td>
<td>137 ± 12</td>
</tr>
<tr>
<td>DB-cAMP</td>
<td>$1 \times 10^{-3}$</td>
<td>147 ± 3</td>
<td>158 ± 15</td>
</tr>
<tr>
<td>theophylline</td>
<td>$5 \times 10^{-3}$</td>
<td>187 ± 12</td>
<td>145 ± 7</td>
</tr>
<tr>
<td>ATP</td>
<td>$5 \times 10^{-3}$</td>
<td>131 ± 6</td>
<td>140 ± 9</td>
</tr>
</tbody>
</table>

<sup>1</sup>Lymphocytes were incubated with or without PHA for 2 hours. <sup>3</sup>H-AIB uptake was then determined with a 15 minute pulse.

<sup>2</sup>The agents were added to the cultures 5 min before the PHA or at an equivalent time before the isotope incubation period was initiated in control cultures.

<sup>3</sup>Each value is the mean uptake in CPM ± SEM of 3 or 4 replicate cultures.
Most of the above agents were found to alter lymphocyte transport properties. Cyclic AMP, DB-cAMP, theophylline and ATP caused a slight stimulation of control Ca$^{++}$ uptake but had no effect on control AIB uptake. They all inhibited both Ca$^{++}$ and AIB uptake by PHA-treated cells at concentrations which also inhibited DNA synthesis. Since caffeine and theophylline normally potentiate cAMP-mediated processes (166), their inhibition of the PHA response must be due to other effects such as the inhibition of transport phenomena.
3.5 Influence of Calcium and Cyclic AMP on the Mixed Lymphocyte Reaction

3.5.1 Introduction

The preceding results have established the importance of Ca\(^{++}\) in the process of lymphocyte transformation induced by PHA. Also they have demonstrated that exogenous cAMP and related agents cannot themselves induce transformation of resting cells, and that they inhibit PHA-induced transformation. Since the PHA response is non-specific and could, therefore, differ from specific immunological transformation processes, it was desirable to extend some of the observations to a process more closely related to real physiological events. Hence, the influence of Ca\(^{++}\) and cAMP on the mixed lymphocyte reaction was studied.

3.5.2 Time-Course Studies

RNA and DNA synthesis and morphological transformation in mixed lymphocyte cultures were followed for 10 days. RNA synthesis was slightly above control levels at 1 day (Fig. 30a), and it then increased rapidly to its maximum level at day 5 where it remained through day 6. Synthesis then decreased rapidly over the next 3 days returning to unstimulated levels by day 9.
As with PHA stimulation DNA synthesis did not rise above the unstimulated level during the first day (Fig. 30b). It then increased progressively to its peak value on day 6 before declining to slightly above control levels of synthesis on day 9. Dye exclusion viability determinations indicated that the rate of cell death in mixed cultures was approximately the same as in unstimulated cultures (see Fig. 5a).

No fully-developed lymphoblasts were observed until day 4 (Fig. 30c). The percentage of transformed cells then increased to a maximum of 20% at 7 days before declining progressively over the last 3 days.

3.5.3 Requirements for Exogenous Calcium and Effects of Cyclic AMP

Increasing concentrations of EDTA beginning at about 10^{-4}M progressively inhibited DNA synthesis (or RNA) with complete inhibition observed at 10^{-3}M (Fig. 31). EDTA inhibited PHA-stimulated DNA synthesis to approximately the same degree over the same concentration range.

EGTA was also an effective inhibitor of DNA synthesis in the MLR. However, the MLR was less sensitive than the PHA response (Fig. 32). No inhibition of the MLR occurred up to 1.3 mM although PHA-induced synthesis was inhibited by 44% at that concentration. Essentially
Time-course studies of the mixed lymphocyte reaction. 

a. RNA synthesis was determined by incubation with $^{3}\text{H}}$-uridine during the final 4 hours of each 24 hour interval. Each point was the mean CPM $\pm$ SEM of 3 replicate cultures expressed as a percentage of the maximum response observed (4250 CPM). 

b. DNA synthesis was determined by incubation with $^{3}\text{H}}$-thymidine during the final 4 hours of each 24 hour interval. Each point was the mean CPM $\pm$ SEM of 3 replicate cultures expressed as a percentage of the maximum response observed (6080 CPM). 

c. The percentage of transformed cells was determined at the end of each 24 hour period. Each point was the mean of duplicate cultures.
Figure 31.

