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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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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

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inhibited both calcium and amino acid uptake in PHA-stimulated cells.

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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-proliferat-
ing 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 pre-
lude 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 phytohemag-
glutinin (PHA), a mucoprotein extract of the red kidney
bean (Phaseolus vulgaris), a significant proportion of
the original small lymphocytes transformed to large im-
mature 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 acti-
vate lymphocytes _in vitro_. They are generally divided
into two main groups. One group consists of agents re-
ferred 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 polypep-
tides 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 con-
canavalin A (31), pokeweed mitogen (32) and lens culin-
aris 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 un-
related donors, the mixed lymphocyte culture reaction
(MLR) (39, 40). The degree of transformation induced by
the various agents is normally quantitated by measure-
ment of the extent of incorporation of radioactive pre-
cursors 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 sim-
ilar in cells exposed to agents from both groups, sev-
eral important distinctions must be recognized. Stimu-
lation by specific agents occurs only when the lympho-
cyte 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 depend-
ing on the criteria used in declaring a cell transformed
(44, 45), compared to the calculated 2% involved in anti-
genic 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 trans-
formation is initiated by a surface rather than an in-
tracellular reaction.

It is known that antigens bind to specific immuno-
globulin receptors on the lymphocyte surface (52), but
the nature of the PHA binding sites is not clearly de-
fined. 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 oligo-
saccharide 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 re-
action 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-agglutin-
ating properties of PHA associated with surface binding
are not essential to the induction of transformation

since removal of the agglutinating factors does not al-
ter 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. How-
ever, it is not yet known which ones are crucial to the
induction of the response and which ones are just sec-
ondary 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 con-
sequent 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 intra-
cellular 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 in-
crease 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 pre-formed 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 con-
densed (83). The cytoplasm increases in size, and in-
tense 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 lympho-
cyte membrane has more than a passive role in the induc-
tion 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 regula-
tor of cell division (86). It is now known that mem-
brane 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 mem-
brane 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 mem-
brane (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 inter-
actions (104) are undoubtedly functions of this extra-
cellular calcium. Alterations in membrane binding of
Ca^{++} can influence permeability to water (105), cations
(106) and proteins (107) possibly by inducing conforma-
tional 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 biolog-
ical processes in which the above specific known func-
tions are undoubtedly involved is clearly recognized.
However, their exact mode(s) of influence is still poor-
ly 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 Ca^{++} to membrane function, and the critical role of Ca^{++} in regulating cell division in bone marrow and thymus cells the present studies were begun with the aim of determining the influence of 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° 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°. 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-^{3}H-thymidine (^{3}HT, 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}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 com-
pletely 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 scin-
tillation fluid, [3 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 $4°$ 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 (10-20×10^6) 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 $37°$ for 30 min after which they were washed 3 times with saline at $4°$ to remove extracellular isotope. They were then diluted to 5×10^5 cells/ml and were dispensed in 0.7 ml volumes to 12 x 75 mm plastic tubes which were incubated at $37°$. 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 super-natant 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_2O. 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: hypaque (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°. 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^{++} and Mg^{++}-free BME supplemented with antibiotics and 2% AB serum to provide a low initial Ca^{++} concentration of approximately 0.025 mM calculated assuming the level of Ca^{++} contamination of the Ca^{++}-free medium to be less than $10^{-5}M$, 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 3.33×10^6 lymphocytes/ml. The cultures were maintained in the CO_2 incubator until used.

For 2-way mixed lymphocyte cultures the cells were diluted to a concentration of 10^6 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}Calcium (^{45}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}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}Ca uptake determination the tubes were transferred to a water bath at the desired temperature, and ^{45}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° 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}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 resuspended 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 µC 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 supplemented with 2% AB serum. At various times thereafter the percentage of isotope in both the cells and supernatant was assayed as described for uptake.

2.8 Measurements of α-Aminoisobutyric Acid Transport

Amino acid transport is commonly studied using α-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 μg/ml. After the pre-incubation period in the CO_2 incubator, the cultures were transferred to a 37°water bath. One microcurie of ^{3}H-AIB (0.2-0.4 Ci/mmole) 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° 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 $5-^3H$-uridine and ^3H-thymidine was Schwarz Bioresearch, Inc., Orangeburg, New York. ^{51}Cr, ^{45}Ca and ^3H-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 (β-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).

Figure 1.

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 $\pm$ SEM of 3 or 4 replicate cultures
expressed as a percentage of the maxi-
mum 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 $\pm$ SEM of 3 or 4 replicate cultures
expressed as a percentage of the maxi-
mum response observed (15,300 CPM).   c.
Calcium uptake was determined at a Ca^{++}
concentration of 1.25 mM with a 60 min-
ute incubation with ^{45}Ca immediately
after PHA addition.   Each point was the
mean uptake in n moles/10^6 lymphocytes $\pm$
SEM of 3 or 4 replicate cultures expres-
sed as a percentage of the maximum re-
sponse observed (135 n moles/10^6 lympho-
cytes).
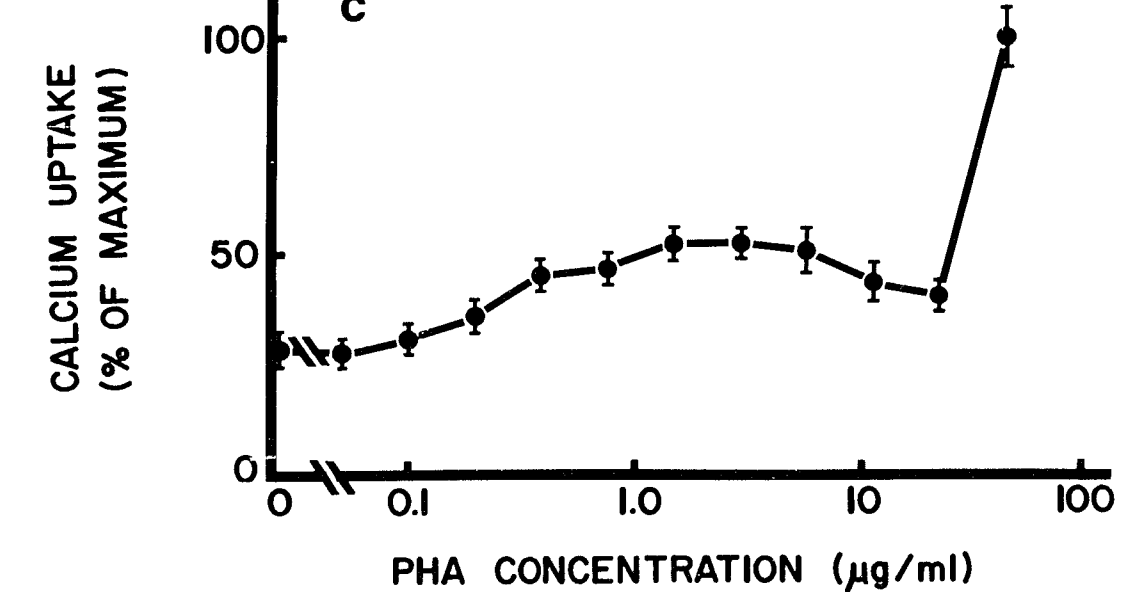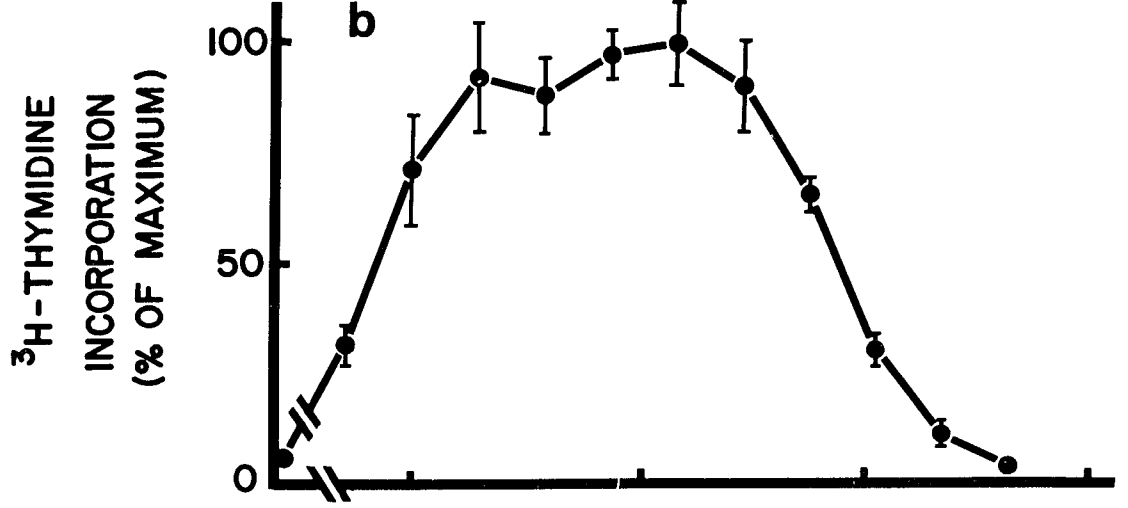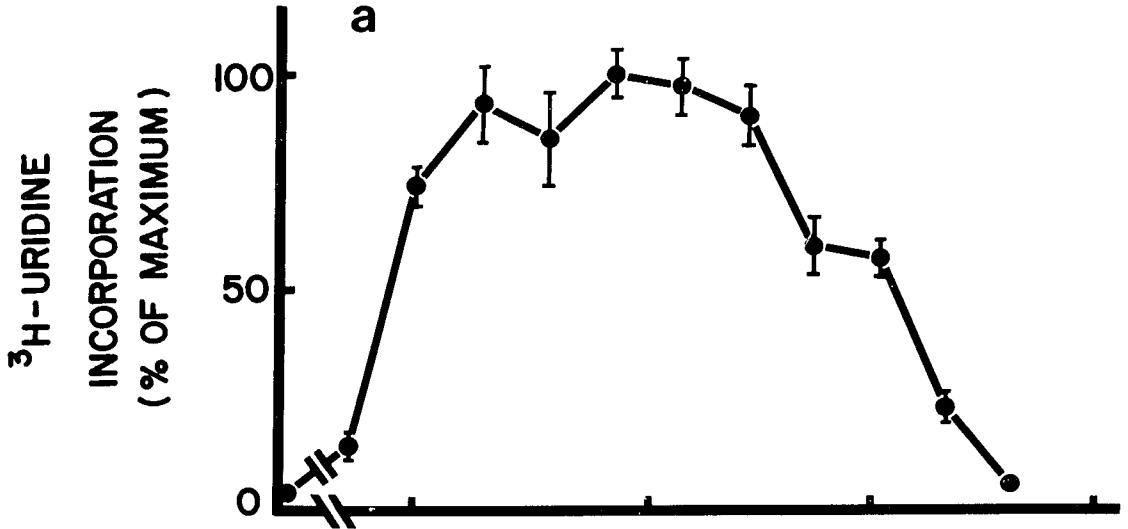
3.1.3 Time-Course of Increased Nucleic Acid Synthesis
and Morphological Transformation

The time-courses of RNA and DNA synthesis and mor-
phological 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 transform-
ation 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 stimu-
lated 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 reach-
ed 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 reaching 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 cultures 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. Lymphoblasts were not commonly observed in unstimulated cultures during the first 4 or 5 days, but an increased degree of spontaneous transformation (up to 4%) occasionally 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 consisted 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 diameter 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 $\pm$ 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 $\pm$ 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.
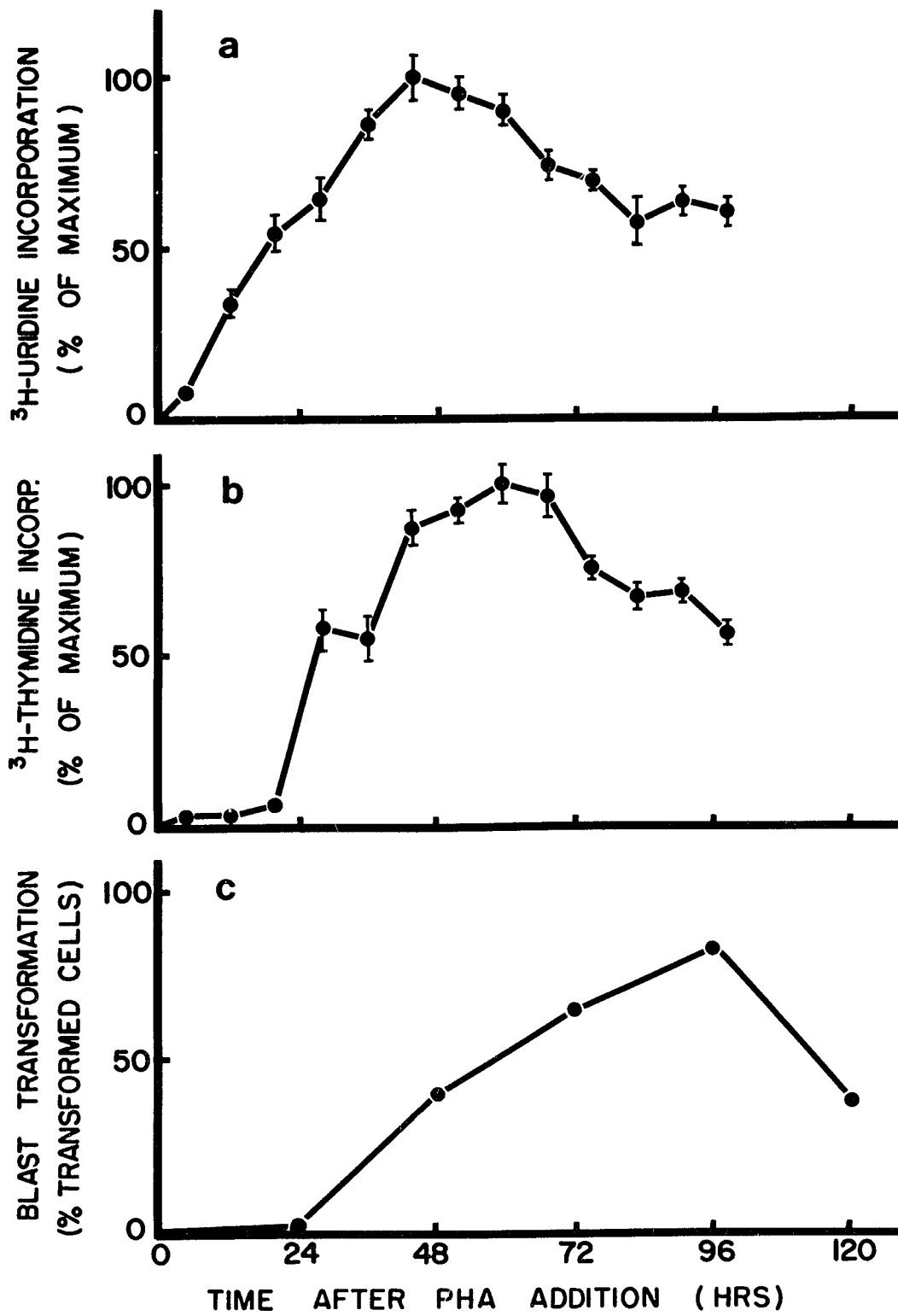
Figure 3.

Photomicrograph of the various morpho-

logical stages of lymphocyte transforma-

tion.   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.

a

b

c

d
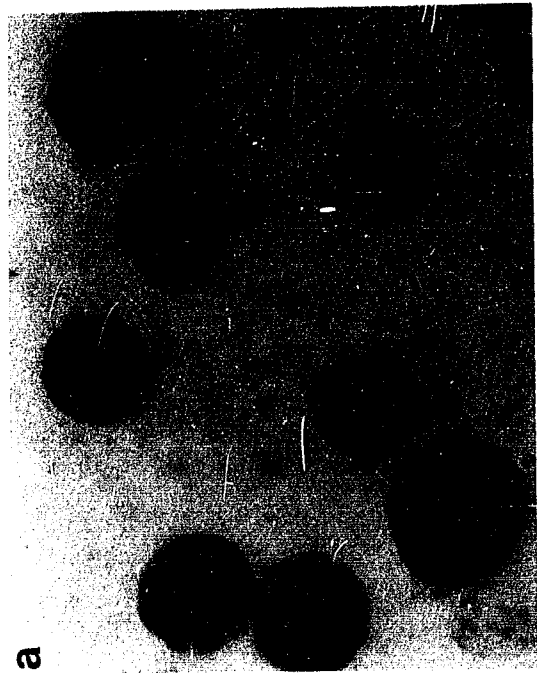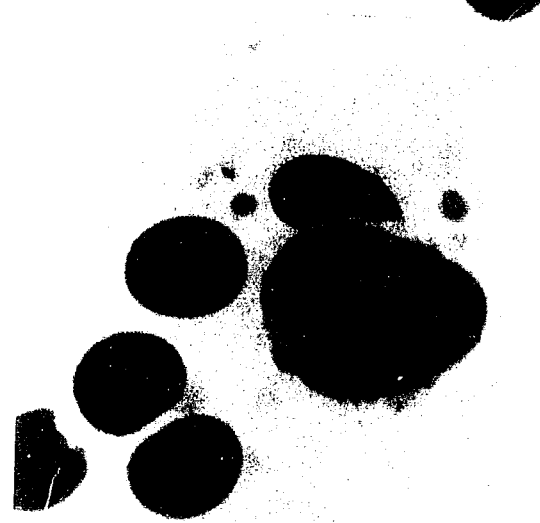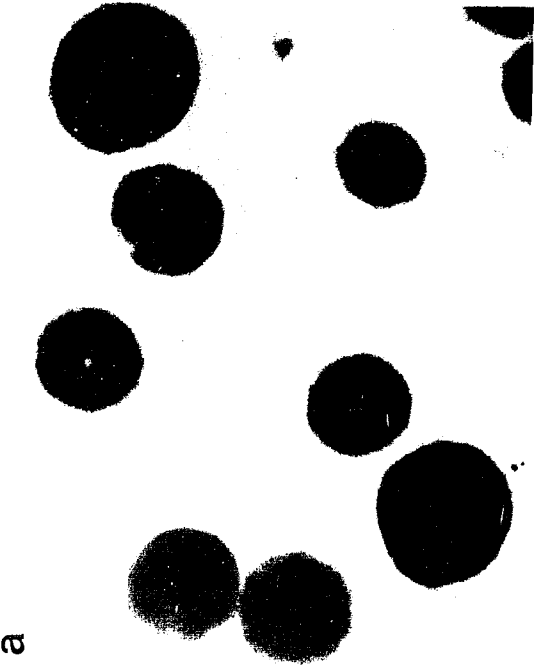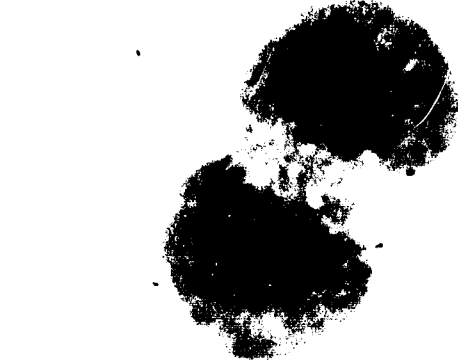
3.1.4 Time-Course of Increased Calcium and Amino Acid Uptake

Stimulation of Ca^{++} 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^{++} 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 lym-
phocytes. ●—● , PHA; O—O , unstimu-
lated. a. The initial rate of calcium
uptake was determined at $37°$ at a Ca^{++}
concentration of 1.25 mM with a 10 min-
ute exposure to ^{45}Ca at the indicated
times. Each point was the mean uptake
in n moles/10^6 lymphocytes/min $\pm$ SEM of
3 replicate cultures expressed as a per-
centage of the maximum rate of uptake
observed (8.7 n moles/min). b. The in-
itial rate of AIB uptake was determined
at $37°$ with a 15 minute exposure to $^3H-$
AIB at the indicated times. Each point
was the mean uptake in CPM/10^6 lympho-
cytes/15 min $\pm$ SEM of 3 replicate cul-
tures expressed as a percentage of the
maximum uptake observed (1190 CPM).
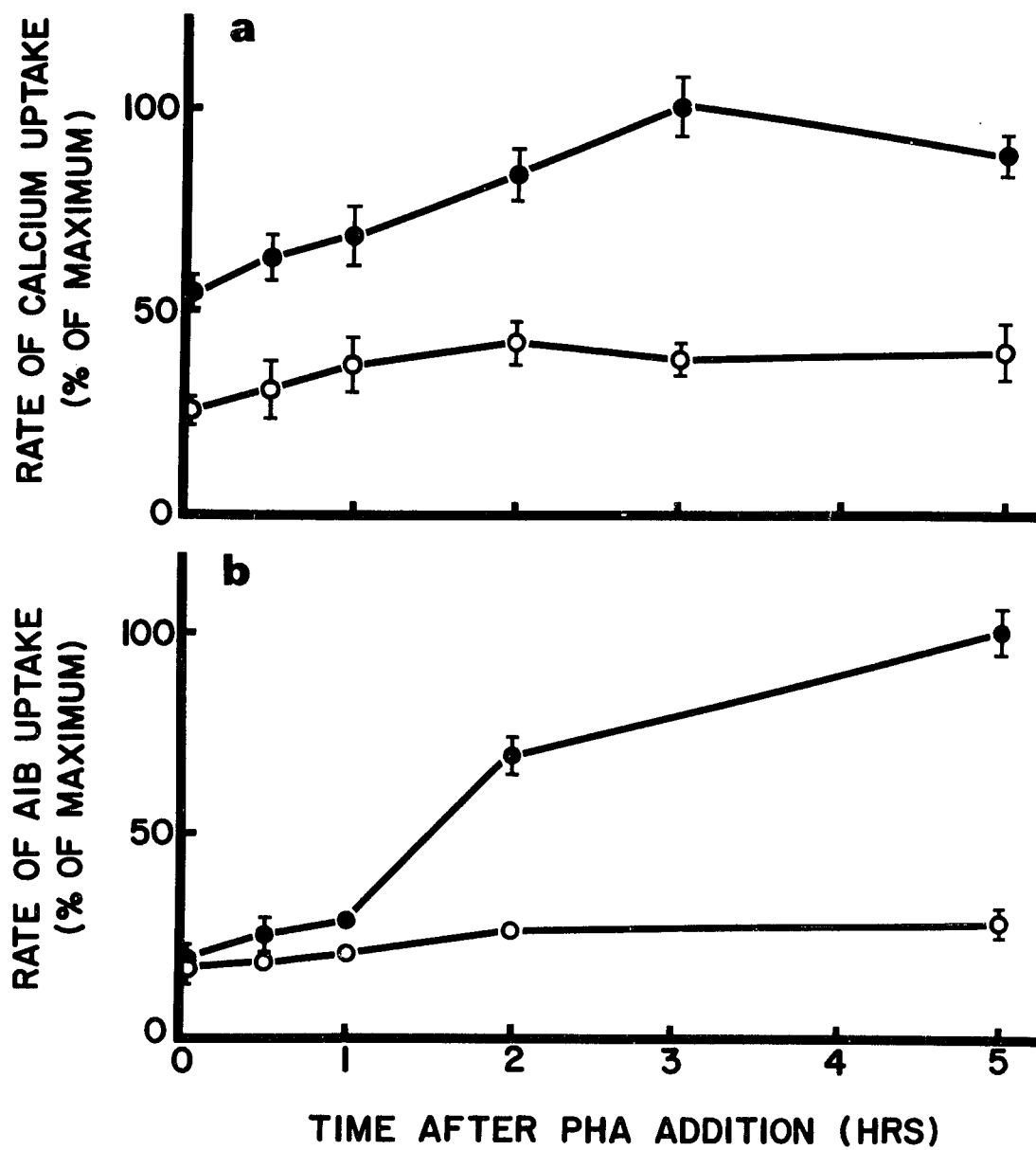
regarding the toxicity of an agent could be made with complete confidence. Consequently, the viability of lymphocytes cultured with or without PHA was followed for 8 days using the three techniques described in the methods.

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-stimu-
lated 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 $\pm$ 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 $\pm$ SEM of 3 replicate
cultures.

TIME AFTER PHA ADDITION (HRS)
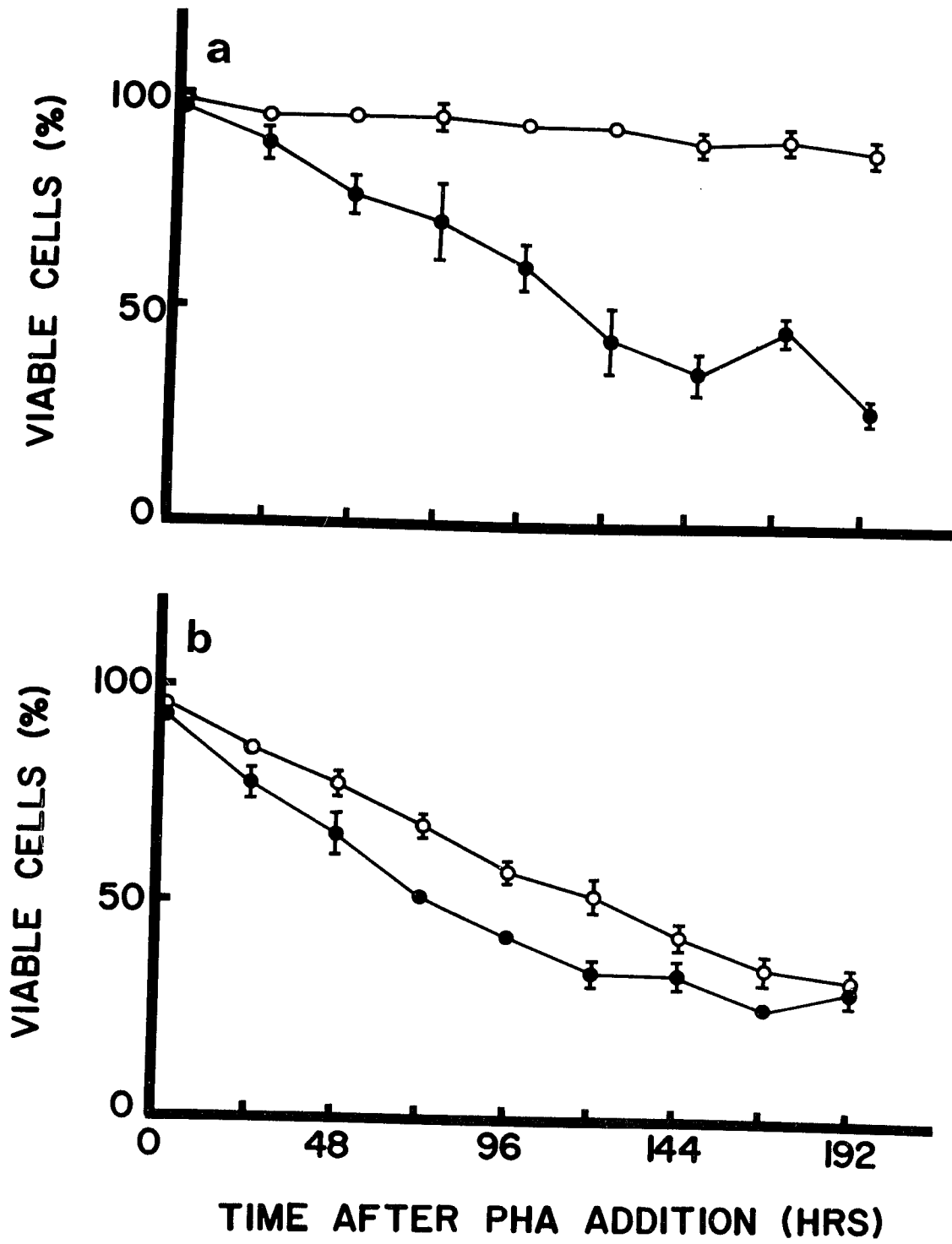
membrane. However, they probably measure different parameters of membrane breakdown. The final method gave an indication of the functional integrity of the cell population as a whole. PHA was added to a set of previously unstimulated cultures every 24 hours, and ^{3}H-uridine incorporation was determined 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]

Time PHA Added (Hrs)	^{3}H-Uridine Incorporation[2] (CPM $\pm$ SEM)
0	15,600 $\pm$ 952
24	18,600 $\pm$ 838
48	17,400 $\pm$ 767
72	13,900 $\pm$ 1980
96	19,900 $\pm$ 789
120	16,000 $\pm$ 469
144	12,700 $\pm$ 352
168	9,590 $\pm$ 271
192	7,980 $\pm$ 433

[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 $\pm$ 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 rapid-
ly 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
cytolytic 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 inter-
action of the stimulant with the cell membrane, and
alterations in membrane properties become apparent soon
after exposure to PHA. Since Ca^{++} are important in main-
taining 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 ini-
tiated first of all to determine if medium Ca^{++} were re-
quired 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 ethyl-
enediaminetetraacetic acid (EDTA). This agent has an

affinity for Ca^{++} which is about 10^2 times greater than
its affinity for Mg^{++} (133). In more recent studies
EDTA has been largely replaced by EGTA. The affinity of
EGTA for Ca^{++} is more than 10^5 times greater than its
affinity for Mg^{++} (133) so the use of this agent pro-
vides much greater assurance that observed effects are
indeed due to a reduction of the level of available Ca^{++}.
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 transforma-
tion was inhibited by EDTA at concentrations above
10^{-4}M (Fig. 6). Complete inhibition of DNA synthesis
occurred at about 10^{-3}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^{-2}M (135).

The previous experiments indicated that divalent
cations were required to permit transformation to pro-
ceed. However, no conclusions could be drawn regarding

Figure 6.

Effect of EDTA concentration on RNA and
DNA synthesis and morphological transfor-
mation in PHA-stimulated lymphocyte cul-
tures. 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 $\pm$ SEM of 3 to 5 replicate
cultures for RNA and DNA and duplicates
for blast transformation expressed as a
percentage of the maximum response ob-
served for each parameter (DNA, 33,630
CPM; RNA, 18,510 CPM; blast cells, 74.2%).

% OF NORMAL RESPONSE

EDTA CONCENTRATION (M)
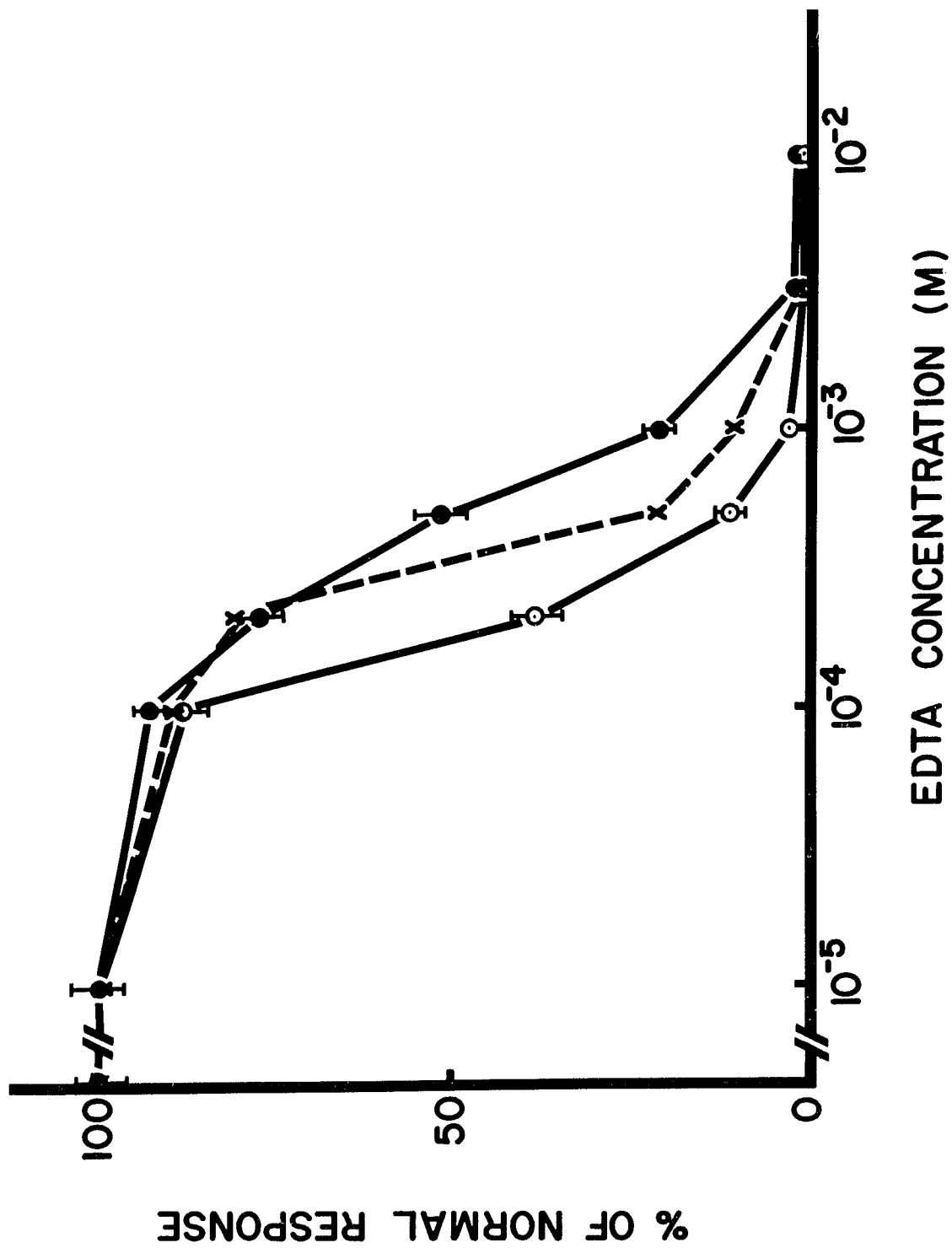
Ca^{++} specifically. Addition of excess Ca^{++} to PHA cultures treated with EDTA at any time up to 3 hours after PHA addition resulted in only a partial reversal of the inhibition of DNA synthesis even with as high a concentration as 7.5 mM (Fig. 7). Magnesium ions were completely ineffective in reversing 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 ^{3}H-thymidine present for the final 16 hours. Each point is the mean CPM $\pm$ SEM of 3 replicate cultures expressed as a percentage of the response obtained with PHA alone (28,300 CPM).
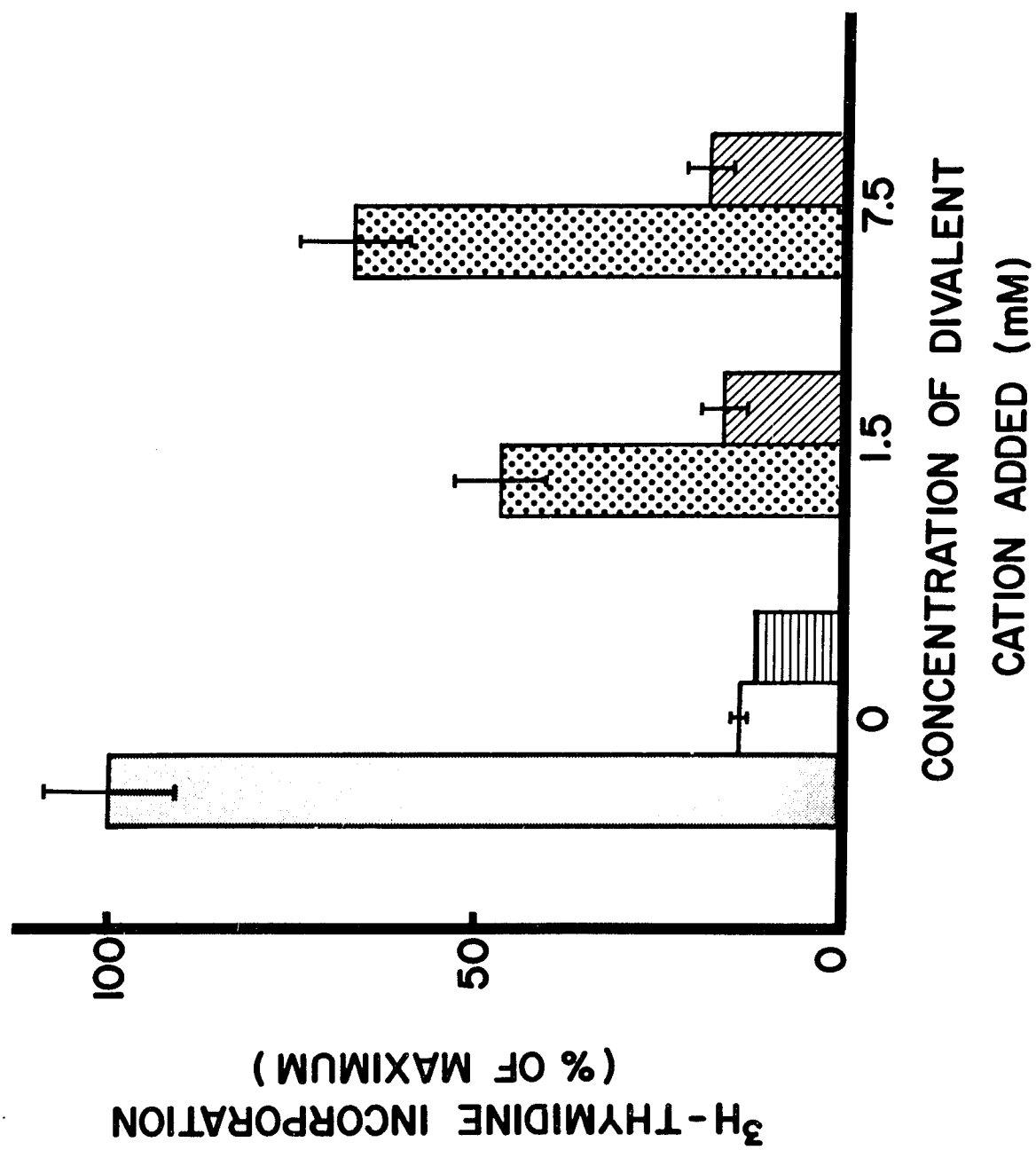
EGTA also inhibited the stimulatory effects of PHA beginning at concentrations slightly greater than the medium Ca^{++} concentration of 1.26 mM (Fig. 8). Complete inhibition of nucleic acid synthesis occurred at an EGTA concentration of 1.39 mM. Morphological transformation was significantly less sensitive 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 transfor-

mation 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 $\pm$ 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%).