Inhibition of DNA synthesis in the mixed lymphocyte response by EDTA. EDTA was added at the start of the experiment, and DNA synthesis was determined at 136 hours with $^3$H-thymidine present for the final 4 hours. Each bar is the mean CPM ± SEM of 4 replicate cultures expressed as a percentage of the mean CPM of mixed cultures not treated with EDTA (2,500 CPM).
Effect of EGTA on DNA synthesis in the mixed lymphocyte response compared to the PHA response. □, mixed lymphocyte cultures; □, PHA cultures. EGTA was added to the mixed cultures at the start of the experiment, and DNA synthesis was determined at 136 hours with $^3$H-thymidine present for the final 4 hours. EGTA was added 15 minutes before PHA, and $^3$H-thymidine incorporation was determined at 72 hours with the isotope present for the final 4 hours. Each bar is the mean CPM ± SEM of 4 replicate cultures expressed as a percentage of the respective amounts of isotope incorporation in cultures not treated with EGTA (2780 CPM, MLR; 8360 CPM, PHA).
total inhibition was observed for both at 1.4 mM.

Attempts to demonstrate stimulation of Ca\(^{++}\) uptake in the MLR during the first 4 days were unsuccessful quite probably because of the relative weakness of the MLR compared to the PHA response, the insufficient sensitivity of the present technique, and the fact that the MLR develops more slowly than the PHA response. However, a highly significant stimulation of uptake was observed in mixed cultures at day 5 when nucleic acid synthesis was nearing its maximum (Table 17).

It was demonstrated previously that cAMP inhibited PHA-induced DNA synthesis, and that this was related to early events since addition of the nucleotide after the response was completely developed did not affect subsequent synthetic levels. The effect of cAMP on the MLR was slightly different. If cAMP at concentrations of 10\(^{-4}\) or 10\(^{-3}\)M was added at the start of the culture, DNA synthesis at 6 days was inhibited (Fig. 33). However, if cAMP at 10\(^{-6}\) and 10\(^{-4}\)M was added to the cultures only during the isotope incubation period for measuring DNA synthesis (final 4 hours), a small but significant stimulation of DNA synthesis was observed (Fig. 33).

3.5.4 Summary

The time-course of the development of DNA synthesis in the mixed lymphocyte reaction has been reported
TABLE 17

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Calcium Uptake$^2$ (n moles ± SEM)</th>
<th>Statistical Significance (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>31.3 ± 2.2</td>
<td>33.3 ± 2.0</td>
</tr>
<tr>
<td>2</td>
<td>45.3 ± 1.2</td>
<td>37.7 ± 1.3</td>
</tr>
</tbody>
</table>

$^1$Lymphocytes from 2 donors (A and B) were cultured individually (10$^6$ cells) or in mixed cultures (0.5 x 10$^6$ cells from each donor) for 5 days at a Ca$^{++}$ concentration of 1.25 mM. Calcium uptake was determined with a 2 hour incubation with $^{45}$Ca at that time.

$^2$The results were expressed as the mean uptake in n moles/10$^6$ lymphocytes ± SEM of 3 replicate cultures.
Differential effects of cyclic AMP on the mixed lymphocyte response depending on time of addition. □, cAMP added 10 minutes prior to the assay period (132-136 hrs); △, cAMP added at the start of the culture; □□□, control with no cAMP added. Each bar is the mean CPM ± SEM of 3 replicate cultures expressed as a percentage of the mean CPM of mixed cultures without cAMP (2230 CPM).
previously (167). Maximum levels of synthesis are observed after about 6 days in contrast to the 3 days required to attain maximum levels in PHA-stimulated cultures. The present results verified that maximum DNA synthesis occurred at day 6, and also that maximum RNA synthesis occurred at day 5 and maximum morphological transformation at day 7.

It was found that transformation in mixed lymphocyte cultures was inhibited by both EGTA and EDTA over approximately the same concentration range that was inhibitory to PHA stimulation. High concentrations of cAMP also inhibited mixed lymphocyte stimulation if present from the start of the culture period. However, in contrast to the PHA response addition of cAMP just before assessing DNA synthesis resulted in a significant stimulation of the level of synthesis.
4. DISCUSSION

4.1 Characteristics of the Transformation Response

The time-course of nucleic acid synthesis and morphological transformation in PHA-stimulated lymphocytes was determined for the culturing technique used. Lymphocyte RNA synthesis increased markedly during the first 8 hours after stimulation with PHA and reached a maximum at 48 hours. DNA synthesis did not begin until after 16 hours of stimulation and then rose to a maximum at 64 hours. Morphological transformation to blast cells was greatest at 96 hours. These observations agreed with previous work which had been done with somewhat different but comparable techniques (41, 42, 43, 81) except that maximum DNA synthesis occurred at 64 rather than 72 hours. The present studies provided the most complete description of RNA and DNA synthesis since they were followed continuously instead of for just two hours at the end of each day as in the other studies. The sequence of events was the same for mixed lymphocyte cultures except that maximum levels for each parameter occurred 3 days later than in the PHA response (167).

It is important to note that the initiation of DNA synthesis does not occur for at least 16 hours after PHA treatment. Thus, these cells are not poised to
immediately enter the DNA synthetic or S phase of the cell cycle. Many preparatory events are necessary to start the lymphocyte on its course to proliferation. Changes in membrane transport properties are almost certainly involved, and the increased uptake of Ca\textsuperscript{++} and AIB may be two crucial ones.

The PHA dose-response relationships for RNA and DNA synthesis exhibited a maximum plateau range with decreased responses at both higher and lower concentrations outside that range as has been observed previously (129, 130). There is no obvious explanation for the high dose inhibition. There is an increased degree of cell death at very high concentrations but not enough to account for the complete inhibition. It is well-known that very high concentrations of antigen can induce tolerance, a state of non-reactivity to that antigen (168). Thus, it is conceivable that large doses of PHA may act similarly, but no evidence is available to support this speculation.

Lymphocytes survived well with the present culture conditions and the use of three viability methods provided complete assurance that the various inhibitory agents studied were non-toxic, a factor which has often been overlooked in past studies.
4.2 The Requirement for Calcium in the Initiation of Transformation

The Ca\textsuperscript{++} requirements of transforming lymphocytes were studied by using EGTA or by adding Ca\textsuperscript{++} to low Ca\textsuperscript{++} medium. EGTA progressively inhibited PHA-induced stimulation with increasing concentrations above the normal medium Ca\textsuperscript{++} level of 1.25 mM. This chelator inhibited the mixed lymphocyte reaction similarly. EDTA also blocked both the PHA and mixed lymphocyte transformation responses. The inhibition could not be attributed to cell death since both EGTA and EDTA were definitely nontoxic to lymphocytes at the concentrations employed as assessed by all three viability methods. The EGTA and EDTA inhibitions were also not due to an isolated effect on thymidine and/or uridine metabolic pathways since RNA and DNA synthesis and morphological transformation were all affected. It was also demonstrated that chelation did not merely induce a delay in the development of the response. Since the EDTA effect was not completely reversible by Ca\textsuperscript{++} alone or in combination with other divalent cations (136), no conclusions regarding Ca\textsuperscript{++} requirements could be drawn. However, the EGTA effect could be totally reversed by adding excess Ca\textsuperscript{++} while other divalent cations were ineffective.

It is important to note again the specificity of the Ca\textsuperscript{++} requirement. Addition of even high concentra-
tions of Mg$^{++}$, another physiologically important divalent cation, to cultures in the absence of Ca$^{++}$ did not permit transformation to proceed nor could Mg$^{++}$ reverse the EGTA effect. Thus, the inhibition was due to the reduction of the medium Ca$^{++}$ level.

The actual concentration of free Ca$^{++}$ in Ca-EGTA buffers can be calculated from the appropriate empirically determined affinity constants. However, it could not be calculated accurately in this work since the affinity constants for the experimental conditions used herein were not available. An estimate of the required level of medium Ca$^{++}$ was obtained from the experiments in which various concentrations of Ca$^{++}$ were added to low Ca$^{++}$ medium. Significant transformation did not occur below about $10^{-5}$ M Ca$^{++}$, and normal transformation was observed at concentrations above about 0.6 mM which is about 50% of the physiological level. The level of transformation was a function of the Ca$^{++}$ concentration between these extremes.

Although these experiments established that medium Ca$^{++}$ were essential to the normal development of the PHA response, a specific Ca$^{++}$ function could not be assumed since all cells require some Ca$^{++}$ to maintain a variety of cellular processes (169). However, it was observed that once the lymphocytes had been stimulated into a population of actively proliferating cells EGTA had no effect on nucleic acid synthesis determined subsequently.
This indicated that sufficient Ca\textsuperscript{++} were still present to permit growth so Ca\textsuperscript{++} had more than a non-specific growth promoting role. It is important to note that both EDTA and ouabain, which alters Na\textsuperscript{+} and K\textsuperscript{+} transport, could rapidly turn off nucleic acid synthesis implying that other divalent and monovalent cations are important in maintaining the transformation response once it is underway. Indeed, it has been reported that high levels of K\textsuperscript{+} are necessary to maintain optimum levels of protein synthesis (170), and the influence of Mg\textsuperscript{++} on the activity of many enzymes and on DNA synthesis in cell-free systems is well-known (154).