EGTA CONCENTRATION (mM)

% OF NORMAL RESPONSE
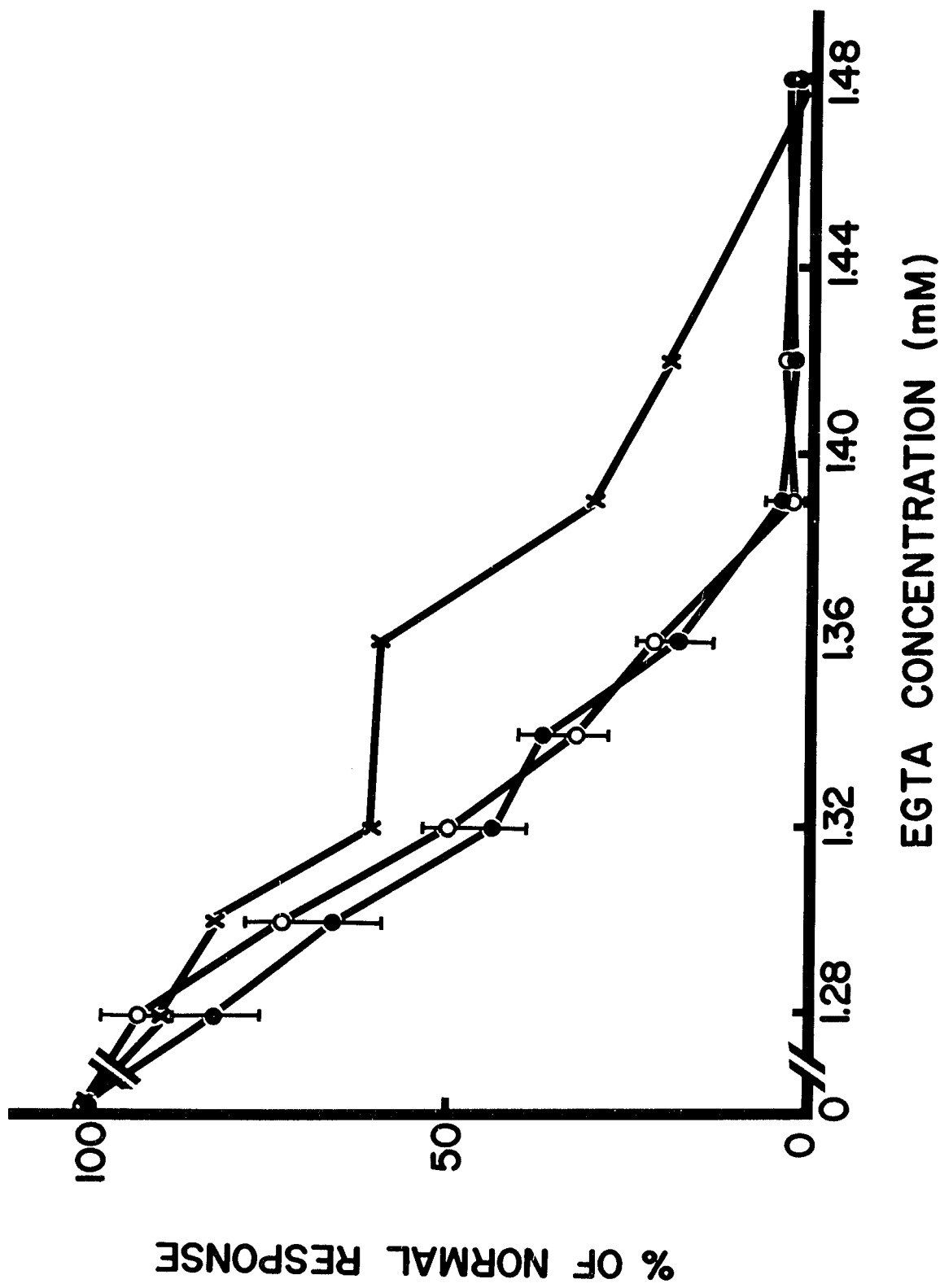
TABLE 2

Effect of EGTA on ^{3}H-Thymidine Incorporation by
Unstimulated Lymphocytes[1]

EGTA Concentration (mM)	^{3}H-Thymidine Incorporation[2] (CPM $\pm$ SEM)
0	385 $\pm$ 14
1.28	387 $\pm$ 16
1.30	378 $\pm$ 16
1.32	408 $\pm$ 10
1.34	372 $\pm$ 6
1.36	371 $\pm$ 30
1.39	374 $\pm$ 19
1.42	233 $\pm$ 15
1.48	206 $\pm$ 29

[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 $\pm$ 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 deter-
mined at 48 hours with ^{3}H-thymidine
present for the final 2 hours. Each bar
is the mean CPM $\pm$ SEM of 3 to 5 replicate
cultures expressed as a percentage of the
maximum level of synthesis observed
(2180 CPM).

CONCENTRATION OF DIVALENT
CATION ADDED (mM)

^{3}H-THYMIDINE INCORPORATION
(% OF MAXIMUM)
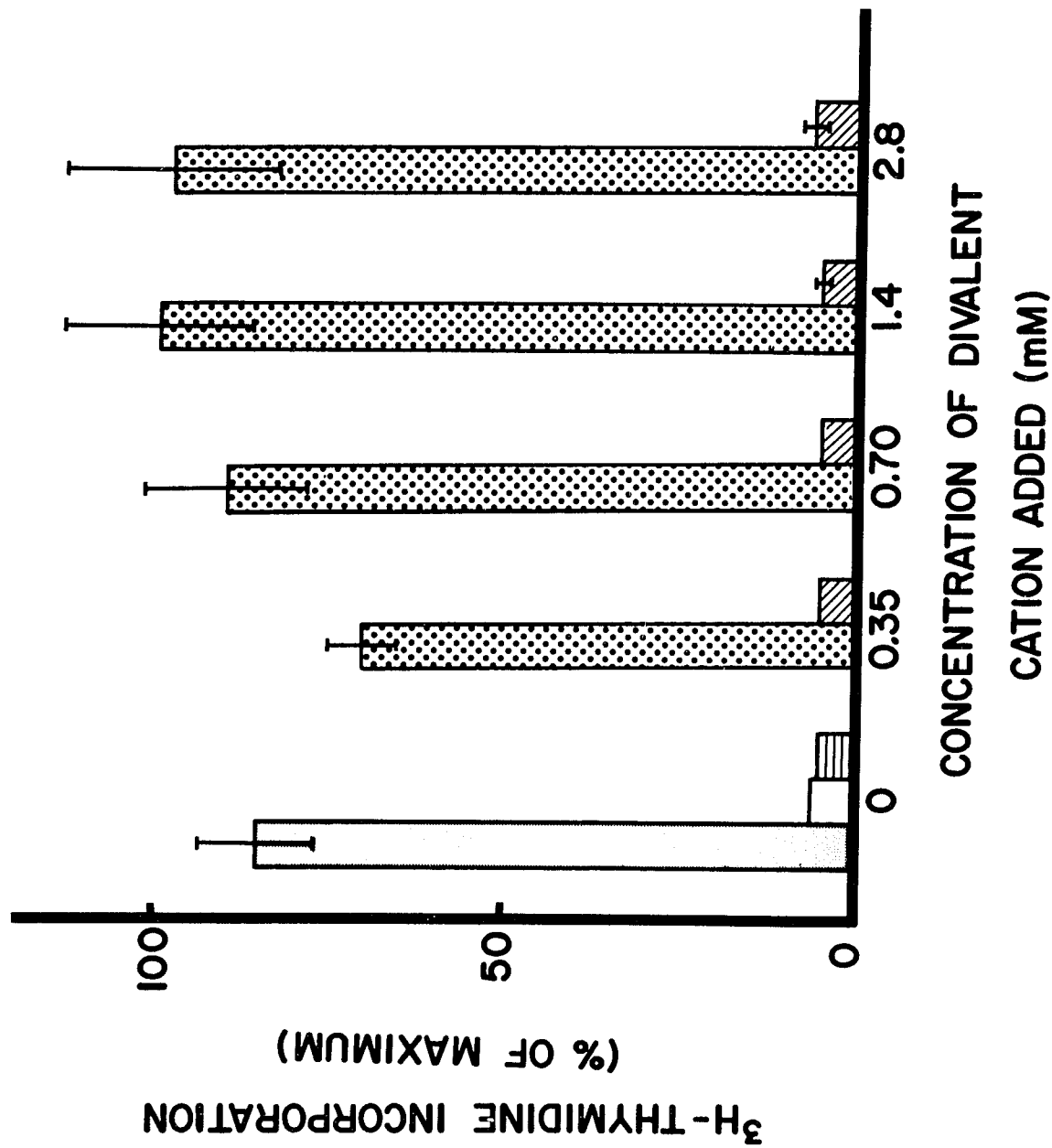
EGTA inhibition even at concentrations higher than are shown in Fig. 9. Manganese ions were similarly ineffective and were toxic at concentrations above 1.0 mM.

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 syn-

thesis observed (6820 CPM).
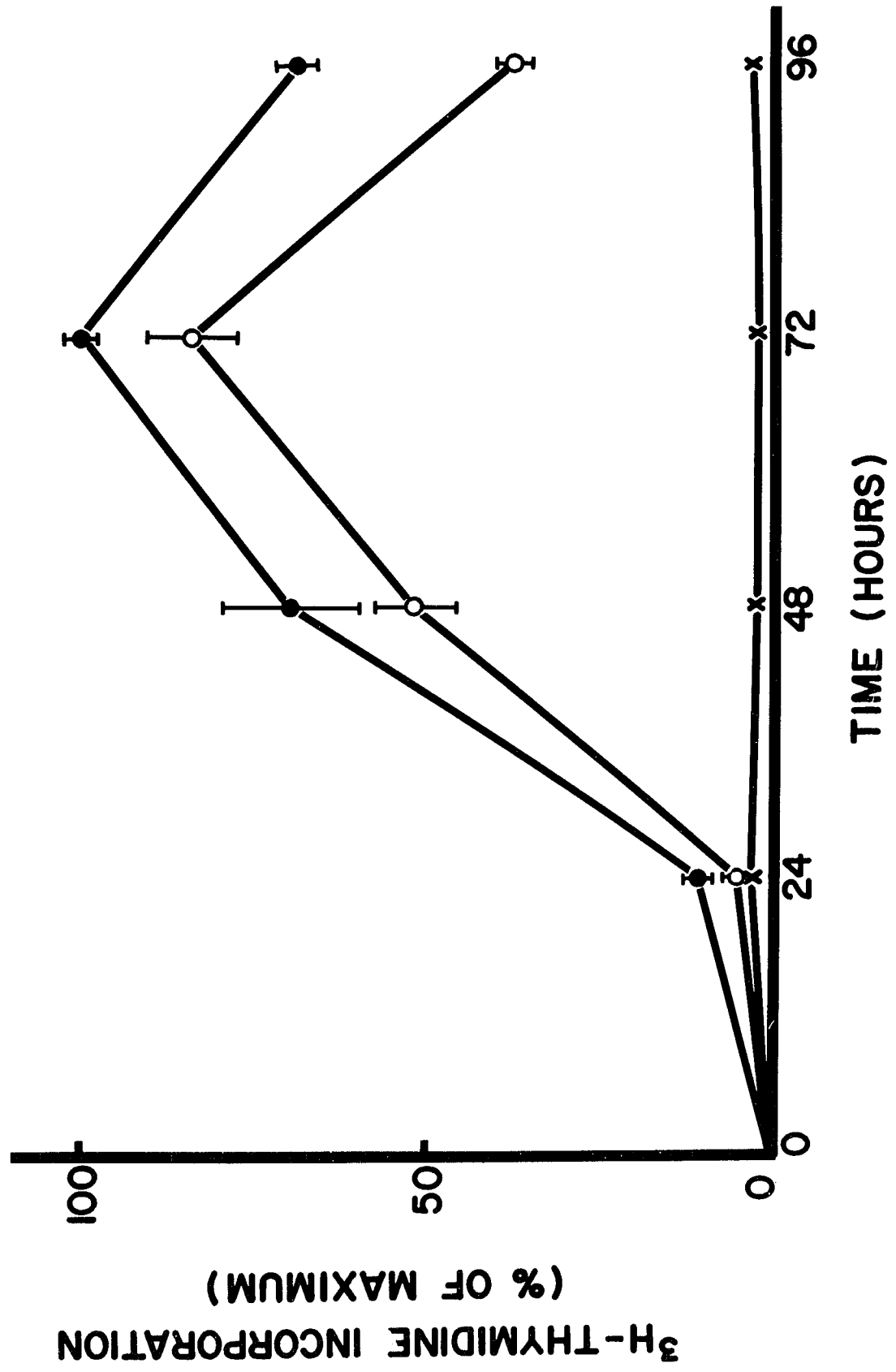
Maximum responses were observed in cultures with 20%

serum. Additional Ca^{++} were then added to the cultures

to provide a range of Ca^{++} concentrations for each serum

concentration.

Cultures containing 0.5% or 1% serum with no added

Ca^{++} gave responses not significantly different from

those of control cultures not treated with PHA. Their

Ca^{++} levels were near 7.0 x 10^{-6}M and 1.4 x 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^{++}

concentration until maximum responses were obtained at

concentrations in the range 6.0 x 10^{-4} to 1.6 x 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 re-

sponse were obtained with no added calcium. However,

these cultures had initial Ca^{++} concentrations of about

1.4 x 10^{-4} and 2.8 x 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^{++} con-

centration after additions to the medium containing 0.5%

and 1% serum. Raising the Ca^{++} concentration above the

physiological level (1.3 mM) resulted in slightly de-

creased isotope incorporation although increased varia-

bility among samples made the differences not significant

Figure 11.

Influence on PHA-induced RNA synthesis of adding Ca^{++} at various concentrations to cultures grown in 4 different concentrations of AB serum. $\square$—$\square$, 20% serum; $\bigcirc$—$\bigcirc$, 10% serum; $\bullet$—$\bullet$, 1% serum; $\times$—$\times$, 0.5% serum. Ca^{++} were added to commercial calcium-free medium plus serum 15 minutes before addition of PHA. RNA synthesis was determined at 48 hours with ^{3}H-uridine present for the final 16 hours. Each point is the mean CPM $\pm$ 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%).
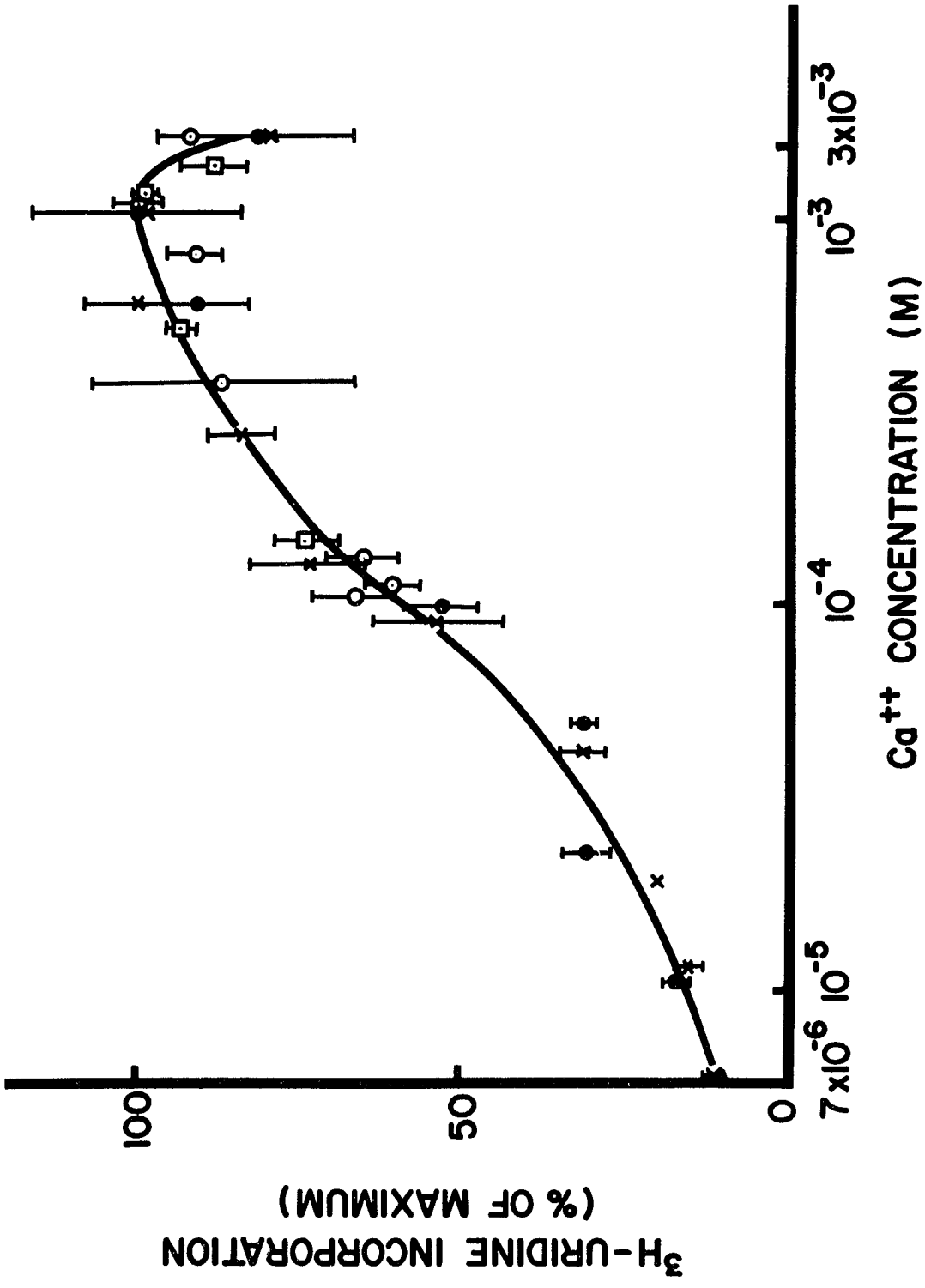
statistically. Precipitation due to serum phosphate and/or toxic effects probably account for the variation in these cases. Magnesium ions could not substitute for Ca^{++} in this experiment.