Further studies revealed that reducing the medium Ca\textsuperscript{++} level with a completely inhibitory concentration of EGTA at any time before, or up to, 2 hours after PHA addition resulted in a complete inhibition of the final response. Addition of EGTA at increasingly later times caused a progressively decreasing inhibition until 12 hours after which time the chelator had no effect on the final response. This inhibition by EGTA added after PHA was not due to a removal of PHA from the membrane. If the cells were washed with medium at various times after PHA addition, some decrease in the eventual response occurred due probably to PHA removal, but the presence of EGTA did not increase the degree of inhibition caused by washing.

The experiments on the time-sequence of EGTA and PHA
addition and the washing experiment together offer
strong evidence that Ca++ have a specific role in the
initiation phase of transformation which refers to the
period between PHA addition and the initiation of DNA
synthesis which is about 16 hours long. Furthermore,
the first 2 hours seems to be the especially critical
period. It is rather difficult at present to explain
the intermediate levels of inhibition observed when EGTA
was added from 2 to 12 hours after PHA. Two reasonable
suppositions would be that sufficient precursors to per-
mit some eventual DNA synthesis had been synthesized
before EGTA was added or else that late Ca++ removal
could only modify already activated processes rather
than being able to completely block them.

One very important point about these effects relat-
ed to Ca++ is that they only affect the initiation stage
of transformation. Many other inhibitory agents have
been studied previously yet EGTA was the only one which
did not inhibit the completely developed response (re-
call the late EDTA and ouabain inhibition) as well as
the initiation stage. Exogenous cAMP also followed the
EGTA pattern of only inhibiting the response when pre-
sent from the start of the culture.

The obvious place to begin investigations of this
Ca++ requirement for initiation of transformation is at
the cell membrane in view of the importance of Ca++ in
cell surface and membrane phenomena (103, 106, 110, 116).
It was reported recently that EDTA inhibited the binding of PHA to the membrane (171). However, other observations reported here suggest a further role(s). The investigations in this thesis have shown that results obtained with EDTA may not be due specifically to Ca\(^{++}\). Further the experiment in which PHA-treated cells were washed to remove some of the bound PHA, carried out as described before (50), showed that EGTA did not increase the extent of inhibition caused by PHA removal. Therefore, it must not be acting on PHA already bound to the membrane. Also the MLR which does not require the binding of an exogenous molecule is also inhibited by EGTA. Finally the observed marked stimulation of Ca\(^{++}\) uptake by PHA argues for an intracellular role.

Calcium ions were shown herein to influence amino acid transport and early RNA synthesis. The inhibitory effect of low Ca\(^{++}\) on PHA-stimulated AIB uptake was predominantly a direct effect on transport rather than an indirect inhibition due to an inhibition of some preceding event required for enhanced transport since addition of EGTA only during the actual 15 minute uptake period still resulted in blocked uptake. It cannot be proved from the available data whether the effect on early RNA synthesis is direct or indirect, but that data definitely strengthens the concept of a very early requirement for Ca\(^{++}\). Further studies will be required to establish the exact intracellular function(s) of Ca\(^{++}\)
in transformation.

4.3 Calcium Accumulation in Unstimulated Lymphocytes

If Ca\(^{++}\) can regulate cellular metabolic events involved in the activation of resting cells, it could reasonably be expected that the cell could regulate the intracellular concentration of the ion. One way by which this regulation could be achieved would be to alter flux between the cytoplasm and a subcellular organelle such as mitochondria which are known to have very large Ca\(^{++}\) accumulating capacities (116, 140). The cells could also control transport across the plasma membrane by influencing influx and/or efflux. Thus, it was of considerable importance to understand the mechanism of Ca\(^{++}\) uptake in lymphocytes.

It was mentioned earlier that Ca\(^{++}\) uptake in most other cell types appears to be an energy-independent process (142, 143, 144), which should not be unexpected in view of the large inwardly directed electrochemical gradient for Ca\(^{++}\), although in chick embryo fibroblasts there may be a very large energy-dependent component (145). However, it has not been clarified whether the process is more than simple diffusion which would offer little regulation of influx.