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 (3×10^{-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 inhib-
itory 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 ^{3}H-thymidine incor-
poration 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 comple-
tely 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 previ-
ously stimulated lymphocytes. ●—●, PHA
only; O—O , PHA + 1.42 mM EGTA; ■—■ ,
PHA + 1.42 mM EDTA; X—X , PHA + 3 x 10^{-7}M
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 deter-
mined at various times thereafter with a
2 hour pulse. Each point is the mean
CPM $\pm$ SEM of 3 to 5 replicate cultures
expressed as a percentage of the maximum
level of synthesis observed (3300 CPM).

TIME AFTER AGENT ADDITION (HRS)

^{3}H-THYMIDINE INCORPORATION
(% OF MAXIMUM)
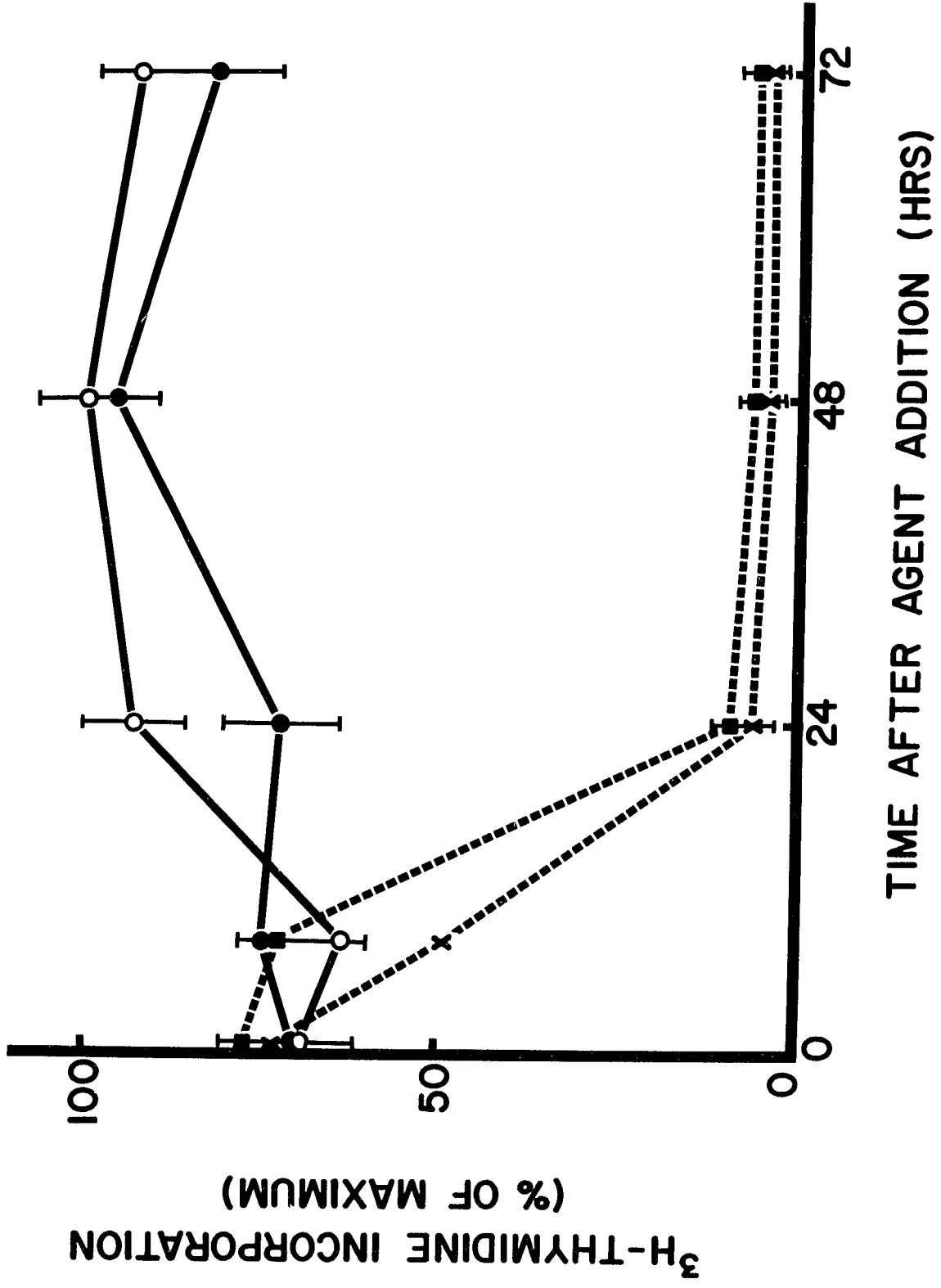
TABLE 3

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

Time of EGTA Addition Relative to PHA (Hrs)	^{3}H-Thymidine Incorporation[2]	
	CPM $\pm$ SEM	% Inhibition
PHA Only	1740 $\pm$ 120	0
-1	231 $\pm$ 34	98
-¼	240 $\pm$ 32	98
0	351 $\pm$ 65	90
+1	382 $\pm$ 20	88
+2	320 $\pm$ 17	92
+4	490 $\pm$ 85	81
+8	722 $\pm$ 168	66
+12	1123 $\pm$ 143	40
+16	1631 $\pm$ 97	0
+24	1852 $\pm$ 97	0
+46	1807 $\pm$ 172	0

[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 $\pm$ 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 com-
pared 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 trans-
formation. 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 ini-
tiation of RNA synthesis, predominantly of a low molecu-
lar weight species. Therefore, the effect of EGTA on
the development of this early RNA synthesis was deter-
mined in order to see if Ca^{++} were required for the
response to proceed to that stage. In PHA-treated cul-
tures 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 slight-
ly above control levels by 2 hours where it remained
through 6 hours. EGTA had no effect on control RNA
synthesis.

TABLE 4

Effect of Washing PHA-treated Lymphocytes in the
Presence or Absence of EGTA[1]

Length of[2] Exposure to EGTA (Hrs)	^{3}H-Uridine Incorporation[3] (CPM $\pm$ SEM) EGTA Concentration	
	0	1.42 mM
1/6	1940 $\pm$ 180	2320 $\pm$ 205
1	2460 $\pm$ 174	2240 $\pm$ 320
3	3480 $\pm$ 236	4005 $\pm$ 362
48 (not washed)	5280 $\pm$ 326	150 $\pm$ 3

[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 min-
utes 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.
RNA synthesis was determined at 48 hours with
[3]H-uridine present for the final 4 hours.

[3]The results were the mean CPM $\pm$ 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 min-
utes prior to adding PHA, and the incor-
poration of ^{3}H-uridine was determined at
the indicated times with a 1 hour pulse.
Each point is the mean CPM $\pm$ SEM of 3 or
4 replicate cultures expressed as a per-
centage of the control response in the
absence of EGTA (165 CPM).

TIME (HOURS)

^{3}H-URIDINE INCORPORATION
(% OF CONTROL LEVEL)
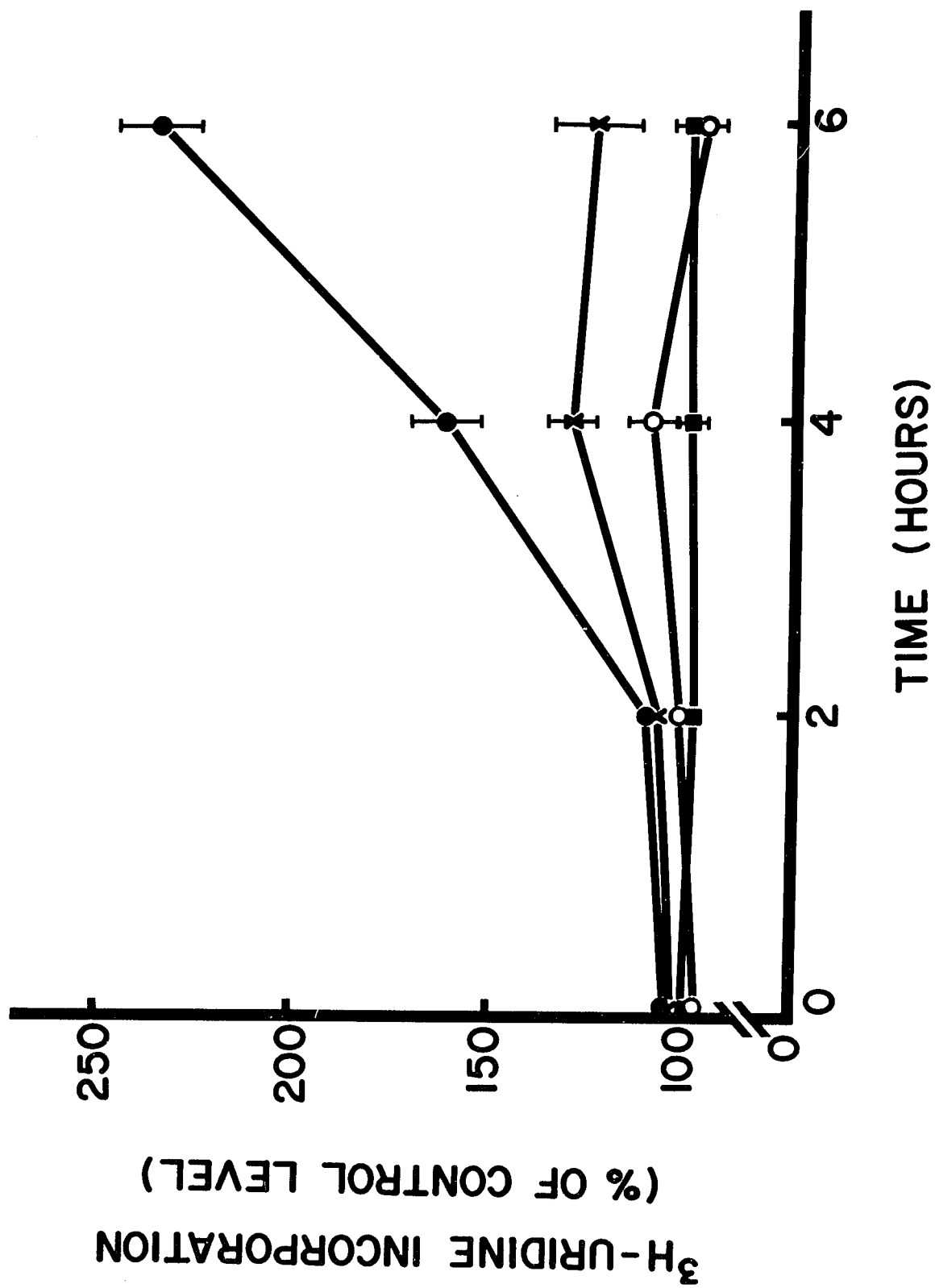
Another early event in PHA-induced transformation
is an enhancement of amino acid uptake which could be a
critical preliminary preparation for the dramatic in-
creases in protein synthesis observed later. As men-
tioned previously Ca^{++} can influence membrane permeabil-
ity to other materials so the effect of lowering the
Ca^{++} concentration on ^{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^{++} 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^{++}. If EGTA
(1.4 mM) was added to the cultures, AIB uptake was in-
hibited by about 62% in both PHA and control cultures.
The inhibitory effect of EGTA could be reversed by add-
ing more Ca^{++}. Addition of 3 mM Ca^{++} to the EGTA-
treated cells resulted in a reversal of most of the in-
hibition of AIB uptake (Table 5). Culturing the cells
in medium containing 0.025 mM Ca^{++} 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^{++} levels was
due to a direct requirement of Ca^{++} 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]

Treatment	AIB Uptake[2] (CPM $\pm$ SEM)	
	PHA	Control
1.25 mM Ca^{++}	694 $\pm$ 7	117 $\pm$ 8
0.025 mM Ca^{++}	284 $\pm$ 42	93 $\pm$ 11
1.40 mM EGTA	123 $\pm$ 6	22 $\pm$ 6
1.40 mM EGTA + 3.00 mM Ca^{++}	635 $\pm$ 27	105 $\pm$ 15

[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 $\pm$ SEM of 3 replicate cultures.

addition. Addition of EGTA ½ hour before or ½ hour
after PHA reduced uptake to control levels (100% inhib-
ition) (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 con-
cluded 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 6×10^{-4}M.
The latter was about one-half the normal physiological
level. Normal responses occurred at higher concentra-
tions. 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 add-
ition of the chelator. Also EGTA treatment was progres-
sively less effective at causing inhibition with pro-

TABLE 6

Effect on AIB Uptake of Adding EGTA at Different Times
Relative to PHA Addition[1]

Time of EGTA Addition Relative to PHA (Hrs)	AIB Uptake[2]	
	CPM $\pm$ SEM	% Inhibition
PHA only	757 $\pm$ 17	0
$-\frac{1}{2}$	123 $\pm$ 6	100
$+\frac{1}{2}$	140 $\pm$ 1	100
+2	260 $\pm$ 10	81
+4	256 $\pm$ 31	80
no PHA	136 $\pm$ 11	100

[1]Lymphocytes were cultured with PHA for 4 hours.
^{3}H-AIB uptake was then determined with a 15 minute pulse.

[2]Each value is the mean uptake in CPM/10^6 lymphocytes/ 15 min $\pm$ SEM of 3 replicate cultures. They were then expressed as % inhibition of the PHA-stimulated up-take. 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_∞ 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 $t_{\frac{1}{2}}$ 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

Figure 14.

Calcium uptake with time in unstimulated
and PHA-treated lymphocytes. ●—● , PHA;
○—○ , unstimulated. a. PHA was added
to cultures at 0 time, and ^{45}Ca was imme-
diately added. The cultures were incu-
bated at 37° at a medium Ca^{++} concentra-
tion 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_∞ equal-
ed uptake at 90 minutes, and $P(t)$ equal-
ed uptake at time t. The slope of the
line equaled k, the rate constant, while
$t_{\frac{1}{2}}$ was the time required to obtain ½ 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 respec-
tively.
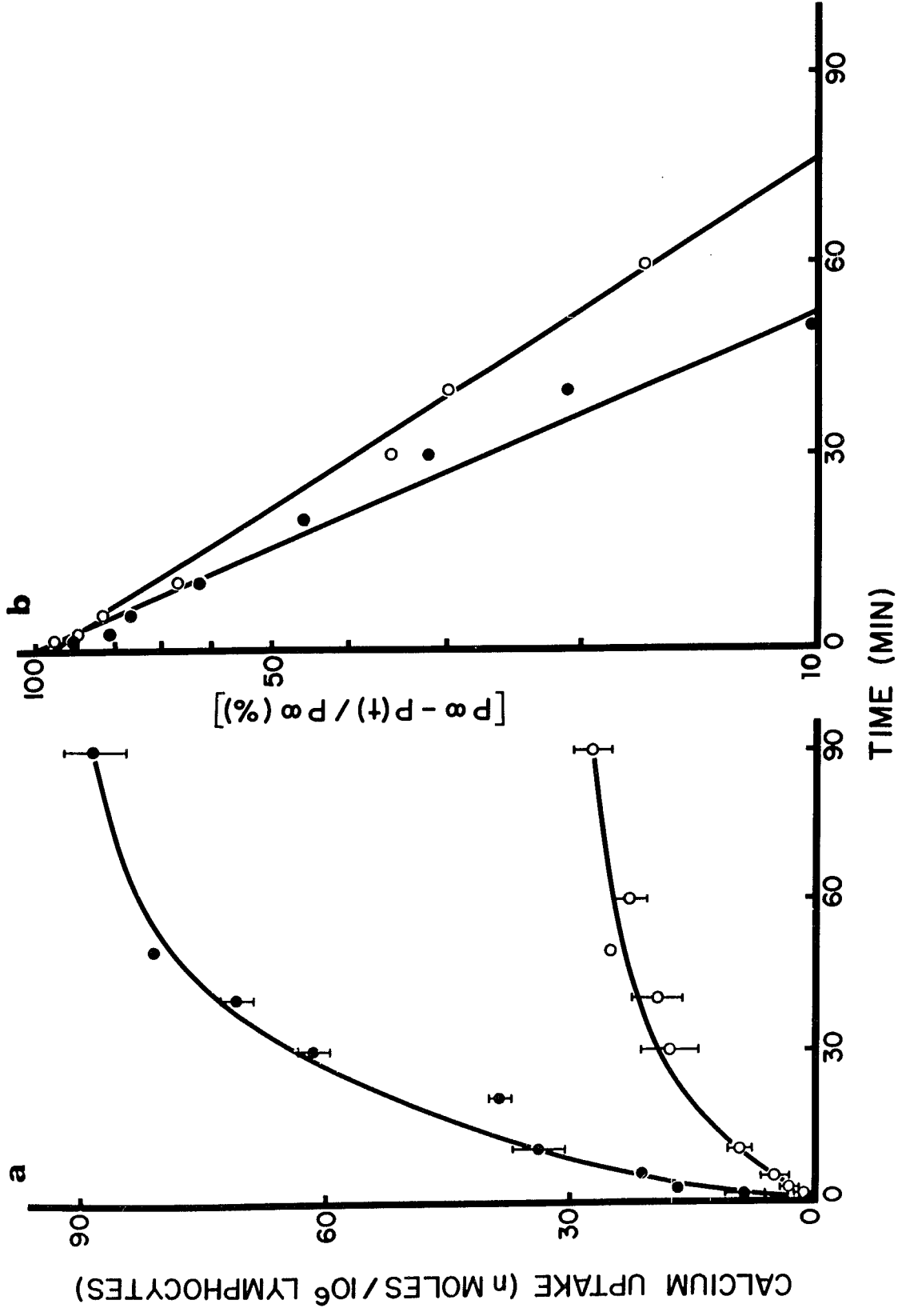
TABLE 7

Enhanced Calcium Uptake by Mouse Spleen Cells Treated with PHA[1]

Experiment	Calcium Uptake[2] (n moles $\pm$ SEM)		Statistical Significance (t-test)
	PHA	Control	
1	64.2 $\pm$ 2.0	41.6 $\pm$ 1.0	$p < 0.01$
2	53.5 $\pm$ 1.9	38.9 $\pm$ 2.7	$p < 0.05$

[1]Mouse spleen cells were cultured with or without PHA for 1 hour at a Ca^{++} concentration of 1.25 mM with ^{45}Ca present.

[2]Results are expressed as the mean uptake in n moles/10^6 nucleated spleen cells $\pm$ SEM of 4 replicate cultures.

by which time both protein and RNA synthesis has increas-
ed considerably (Fig. 15).

The pH of the incubation medium had no effect on
Ca^{++} 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 sim-
ilar pattern was observed in unstimulated cultures ex-
cept that uptake was significantly lower at pH 6.5 than
it was at pH 7. These observations probably indicate
a competition of H^+ with Ca^{++} for binding sites or pos-
sibly 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^{++} uptake by lymphocytes required expen-
diture of metabolic energy.

The temperature coefficient (Q_{10}) for Ca^{++} uptake
was determined by measuring the rate of accumulation at
7°, 17°, 27° and 37°. Calcium accumulation increased lin-
early with increasing temperature in both unstimulated
and PHA-treated cultures (Fig. 17a). The average Q_{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); ▨ , unstim-
ulated; ☐ , PHA-treated (uptake de-
termined from 11 to 12 hours after ex-
posure). Results are expressed as the
mean uptake in n moles/10^6 lymphocytes $\pm$
SEM of 3 replicate cultures.

CALCIUM UPTAKE
(n MOLES / 10^6 LYMPHOCYTES)

NO
ADDITIVE

ACTINOMYCIN D
4×10^{-7} M

CYCLOHEXIMIDE
1.1×10^{-4} M
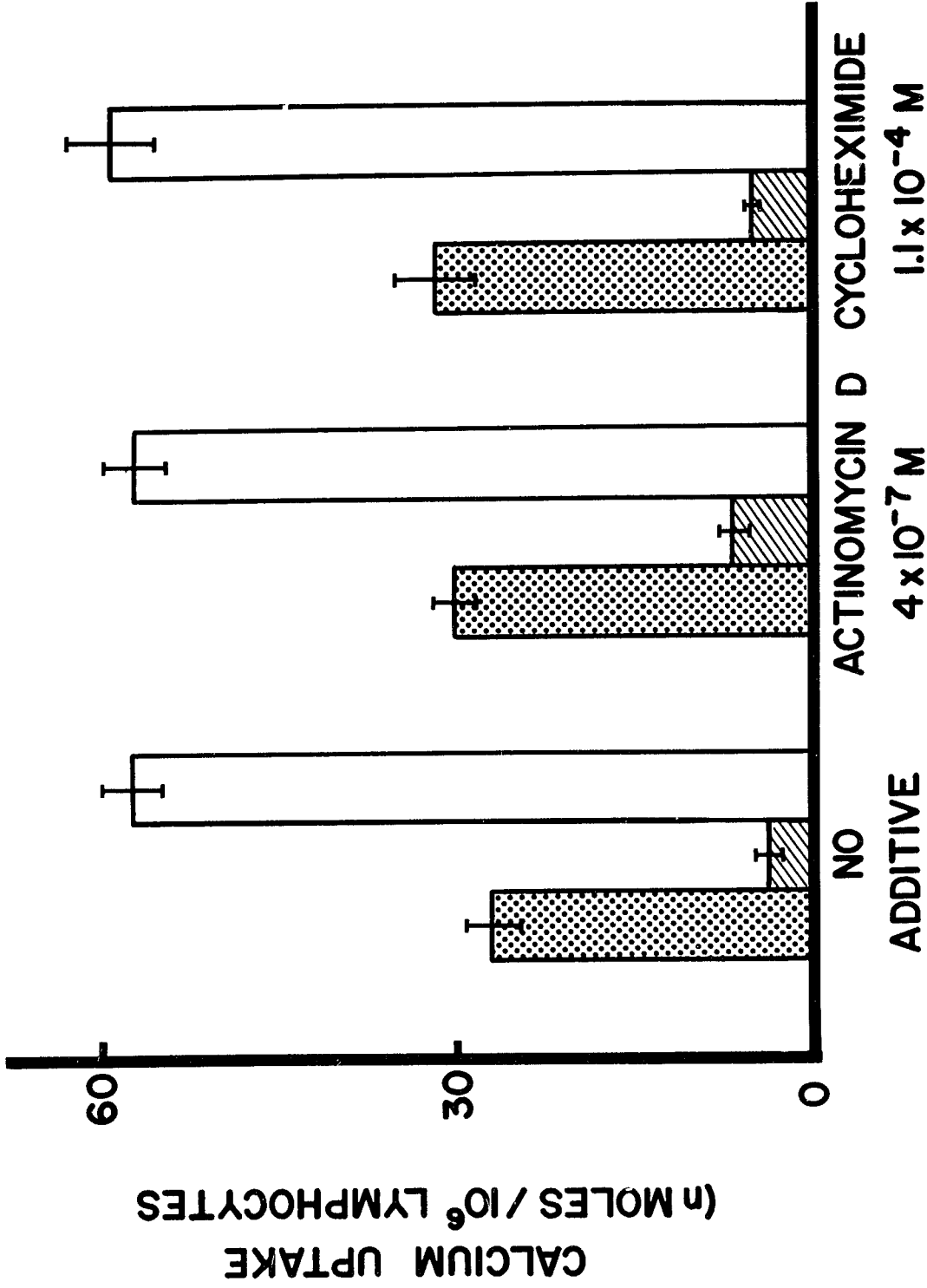
Figure 16.

Variation in lymphocyte calcium transport
with pH of the incubation medium. ●—● ,
PHA; O—O , unstimulated. Lymphocytes
were suspended in media of different pH.
PHA was added to the appropriate cultures,
and calcium uptake was determined im-
mediately at $37°$with a 30 minute incuba-
tion with ^{45}Ca. Uptake was expressed as
n moles/10^6 lymphocytes $^+_-$ SEM of 3 rep-
licate cultures.
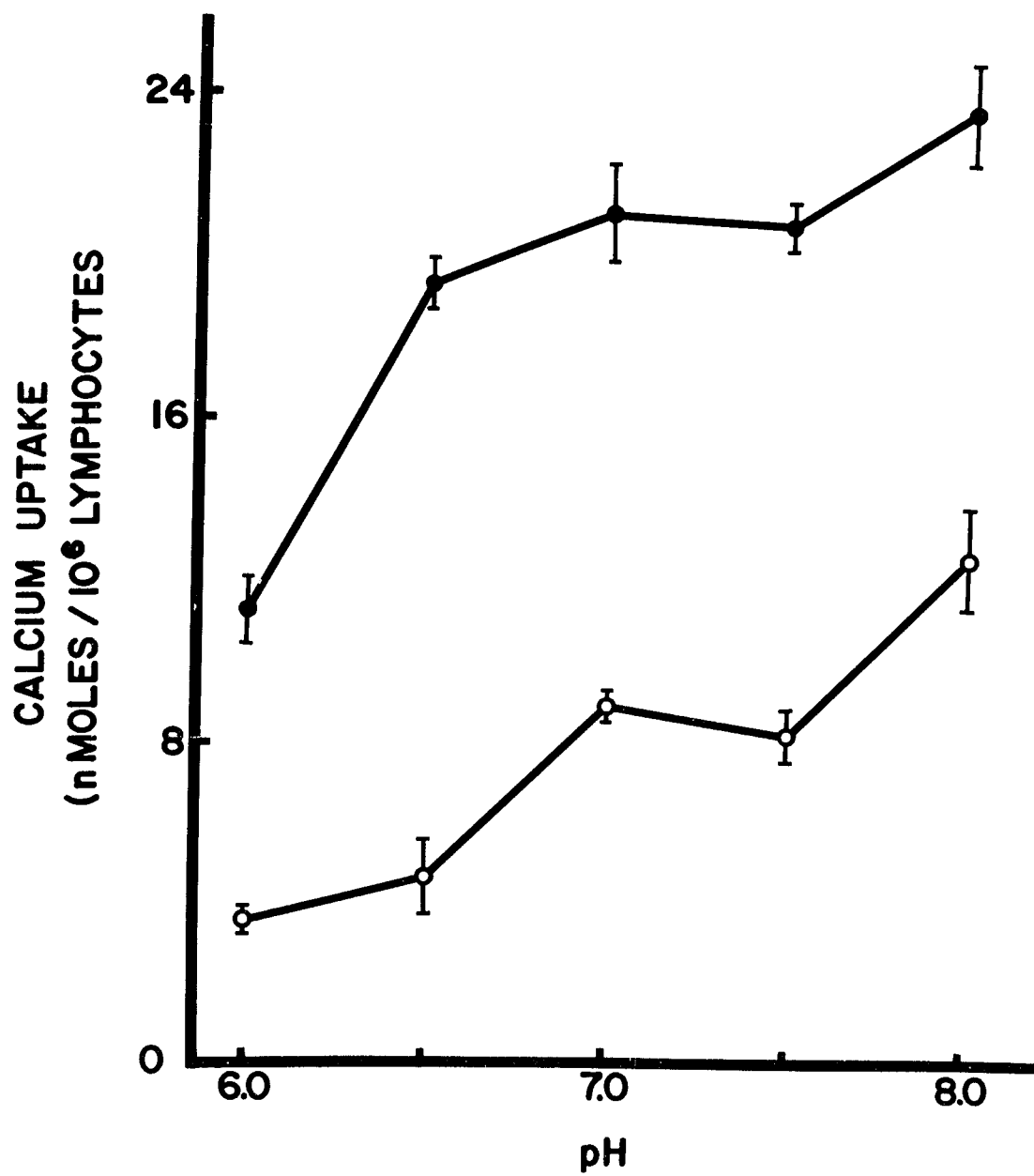
and 1.58 respectively. Fig. 17b is an Arrhenius plot of
the data in Fig. 17a. He showed that the activation
energy (E) could be determined experimentally. The rate of
a reaction depends on the number of molecules having the
required act'va.'on energy. By plotting the natural log
of the rates o. the reaction at different temperatures
against the reciprocal of the absolute temperature, E can
be calculated from: $E = 2.303 \ (\log k_2 - \log k_1) \ R/T_1 - T_2$, and
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 up-
take by unstimulated and PHA-treated
lymphocytes. ●—● , PHA; O—O , unstim-
ulated. a. The initial rate of calcium
uptake was determined with a 15 minute
incubation with ^{45}Ca at a Ca^{++} concen-
tration of 0.025 mM. The cultures were
pre-cooled to the respective temperature
before the isotope was added. Uptake was
determined immediately after PHA addi-
tion. The rate of uptake was expressed
as n moles/10^6 lymphocytes/min $\pm$ 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 un-
stimulated and PHA-stimulated cells
respectively. For b. they were ln y =
-4.02x + 15.9 and ln y = 3.29x + 14.2
respectively.
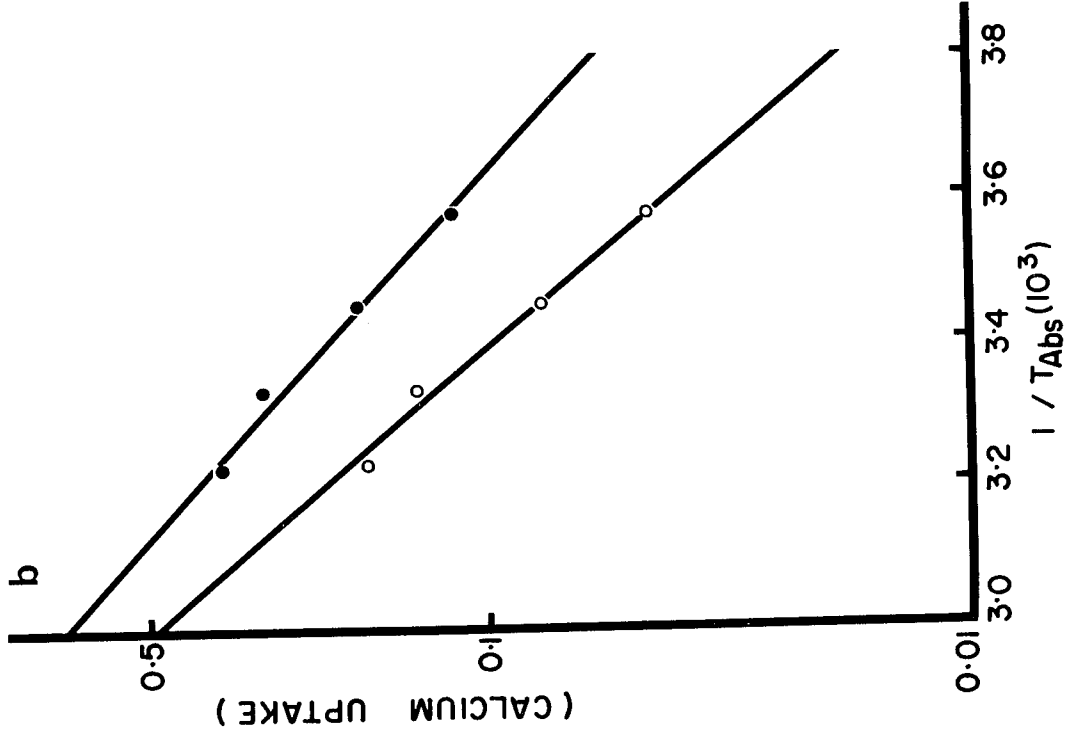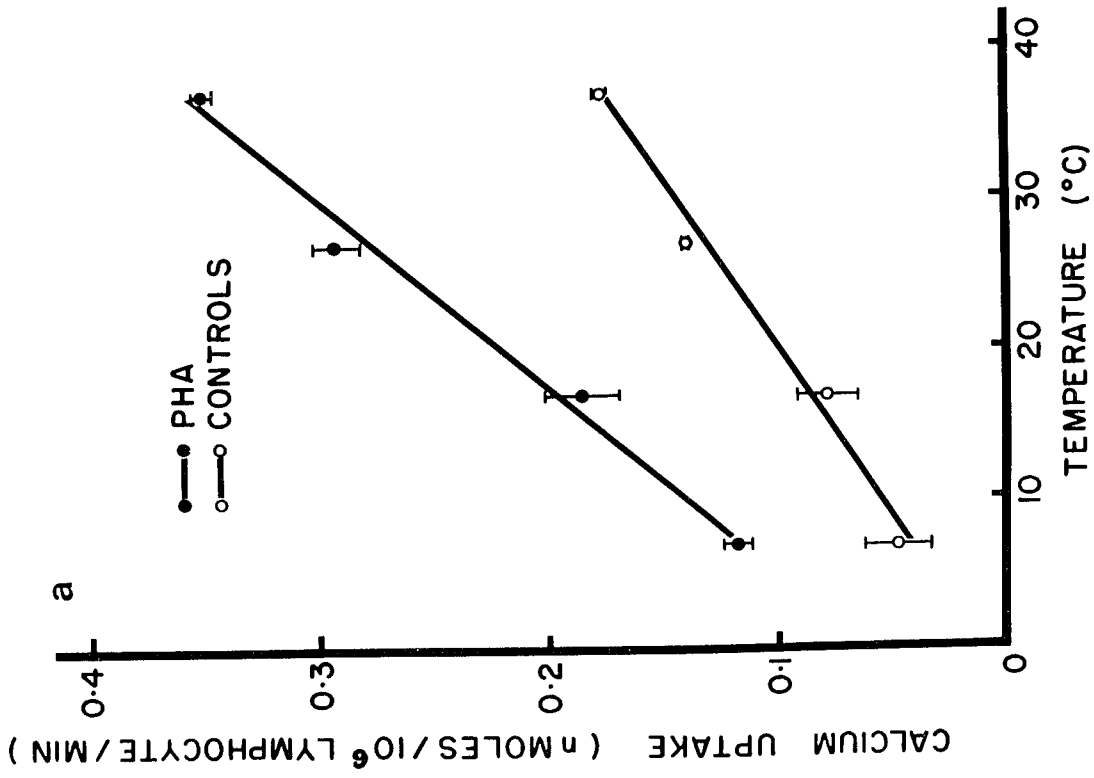
TABLE 8

Effect of Metabolic Inhibitors on
Calcium Uptake by Lymphocytes[1]

Agent[2]	Calcium Uptake[3] (n moles $\pm$ SEM)	
	PHA	Control
none	12.5 $\pm$ 0.49	8.22 $\pm$ 0.29
Azide	12.3 $\pm$ 0.17	8.09 $\pm$ 0.20
Ouabain	12.8 $\pm$ 0.45	7.97 $\pm$ 0.16
Iodoacetamide	9.03 $\pm$ 0.29	7.32 $\pm$ 0.13
N-Ethylmaleimide	7.16 $\pm$ 0.52	5.38 $\pm$ 0.13
Fluoride	26.8 $\pm$ 1.2	18.1 $\pm$ 0.87

[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 $\pm$ SEM of 3 replicate cultures.

low Q_{10} and is relatively insensitive to metabolic in-
hibitors 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 de-
scribes a rectangular hyperbola (154).

$$ v = \frac{S \ V_{max}}{S + K_m} $$

In this equation for Ca^{++} transport v = the initial rate
of uptake, V_{max} = the maximum rate of uptake which can
be attained under the specified conditions, S = sub-
strate concentration (Ca^{++}) and K_m = the apparent
Michaelis constant which is numerically equivalent to
the concentration at which $\frac{1}{2} \ V_{max}$ 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_{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.