Calcium accumulation by lymphocytes was also predominantly energy-independent since it had a \(Q_{10}\) far
below that observed for any process requiring expenditure of metabolic energy (151). Also the metabolic inhibitors azide and ouabain had no effect. The potent inhibitor of glycolysis, iodoacetamide, only caused a slight inhibition which can be explained on the basis of its being a sulfhydryl reagent. Since the classical sulfhydryl reagent, N-ethylmaleimide, strongly inhibited uptake it seems quite likely that sulfhydryl groups, which are numerous in the lymphocyte membrane (96, 97), are involved in Ca\(^{++}\) transport in some as yet undefined manner. Other recent evidence with other cell types supports this contention (145, 172). The strongly potentiated uptake observed in the presence of the metabolic inhibitor sodium fluoride, has been observed in muscle granules (173) and probably depends on co-transport of the fluoride anion. This would result in precipitation of the Ca\(^{++}\) taken up thereby preventing the rise in intracellular Ca\(^{++}\) concentration which would eventually halt uptake. The lack of a ouabain effect negates the possibility that Ca\(^{++}\) uptake might be coupled to Na\(^{+}\) and K\(^{+}\) transport via the Na\(^{+}\)-K\(^{+}\)ATPase although it is quite possible that Ca\(^{++}\) flux might be linked to passive Na\(^{+}\) influx as is the case in nerve cells (149).

In spite of the lack of an active Ca\(^{++}\) accumulation mechanism similar to that in mitochondria (139, 140) and sarcoplasmic reticulum (141), the passive nature of the process did not rule out regulation by the participation
of a membrane carrier. It was found that Ca$^{++}$ uptake was a saturable process which is consistent with a carrier-mediated mechanism. Also Mn$^{++}$ competitively inhibited uptake which suggests that Ca$^{++}$ and Mn$^{++}$ compete for a common site. However, exchange diffusion was not observed indicating either that the system is not reversible or that the carrier affinity on the internal side of the membrane is considerably greater than that on the external side so that no effect can be detected (128). Magnesium ions had no effect on Ca$^{++}$ influx showing that they do not compete with Ca$^{++}$ nor can they potentiate uptake as they do in some other Ca$^{++}$ transporting systems (141, 145, 155).

These studies on the kinetics of lymphocyte Ca$^{++}$ uptake provide very strong evidence that Ca$^{++}$ uptake is mediated by a membrane carrier or some other membrane constituents which bind Ca$^{++}$ which consequently indicates that the process is of the facilitated diffusion type.

It may be significant that maximum Ca$^{++}$ uptake occurs at a medium Ca$^{++}$ concentration of about 0.8 mM or higher. This is approximately the minimum Ca$^{++}$ level necessary to obtain maximum RNA synthesis in PHA-treated cells (refer to section 3.2.3). Thus, it could be that the degree of transformation is related to the amount of Ca$^{++}$ which can be accumulated although this is just speculation as yet.
4.4 Stimulation of Calcium Uptake by PHA

PHA was found to significantly enhance $\text{Ca}^{++}$ accumulation within 1 minute after treatment which makes it the earliest event to occur after exposure of the cells to PHA along with the reported activation of adenylate cyclase (59) which also occurs within 1 to 2 minutes. The maximum uptake obtainable was a 3-fold increase. The stimulated uptake also exhibited saturation kinetics indicating that PHA acted by influencing the proposed membrane carrier or $\text{Ca}^{++}$ binding sites. Indeed PHA produced an immediate decrease in the $K_m$. After the PHA response had developed for 3 hours, the rate of $\text{Ca}^{++}$ uptake had increased further probably as a result of a small increase in $V_{\text{max}}$ in addition to the decreased $K_m$.