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

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

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_{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[1]

Time (Min)	Calcium Uptake[2] (nmoles ± SEM)			
	0.025 mM Ca++		1.25 mM Ca++	
	PHA	Control	PHA	Control
4	2.46 ± .19	0.98 ± .05	26.6 ± 1.6	10.3 ± 1.2
8	3.07 ± .10	1.46 ± .04	39.8 ± 2.2	17.2 ± 1.4
12	3.87 ± .32	1.62 ± .07	47.5 ± .81	21.2 ± .35
16	4.32 ± .22	1.90 ± .09	64.2 ± 1.0	31.0 ± 4.7
correlation[3] coefficient	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 lympho-
cytes. a. The initial rate of calcium
uptake (v) was determined at 37° with a
10 minute incubation with ^{45}Ca at dif-
ferent 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 max-
imal velocity (V_{max}) and the apparent
Michaelis constant (K_{m}). The regres-
sion line was fitted by least squares
analysis and was found to be y =
-0.728x + 13.0.
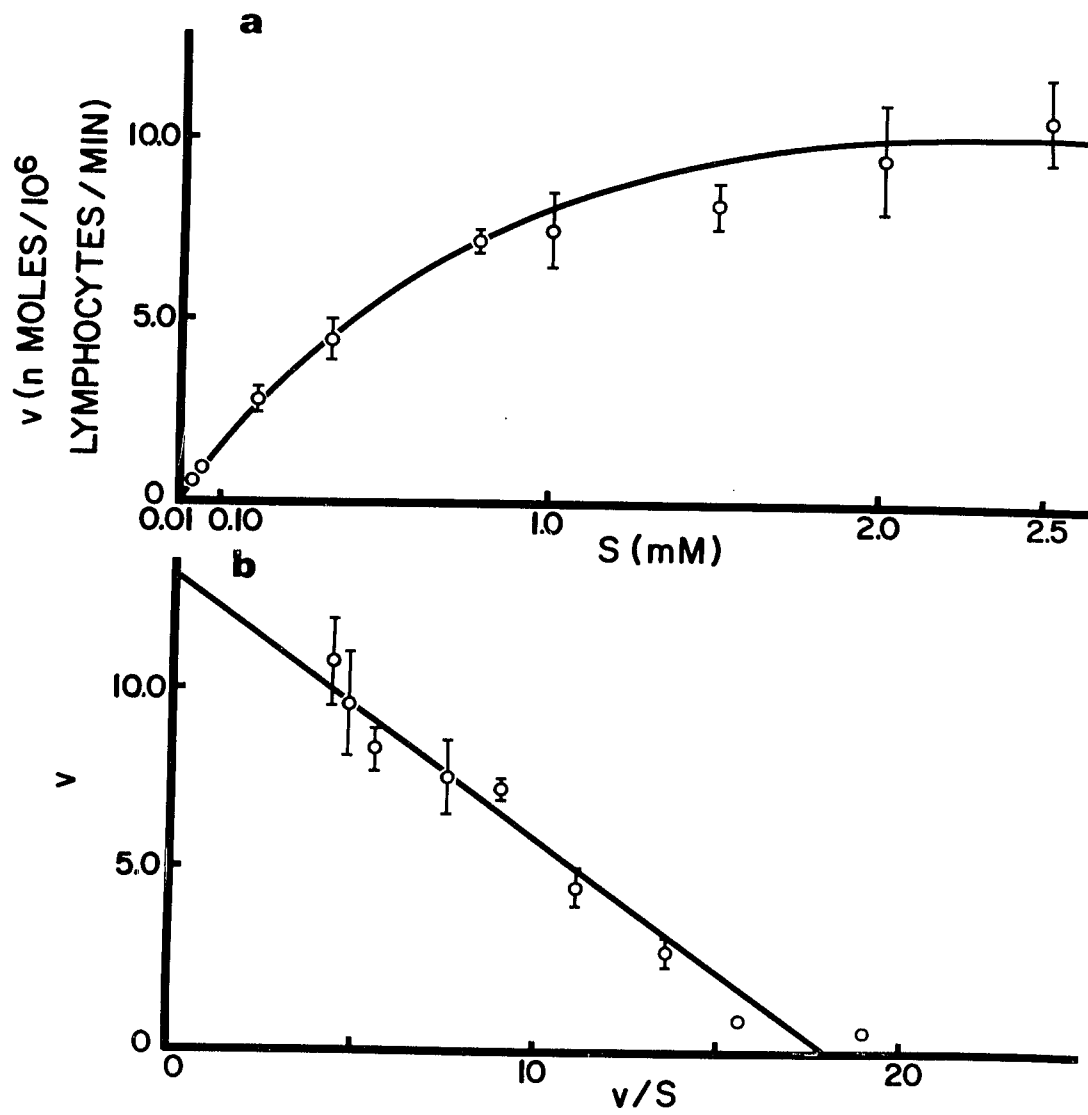
similar molecules, other divalent cations in this case, can also be taken as evidence for the mediation of a carrier. The effect of manganese (Mn^{++}) was tested since it has been found to inhibit Ca^{++} accumulation by chick embryo fibroblasts (145), and since it seems to be accumulated by the same 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_{max} of Ca^{++} uptake (2.77 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.

$$\frac{1}{v} = \frac{K_m}{V_{max}} (1 + I/K_I) \cdot \frac{1}{S} + \frac{1}{V_{max}}$$

Thus, the intercept on the l/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

Figure 19.

Inhibition of calcium uptake in unstimu-

lated lymphocytes by manganese. ●—● ,

no Mn^{++}; O—O , 5 mM Mn^{++}. a. The ini-

tial rate of calcium uptake (v) was det-

ermined at $37°$ with a 10 minute incuba-

tion with ^{45}Ca at different Ca^{++} con-

centrations (S in mM). The rate of up-

take was expressed as n moles/10^6 lym-

phocytes/min $\pm$ SEM of 3 replicate cul-

tures. b. A Lineweaver-Burk plot

(1/v versus 1/S) of the data in a. from

which V_{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.
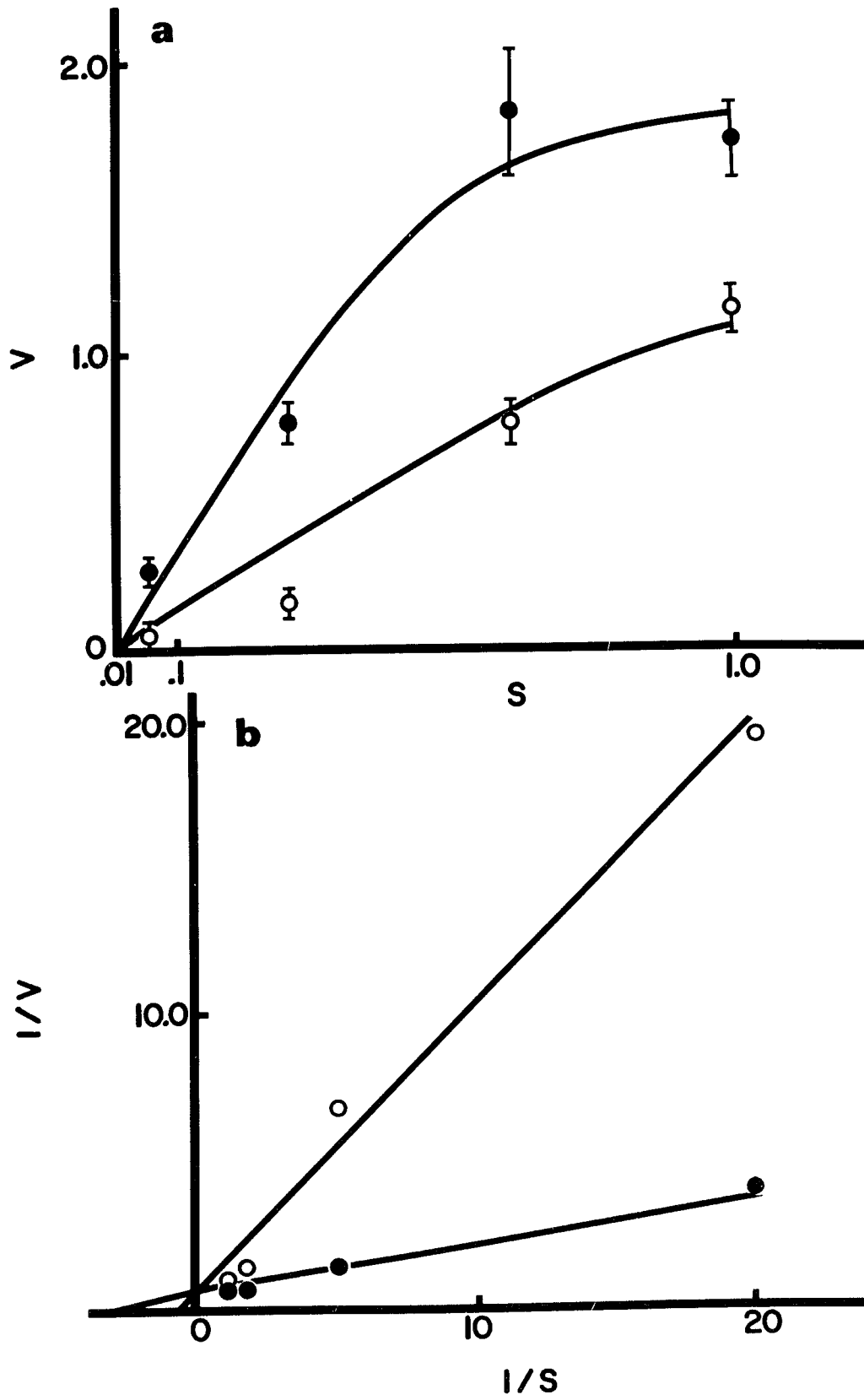
in medium containing 0.025 mM Ca^{++} plus ^{45}Ca. No significant differences in uptake were observed in the pre-loaded cells (Fig. 20a).

In the other experiment the rate of Ca^{++} uptake under steady state conditions was compared to the net rate of uptake at the same exogenous Ca^{++} level; that is, one set of cultures was incubated at normal Ca^{++} levels (1.25 mM) for several hours to pre-load the cells. Calcium uptake at 1.25 mM exogenous Ca^{++} 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^{++} 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^{++} accumulation at all external Ca^{++} 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_{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^{++} concen-
tration 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/10^6 lymphocytes $\pm$
SEM of 3 replicate cultures.
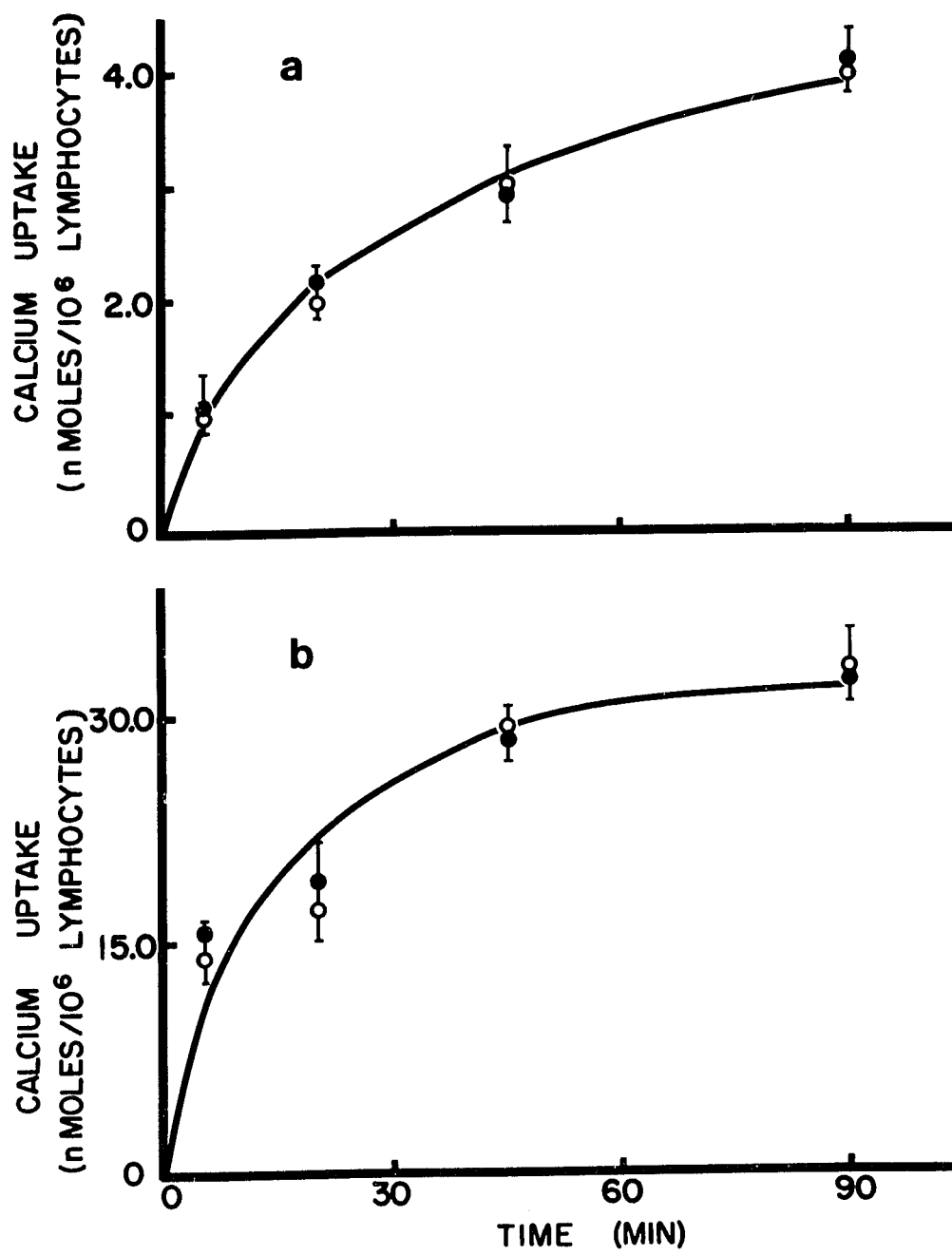
Figure 21.

Effect of medium Ca^{++} concentration on
calcium uptake in unstimulated lympho-
cytes and PHA-stimulated lymphocytes im-
mediately after PHA addition. ●—● ,
PHA; O—O , unstimulated. a. The ini-
tial 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 ex-
pressed as n moles/10^6 lymphocytes/min $\pm$
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.
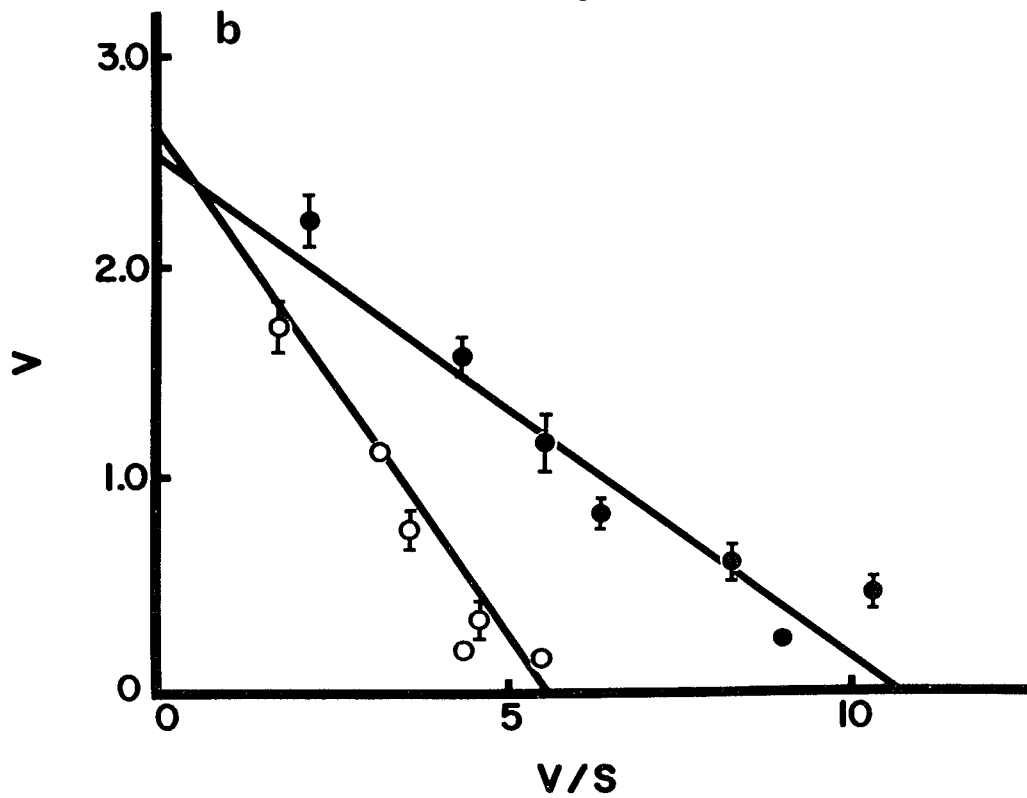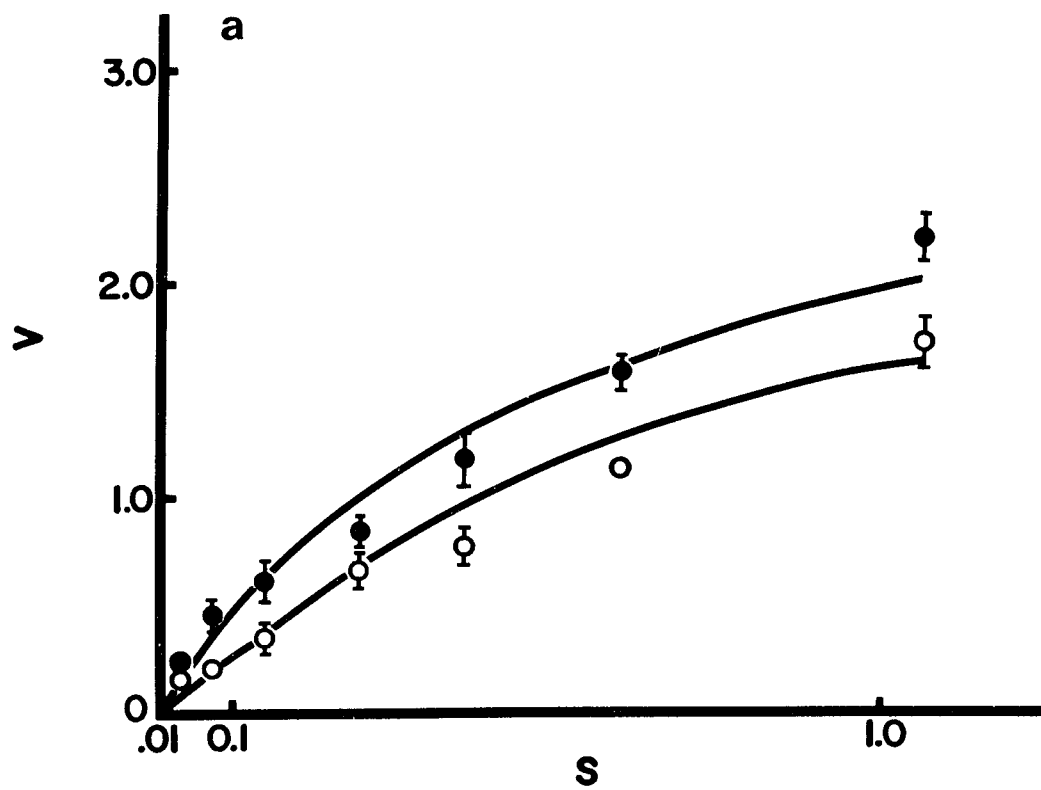
respectively which were not significantly different.

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 process-

es 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 lym-

phocytes were also made essentially as described pre-

viously for unstimulated cells. In one type of experi-

ment lymphocytes were pre-incubated with 0.025 or 1.25

mM Ca^{++} or with 1.4 mM EGTA to remove most of the resi-

dual medium Ca^{++}. They were then washed and resuspended

in 0.025 mM Ca^{++}, and uptake was determined for 20

Figure 22.