These observations are interpreted to mean that PHA enhances $\text{Ca}^{++}$ uptake mainly by increasing the affinity of the hypothetical $\text{Ca}^{++}$ carrier or membrane sites rather than by increasing the number of sites or the rate of transport by each site both of which would be characterized by an increased $V_{\text{max}}$. This conclusion is supported by the fact that cycloheximide did not alter uptake indicating that synthesis of new sites associated with proteins was not occurring. The PHA effect was also not due to a stimulation of a normally unobservable exchange diffusion mechanism, nor was it due to an inhibition of efflux.
Recent studies on $K^+$ (76) and AIB (51) transport by PHA-stimulated lymphocytes have indicated that in these cases there is an increased $V_{max}$ while $K_m$ is not affected. Several points of dissimilarity may account for this major difference in the PHA effect on $Ca^{++}$ compared with $K^+$ and AIB transport. The stimulation of $Ca^{++}$ transport occurs essentially immediately after PHA treatment while enhanced AIB (51) and $K^+$ (76) uptake is not observed for 30 and 60 minutes respectively. This implies that substantial alterations in membrane properties must precede the stimulation of transport of these factors. Changes in membrane structure as manifested by increased phospholipid turnover are observable by 10 minutes after exposure to PHA (62). In order to increase the $V_{max}$ new sites would have to be either synthesized, which is unlikely since increased uptake occurred in the absence of protein synthesis, or uncovered from normally masked areas. Both of these possibilities would probably require more extensive membrane changes than would be necessary to increase the affinity of already available sites. Also both $K^+$ (149) and AIB (128) uptake are active transport processes in contrast to the energy-independent $Ca^{++}$ accumulation. Further calcium is an integral component of the membrane (116) in addition to being transported across it.

In addition to an absolute requirement for exogenous $Ca^{++}$ the PHA response is characterized by a large, rapid
increase in Ca\(^{++}\) accumulation. The evidence is not yet sufficient to propose a cause and effect relationship between increased Ca\(^{++}\) uptake and subsequent events in stimulation. However, the fact that it is the earliest observed change in transport function is significant in that it correlates with the proposed early function of Ca\(^{++}\). Also it has been shown that inulin uptake is not stimulated by PHA (51), and other workers have reported that there is selective stimulation of uptake of various nucleotides (69). It seems likely then that the enhanced Ca\(^{++}\) uptake is an important early event and not just part of a general PHA-induced permeability change.

4.5 Influence of Cyclic Nucleotides and Related Agents on Nucleic Acid Synthesis and Transport Properties in Unstimulated and Transforming Lymphocytes

It was reported during the course of the present work that PHA rapidly stimulated adenylate cyclase activity in the membranes of human peripheral blood lymphocytes (57) and mouse spleen cells (58). In the former case this was accompanied by a corresponding increased production of cAMP within 1 to 2 minutes (59). Cyclic AMP levels in the stimulated cells remained above control levels for 1 hour before falling to below control levels at 6 hours (59).

These observations suggested that cAMP could be an
important mediator of transformation. If this cyclic nucleotide is indeed a "second messenger" (175) mediating the transformation process, it might be able to mimic the action of the mitogen when added exogenously in the absence of any stimulant (165, 166). Further it might be able to potentiate the stimulated response.

The data reported in this thesis demonstrated that cAMP and a dibutyryl derivative were completely ineffective at stimulating either RNA or DNA synthesis in unstimulated lymphocytes. Also they could not stimulate amino acid uptake (AIB), and Ca++ uptake was only enhanced to a slight extent by their presence. In contrast PHA strongly stimulated all of these processes. Thus, exogenous cAMP could not mimic the action of PHA on human lymphocytes. This agrees with several other recent reports that exogenous cAMP or DB-cAMP alone cannot stimulate normal transformation in human lymphocytes (176, 177, 178), or horse lymphocytes (179).

This failure to stimulate could possibly be attributed to the very limited ability of cAMP to cross the cell membranes (164, 180). However, the similar inability of the analog, DB-cAMP, which readily traverses the membrane (164), to activate human lymphocytes argues against that explanation. Of course this molecule would be degraded intracellularly to cAMP and a dibutyryl moiety the latter of which might somehow be inhibitory to stimulation. The possibility that cAMP might have
been degraded extracellularly by some enzymes in the serum was ruled out by the recent work of Webb et al. who cultured their cells without serum and still found no stimulation (181). In contrast to the above results are the findings of Cross and Ord who observed that DB-cAMP was as effective as PHA at stimulating several parameters of transformation in pig lymphocytes (60). However, in these studies PHA-induced stimulation was of a much smaller magnitude than is observed in other types of lymphocytes (only a 3-fold increase above control levels) indicating that these cells may be atypical.

Cyclic AMP and DB-cAMP have been found to stimulate transport of Na$^+$ (182), Ca$^{++}$ (183) and AIB (174) in other systems, and ATP is in many other cases a potent stimulant of Ca$^{++}$ accumulation (140, 141, 145). However, they each could only slightly stimulate Ca$^{++}$ uptake by resting lymphocytes, and they had no effect on AIB transport. It may be that their failure to induce transformation is a consequence of their inability to enhance transport of these other essential ions and molecules.