Effect of medium Ca^{++} concentration on
calcium uptake in unstimulated lympho-
cytes and lymphocytes exposed to PHA for
3 hours. ●—● , PHA; O—O , unstimulated.
a. The initial rate of uptake (v) was
determined at $37°$ with a 10 minute expo-
sure to ^{45}Ca at different Ca^{++} concen-
trations (S in mM) 3 hours after PHA
treatment. The rate of uptake was ex-
pressed 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 V_{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.
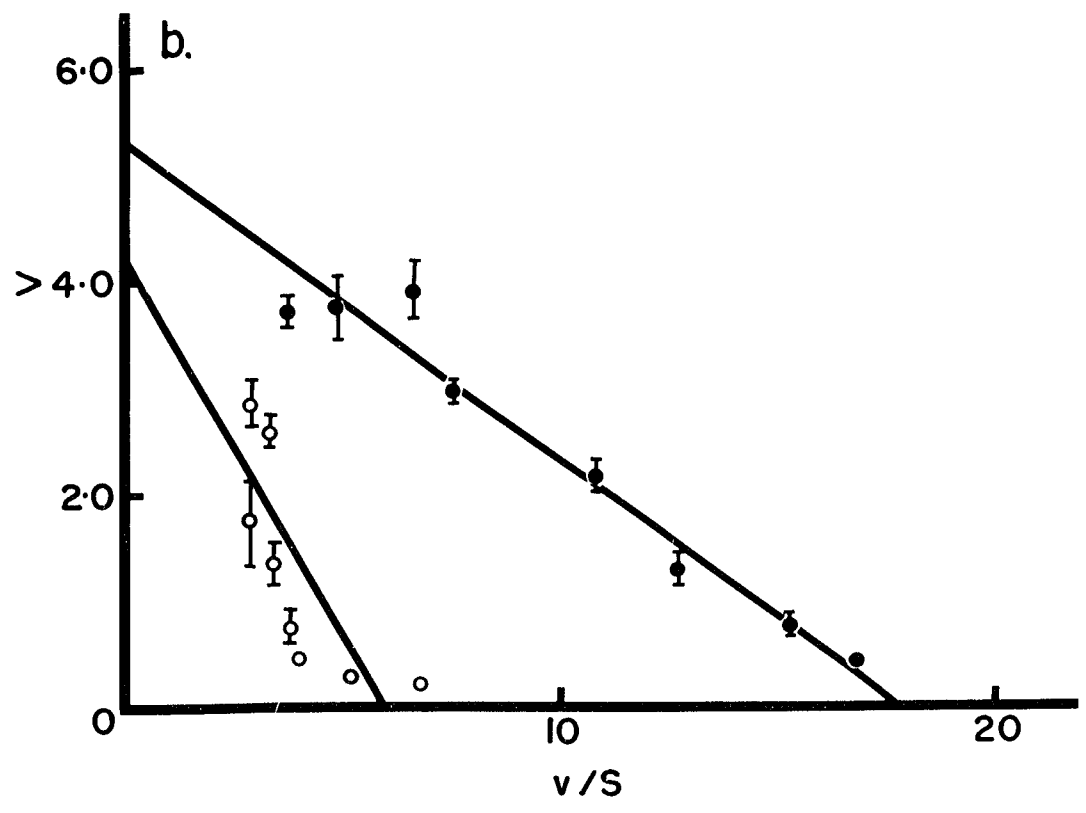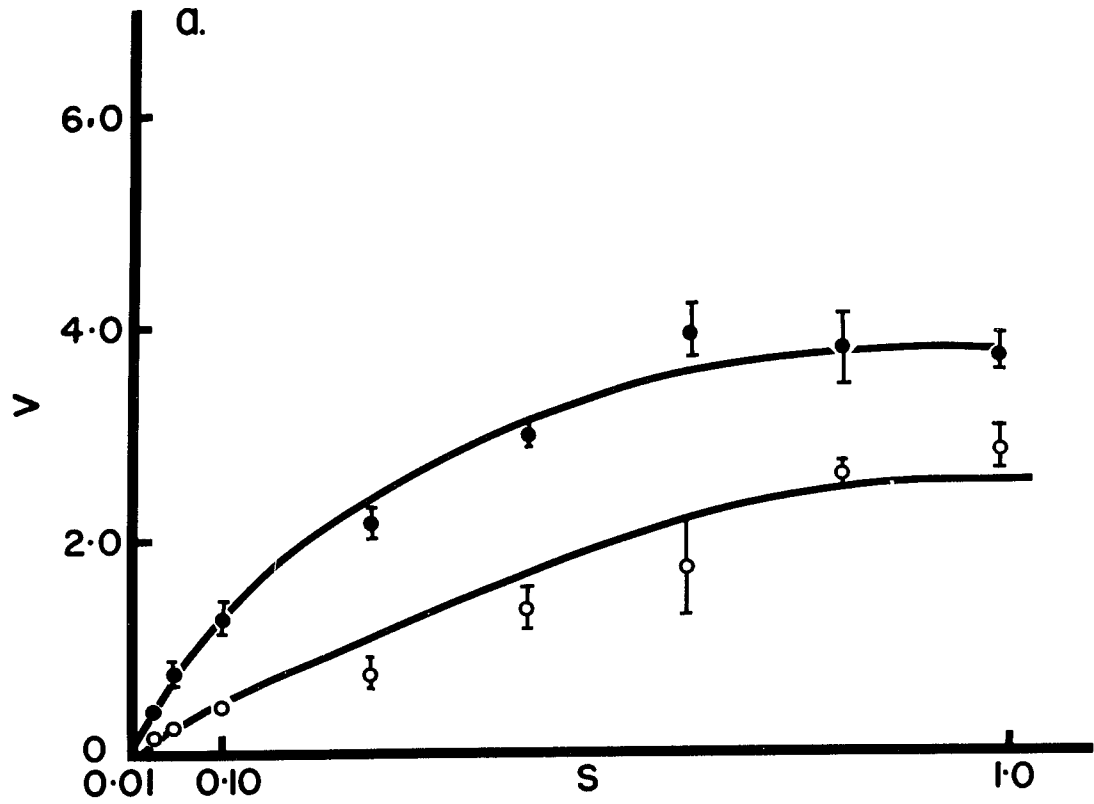
Figure 23.

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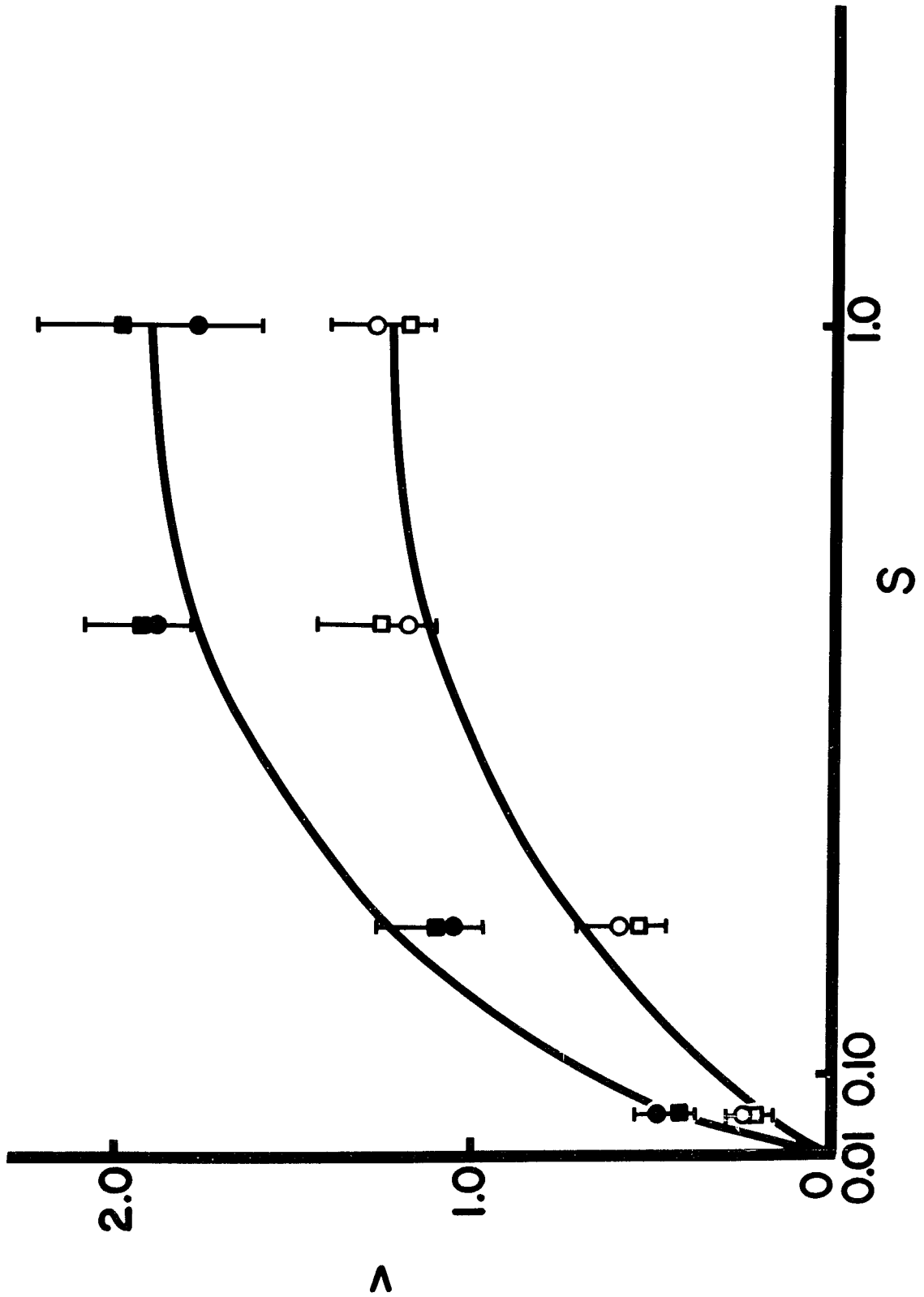
minutes. There were no significant differences in either stimulated or unstimulated uptake regardless of the pre-incubation conditions (Table 10).

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

| Pre-incubation | Calcium Uptake[2] (n moles $\pm$ SEM) | |
Conditions	PHA	Control
1.25 mM Ca^{++}	0.867 $\pm$ 0.06	0.477 $\pm$ 0.11
.025 mM Ca^{++}	0.888 $\pm$ 0.07	0.527 $\pm$ 0.04
1.40 mM EGTA	0.895 $\pm$ 0.04	0.494 $\pm$ 0.03

[1]Unstimulated lymphocytes were incubated for 2

hours in medium containing 1.25 or 0.025 mM

Ca^{++} or at the even lower concentration afford-

ed 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 incu-

bation period.

[2]The results were expressed as the mean uptake

in n moles/10^6 lymphocytes $\pm$ SEM of triplicate

cultures.

TABLE 11

Test for Exchange Diffusion Transport of Calcium by Lymphocytes at the Physiological Calcium Ion Concentration[1]

Pre-incubation Ca^{++} Concentration (mM)	Calcium Uptake[2] (n moles $\pm$ SEM)			
	Time			
	10		30	
	PHA	Control	PHA	Control
0.025	54.8 $\pm$ 2.3	27.9 $\pm$ 3.1	86.3 $\pm$ 2.6	49.4 $\pm$ 5.7
1.25	52.0 $\pm$ 4.6	27.0 $\pm$ 3.8	83.0 $\pm$ 10	48.7 $\pm$ 4.9

[1] Unstimulated 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.

[2] The results were expressed as the mean uptake in n moles/10^6 lymphocytes $\pm$ SEM of 3 replicate cultures.

TABLE 12

Effect of PHA on Calcium Efflux from
Pre-labeled Lymphocytes[1]

| Time | ^{45}Ca in supernatant[2] (%) | |
(Min)	PHA	Controls
10	62.3 ± 1.4	64.7 ± 2.3
30	77.4 ± 2.0	79.0 ± 2.5
60	82.7 ± 1.8	84.3 ± 1.4
90	84.3 ± 2.0	85.3 ± 2.6

[1]Unstimulated lymphocytes were incubated for 2 hours with 5 μCi of ^{45}Ca. They were then washed 3 times at $4°$ 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 $37°$. 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 sulfhydryl 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 regu-
latory influence on the transcription of certain genes
(156) and on the translation of mRNA (156) which sug-
gests that the nucleotide may be an important regula-
tor of cell proliferation. Indeed Whitfield et al. have
provided strong evidence that cAMP can regulate prolifera-
tion of rat thymocytes (157, 158, 159, 160, 161) and it
has been shown that contact inhibition of growth is re-
stored to virally transformed fibroblasts by treating
them with cAMP (162, 163).

Therefore, the effects of exogenous cAMP and re-
lated agents on unstimulated and PHA-stimulated lympho-
cytes were tested in order to provide information on
the possible role of cAMP in the mediation of transfor-
mation. These experiments were particularly relevant
since the previous results had established that exogen-
ous 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 pro-
liferation (157, 158, 159, 160, 161).

3.4.2   Effects on Unstimulated and PHA-Stimulated Nucleic
        Acid Synthesis

Exogenous cAMP and a dibutyryl derivative, $N^6,O^{2'}$-
dibutyryl adenosine $3',5'$-cyclic monophosphate (DB-cAMP),
which penetrates the membrane more readily than cAMP
(164) and which is less susceptible to enzymatic degra-
dation (165), had no effect on PHA-stimulated DNA syn-
thesis in the physiological concentration range of 10^{-8}
to 10^{-6}M (Fig. 24). However, with increasing concentra-
tion 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
3×10^{-3} and 8×10^{-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 inhib-
iting the cAMP-degrading enzyme, phosphodiesterase,
while imidazole decreases the intracellular cAMP concen-
tration by stimulating the phosphodiesterase (Fig. 25)
(166).   Both caffeine and theophylline progressively

Figure 24.

Inhibition of PHA-stimulated lymphocyte

transformation by exogenous nucleotides.

●—● , DB-cAMP; O—O , cAMP; □—□ ,

ATP. The agents were added 15 minutes

before PHA. DNA synthesis was deter-

mined 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 per-

centage of the PHA response observed

with no agent added (5230 CPM).
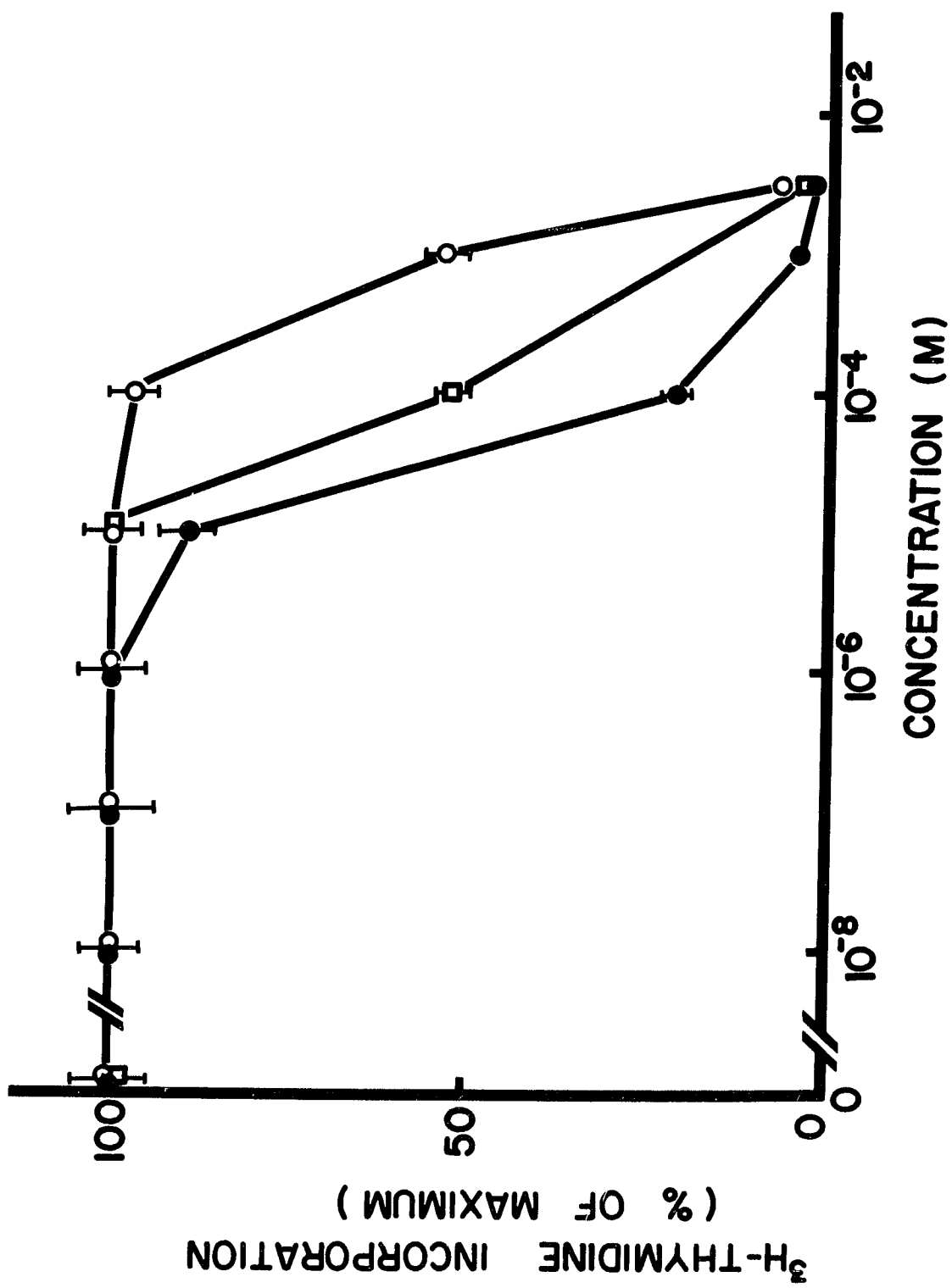
Figure 25.