In spite of their lack of effect on unstimulated cells, cAMP, DB-cAMP and ATP at higher than physiological concentrations all strongly inhibited RNA and DNA synthesis by PHA-stimulated cells also agreeing with the majority of the recent studies mentioned earlier (176, 178). However, in one report cAMP produced a 2.5 fold
increase in the PHA response (181), and in another low concentrations ($10^{-7}$ to $10^{-6}$M) of DB-cAMP caused a 50% increase in DNA synthesis (177). The experiments in this thesis did not show any stimulation of the PHA-response in the physiological concentration range of ($10^{-8}$ to $10^{-6}$M).

The inhibition of the PHA response by cyclic nucleotides and related agents except for ATP is similar to that caused by EGTA in that they are only inhibitory to the initiation of the response. When they are added to proliferating lymphocytes, they have no effect. However, in the MLR addition of cAMP just before determining the level of synthesis resulted in a significant stimulation of DNA synthesis while adding it at the start of the culture resulted in an eventual inhibition just as it did in the PHA case.

These observations suggest that like EGTA these substances interfere with some early event(s) in the process, and since at least one of these, cAMP, does not enter cells, an effect on membrane events seems a likely locus of action. Indeed the present work demonstrated that the cyclic nucleotides as well as ATP and theophylline were potent inhibitors of PHA-stimulated transport of Ca$^{++}$ and AIB which are both very early events in the response. Dibutyryl cAMP was found to alter the kinetics of PHA-stimulated Ca$^{++}$ uptake by reducing the $V_{\text{max}}$ to that found for unstimulated cells. However, the
inhibition was not strictly competitive or non-competitive.

At present the general consensus is that exogenous cAMP has little or no effect on the response at physiological levels. Since PHA at the concentrations studied stimulates the majority of lymphocytes, it would be reasonable to expect that cAMP could not enhance the already maximal response. However, in the present work cAMP also did not stimulate the low level of transformation induced by a very sub-optimal concentration of PHA so the lack of stimulation is not merely due to an inability to further stimulate an already maximal response.

Also cAMP is inhibitory to the transformation process at higher than physiological concentrations. This inhibition is not due to cell death, a direct effect on DNA synthesis, or an inhibition of PHA binding (178). Further since it only inhibits when present from the start of the culture or shortly thereafter, it must exert its effects on early events subsequent to binding. Hence, the most plausible explanation for the cAMP inhibition is that it, and the other agents, act by blocking uptake of essential cofactors specifically Ca++ and amino acids.

Although exogenous cAMP cannot induce transformation of human lymphocytes and either inhibits or has no effect on the PHA response depending on concentration, its
possible role as a mediator of transformation cannot yet be ruled out. However, it is fairly clear that cAMP is not the sole initiator of the process. Smith et al. (178) showed that treating the cells with agents that increased intracellular cAMP levels also did not induce transformation supporting this contention. More studies on the influence of PHA on intracellular cAMP levels will be necessary to verify the observations of Smith et al. (59) that PHA does increase cAMP production. Several measurements of lymphocyte cAMP levels were made in this work, and it was found that PHA caused a very marginal stimulation of cAMP production. However, a technique developed for other cells was used which may account for the negative results obtained.

4.6 General Discussion

The present results have established the requirement for exogenous Ca++ in the initiation of cell proliferation in normally resting lymphocytes which have been treated with the mitogen, phytohemagglutinin. However, it is crucial to note that the actual proliferation of the cells after DNA synthesis has begun is independent of the Ca++ levels which are necessary during the initiation phase of the response. The additional observation that the mixed lymphocyte reaction, a process having physiological significance (8), also
requires Ca$^{++}$ lends credence to the proposal that Ca$^{++}$ are of general importance in the initiation of cell division in normally non-cycling ($G_0$) cells. It seems reasonable to speculate that such a basic process as mitosis would have passed through the process of evolution essentially intact so that the same mitotic mechanisms would operate in different species as well as in different cell types.

The important regulatory role of Ca$^{++}$ on the proliferation of rat thymus and bone marrow cells which are already in a normal cell cycle is well-known (117, 118, 119, 120). The recent observation that Ca$^{++}$ have an important role in controlling the proliferation of normal but not virally-transformed chicken fibroblasts (184) offers further evidence for a regulatory role(s) of Ca$^{++}$ in cell proliferation. However, the role of Ca$^{++}$ in these processes may differ from their role in transformation since these cells are already in an active cell cycle. Also quite a considerable amount of recent work has shown that Ca$^{++}$ have profound influences on antibody formation both in vivo and in vitro (185), a process which involves lymphocyte transformation. Thus, it is conceivable that this influence was due to an effect on transformation.

All of the above observations point out the significance of Ca$^{++}$ in cell proliferation, but they offer little more than circumstantial evidence to suggest the
mode of action of Ca\textsuperscript{++}. It was demonstrated that Ca\textsuperscript{++} affect PHA-stimulated amino acid transport in lymphocytes showing one possible locus of control, and it is probable that other transport functions which are essential to proliferation are influenced by Ca\textsuperscript{++} (110).

Whitfield and his colleagues found that in the rat thymocyte system Ca\textsuperscript{++} can influence cAMP production possibly by altering the activity of the adenylate cyclase enzyme, which is membrane-bound, or the specific phosphodiesterase found in the cytoplasm (161). Hence, controlling alterations in membrane transport and in the activity of membrane-associated enzymes appear to be likely points for Ca\textsuperscript{++}-mediated regulation.

However, the fact that PHA produces a rapid, marked enhancement of Ca\textsuperscript{++} accumulation argues that Ca\textsuperscript{++} may be important to intracellular events in resting cell activation also. Although there is no direct evidence as yet for or against this proposal, many of the known events occurring during the induction of cell cycling are influenced by Ca\textsuperscript{++} in other systems.

Inhibition of the cAMP-degrading phosphodiesterase from rat thymocytes by Ca\textsuperscript{++} would provide a means of subsequent regulation of cAMP production (161). The ability of Ca\textsuperscript{++} to modify membrane properties particularly in the case of protein leakage (107) could be very important if the observed release of lysosomal hydrolases by PHA (75) is a critical event. Calcium
ions could also have direct effects on DNA replication since they can bind to nucleoproteins (112), and since they can cause chromosome condensation (122). However, this would be rather unlikely in the transformation process since DNA synthesis does not begin for many hours after PHA treatment, and exogenous Ca\(^{++}\) are no longer required by that time. An early effect on transcription or translation might still be possible though. Another finding linking Ca\(^{++}\) to cell division showed that in the sea urchin there is a Ca\(^{++}\)-activated ATPase which varies in activity during the cell cycle and which may be involved with chromosome condensation (186). It was also reported recently that Ca\(^{++}\) enhance protein synthesis by augmenting the transfer of amino acids from transfer RNA to protein (187). Probably the most reasonable proposal with the greatest experimental support would be that Ca\(^{++}\) act through selective inhibitory or stimulatory effects on metabolic sequences i.e. on enzymes. In a review Bygrave has indicated numerous loci of Ca\(^{++}\) action particularly those which are essential to the energy metabolism of the cell (111). These possible points of intracellular action offer direction for extensive future work on the role of Ca\(^{++}\) in the activation of resting cells.

The role of cAMP in initiating lymphocyte proliferation is much less certain. It is fairly obvious though that little is going to be learned by administering cAMP
exogenously. The more recent work of Whitfield et al. which clearly implicates cAMP along with Ca\textsuperscript{2+} as mediators of thymus cell proliferation is, however, sufficient to warrant further studies on endogenous cAMP production in stimulated lymphocytes and other activated cell types. Mediation by cAMP is very attractive because of the ability of the cyclic nucleotide to activate protein kinases and other key enzymes involved in energy-producing metabolic sequences (111). Cyclic AMP can also stimulate histone phosphorylation (188), and it influences many transport functions (174, 182, 183). When studies on cAMP production are performed, one should realize that it is possible that a reduction rather than an increase in cAMP concentration may be necessary to achieve escape from control mechanisms (189).

Activation of lymphocytes or other resting cells is undoubtedly a very complex process. It is tempting to speculate that the main mediators of the process are Ca\textsuperscript{2+} and cAMP as they are in the process of hormonal stimulation (116) and in the regulation of rat thymocyte proliferation (161). However, more studies on the influence of mitogens on the intracellular production of cAMP are necessary before any definite conclusions can be made. A main question to be elucidated is whether transformation is a result of a sequential series of events leading eventually to DNA synthesis or whether numerous events occur in parallel some of which converge
later to influence one rate limiting step which eventually leads to the initiation of DNA synthesis and normal cell proliferation.
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