Diagram of the intracellular metabolism
of cyclic AMP. — — — , inhibition;
——— , potentiation.
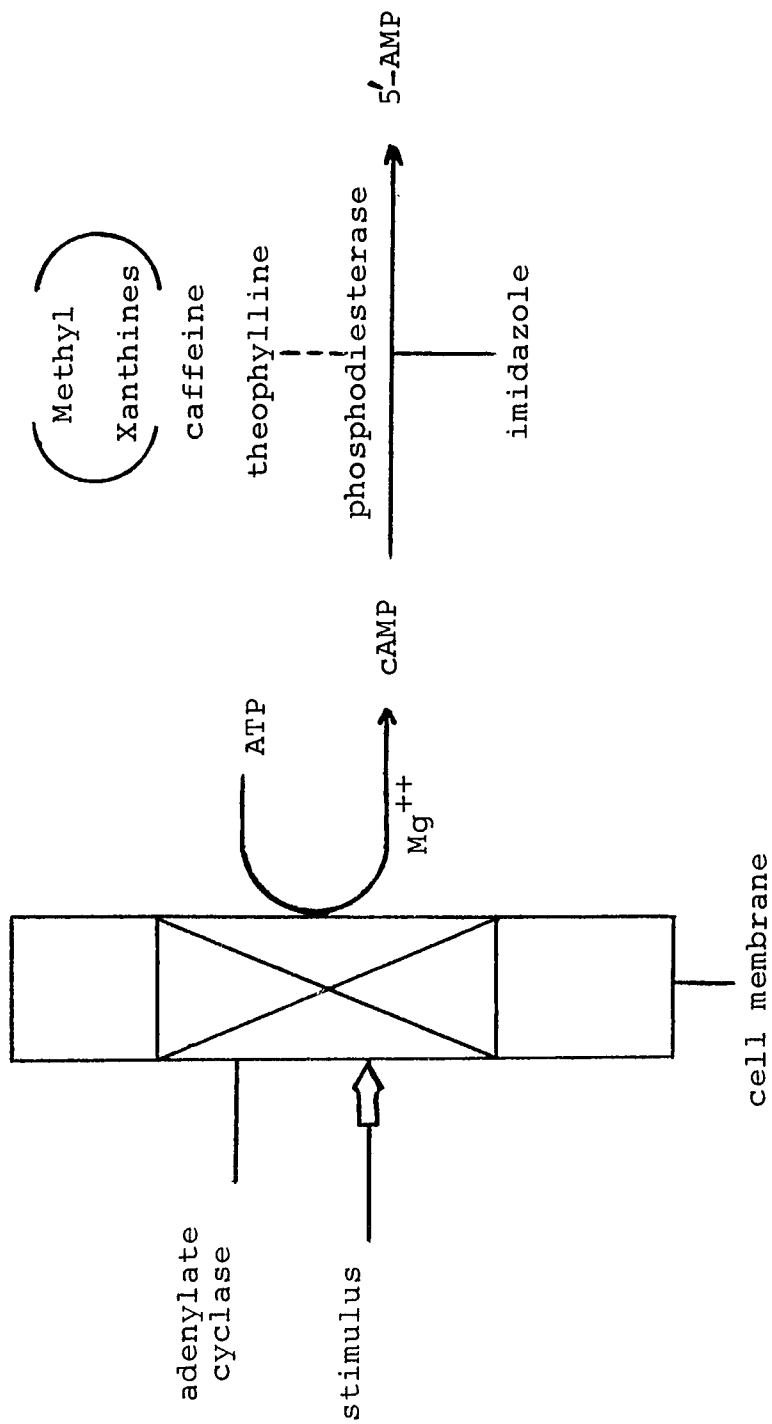
inhibited PHA-stimulated DNA synthesis (Fig. 26). Theo-
phylline was a more effective inhibitor, but complete
inhibition was obtained with both at about 5×10^{-3} to
7×10^{-3}M. Imidazole had no effect on DNA synthesis up
to 10^{-3}M (Fig. 26). However, concentrations from
2×10^{-3} to 8×10^{-3} produced 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 con-
trast 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 concen-
tration of 10^{-4}M or at 10^{-7}M had no effect on the late
synthetic phase of the response even after 72 hours

Figure 26.

PHA response in the presence of agents
which influence intracellular cyclic AMP
metabolism. ●—● , theophylline; O—O ,
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 $\pm$
SEM of 3 or 4 replicate cultures express-
ed as a percentage of the PHA response
observed with no agent added (5230 CPM).

^{3}H-THYMIDINE INCORPORATION
(% OF MAXIMUM)
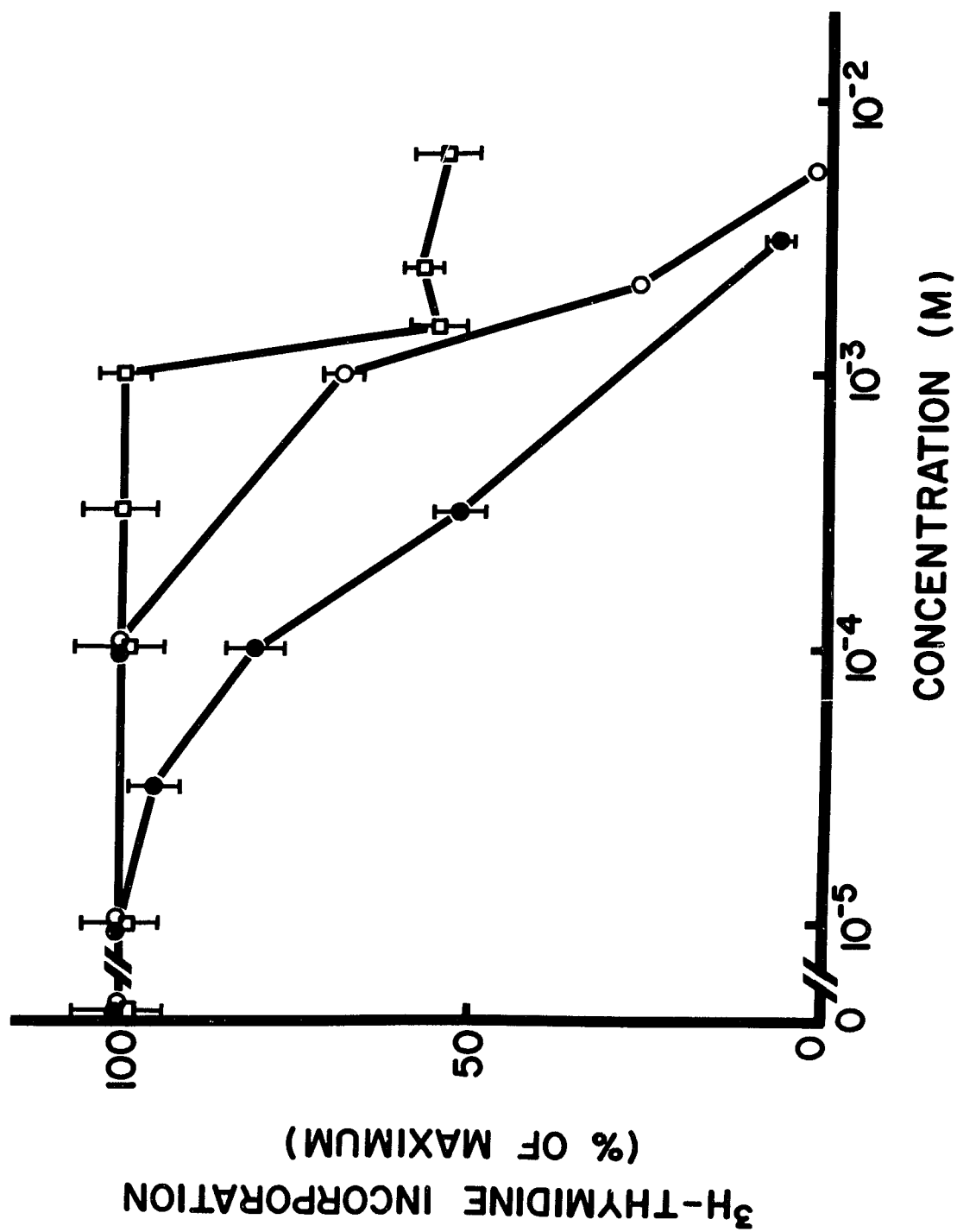
CONCENTRATION (M)

TABLE 13

Effect of Cyclic AMP and Dibutyryl Cyclic AMP
on ^{3}H-Thymidine Incorporation
by Unstimulated Lymphocytes[1]

Concentration (M)	^{3}H-Thymidine Incorporation[2] (CPM $\pm$ SEM)	
	cAMP	DB-cAMP
0	409 $\pm$ 20	———
10^{-8}	418 $\pm$ 13	407 $\pm$ 6
10^{-7}	431 $\pm$ 9	412 $\pm$ 12
10^{-6}	397 $\pm$ 6	438 $\pm$ 10
10^{-4}	460 $\pm$ 15	407 $\pm$ 12
PHA	29,000 $\pm$ 1100	———

[1]Unstimulated 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.

[2]Each value is the mean CPM $\pm$ 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 ^{3}H-thymidine was deter-
mined at various times thereafter with a
2 hour pulse. Each point is the mean
CPM $\pm$ SEM of 3 or 4 replicate cultures
expressed as a percentage of the maximum
level of synthesis observed (4120 CPM).
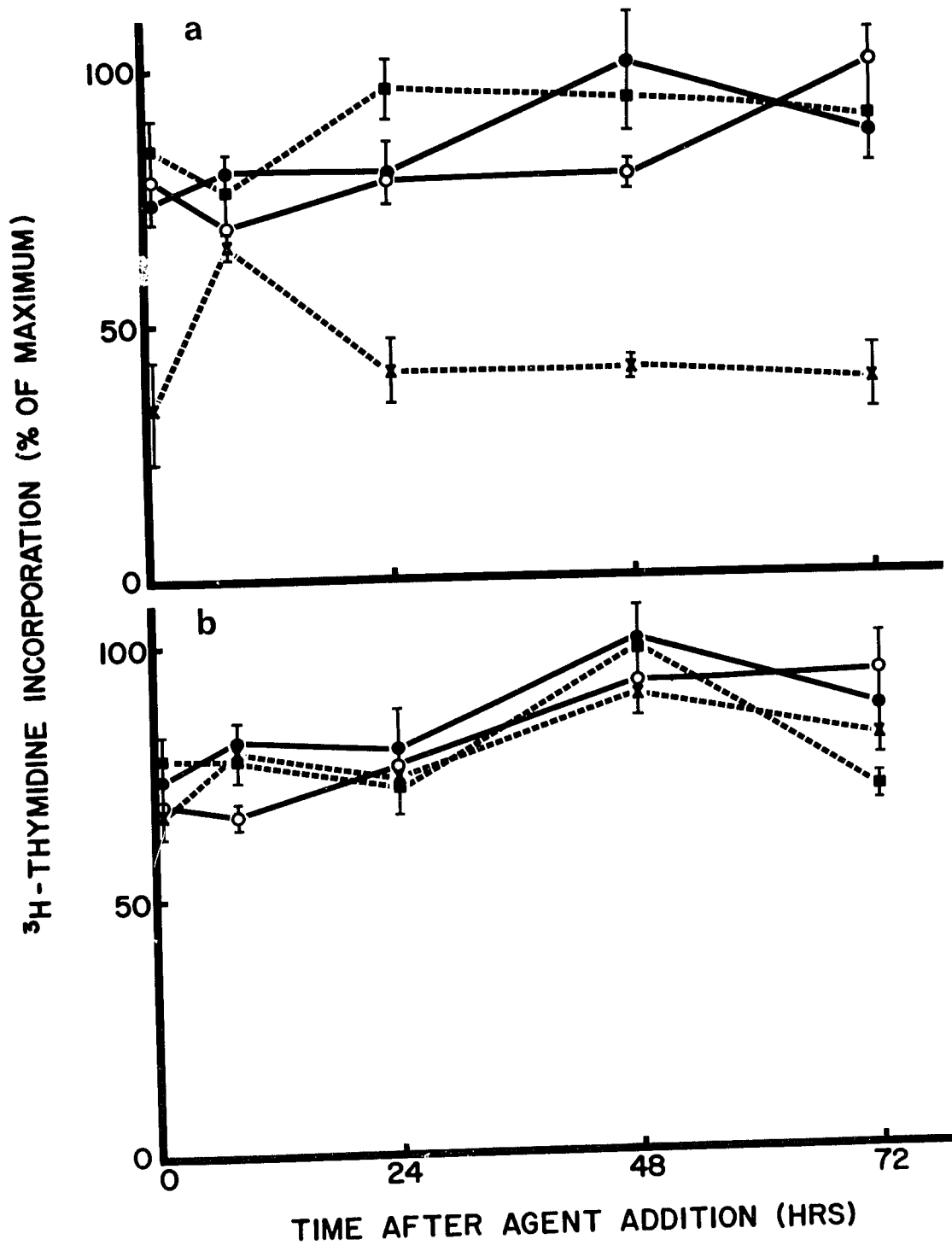
TABLE 14

Influence of Cyclic AMP on the Early Incorporation of ^{3}H-Uridine by PHA-Stimulated and Unstimulated Lymphocytes[1]

Concentration (M)	^{3}H-Uridine Incorporation[2] (CPM $\pm$ SEM)		
	PHA Concentration (μg/ml)		
	0	0.038	0.750
0	114 $\pm$ 9	137 $\pm$ 5	358 $\pm$ 8
10^{-8}	110 $\pm$ 4	126 $\pm$ 11	328 $\pm$ 18
10^{-7}	110 $\pm$ 4	127 $\pm$ 9	332 $\pm$ 21
10^{-4}	99 $\pm$ 3	125 $\pm$ 6	322 $\pm$ 12
10^{-3}	105 $\pm$ 8	119 $\pm$ 10	225 $\pm$ 17

[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 $\pm$ 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 μg/ml) were unaffected by DB-cAMP at either 2×10^{-7} or 10^{-3}M or by cAMP at 10^{-3}M while addition of 2×10^{-7}M cAMP, 5×10^{-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 μg/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 5×10^{-4} to 4×10^{-3}M although it inhibited uptake at 8×10^{-3}M (Fig. 28). However, it progressively inhibited PHA-stimulated Ca^{++} accumulation with increasing concentration above 5×10^{-4}M.

TABLE 15

Alterations in Calcium Uptake by Lymphocytes in the Presence of Cyclic AMP, Dibutyryl Cyclic AMP and Theophylline[1]

Agent[2]	Concentration (M)	Calcium Uptake[3] (nmoles ± SEM) PHA Concentration (µg/ml)		
		0	0.19	1.5
none	——	17.0 ± .17	36.3 ± 1.3	64.0 ± 1.4
cAMP	1×10^{-3}	18.9 ± .78	38.3 ± 2.4	59.8 ± 1.6
DB-cAMP	1×10^{-3}	19.6 ± .61	37.0 ± .87	55.8 ± 2.0
cAMP	2×10^{-7}	19.0 ± .61	40.0 ± .35	54.1 ± 1.3
DB-cAMP	2×10^{-7}	20.3 ± .70	36.0 ± .78	54.5 ± .87
theophylline	5×10^{-3}	19.7 ± .87	40.3 ± .43	46.9 ± 1.2
DB-cAMP + theophylline	2×10^{-7} 5×10^{-3}	22.6 ± 1.0	42.1 ± 2.9	46.6 ± 1.3

[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.

Figure 28.

Influence of exogenous ATP on calcium up-

take in unstimulated and PHA-treated lymph-

ocytes. ●—● , PHA; O—O , unstimulated.

ATP was added 15 minutes before the PHA,

and uptake was determined with a 30 min-

ute incubation with ^{45}Ca initiated imme-

diately after PHA addition. Results are

expressed as the mean uptake in n moles/

10^6 lymphocytes $\pm$ SEM of 3 replicate cul-

tures.
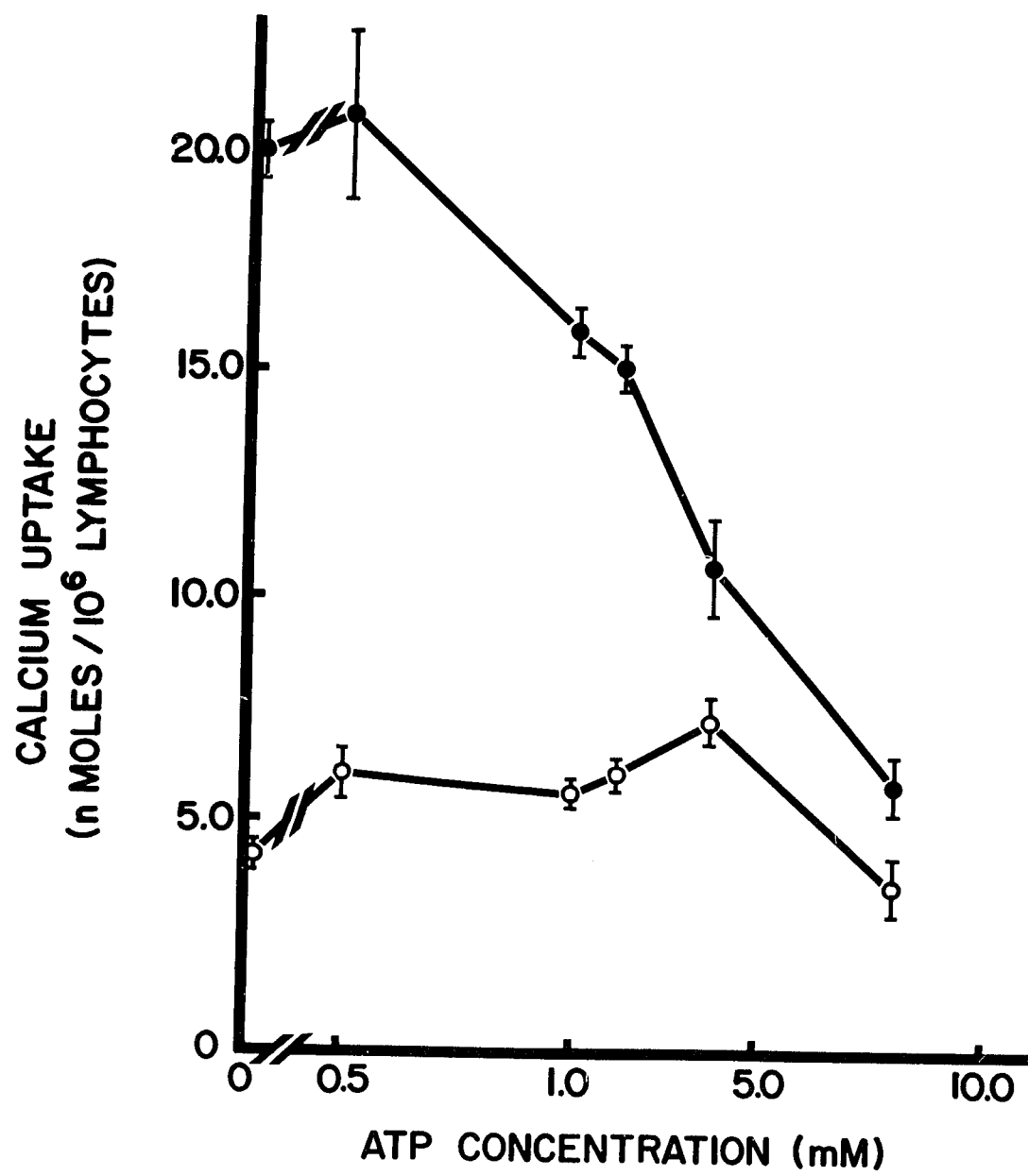
In order to better characterize the manner in which the cyclic nucleotides affected Ca^{++} uptake the effect of DB-cAMP on the kinetics of Ca^{++} accumulation was studied. Dibutyryl cyclic AMP at a concentration of 2 mM significantly inhibited Ca^{++} uptake by PHA-stimulated lymphocytes up to a medium Ca^{++} 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_{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_{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^{++} uptake in controls at 0.05 and 1.0 mM Ca^{++}, but did not significantly alter it at 0.2 and 0.6 mM Ca^{++} (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_{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^{++} (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_{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. ●—● , PHA
(no DB-cAMP); O—O , PHA + 2 mM DB-cAMP;
■—■ , unstimulated (no DB-cAMP); □—□ ,
unstimulated + 2 mM DB-cAMP. a. The
initial rate of calcium uptake (v) was
determined at 37° with a 10 minute incu-
bation with ^{45}Ca at different Ca^{++} con-
centrations (S in mM). The rate of
uptake was expressed as n moles/10^6 lym-
phocytes/min $\pm$ SEM of 3 replicate cul-
tures. b. Lineweaver-Burk plot (1/v
versus 1/S) of the data in a. The re-
gression 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.
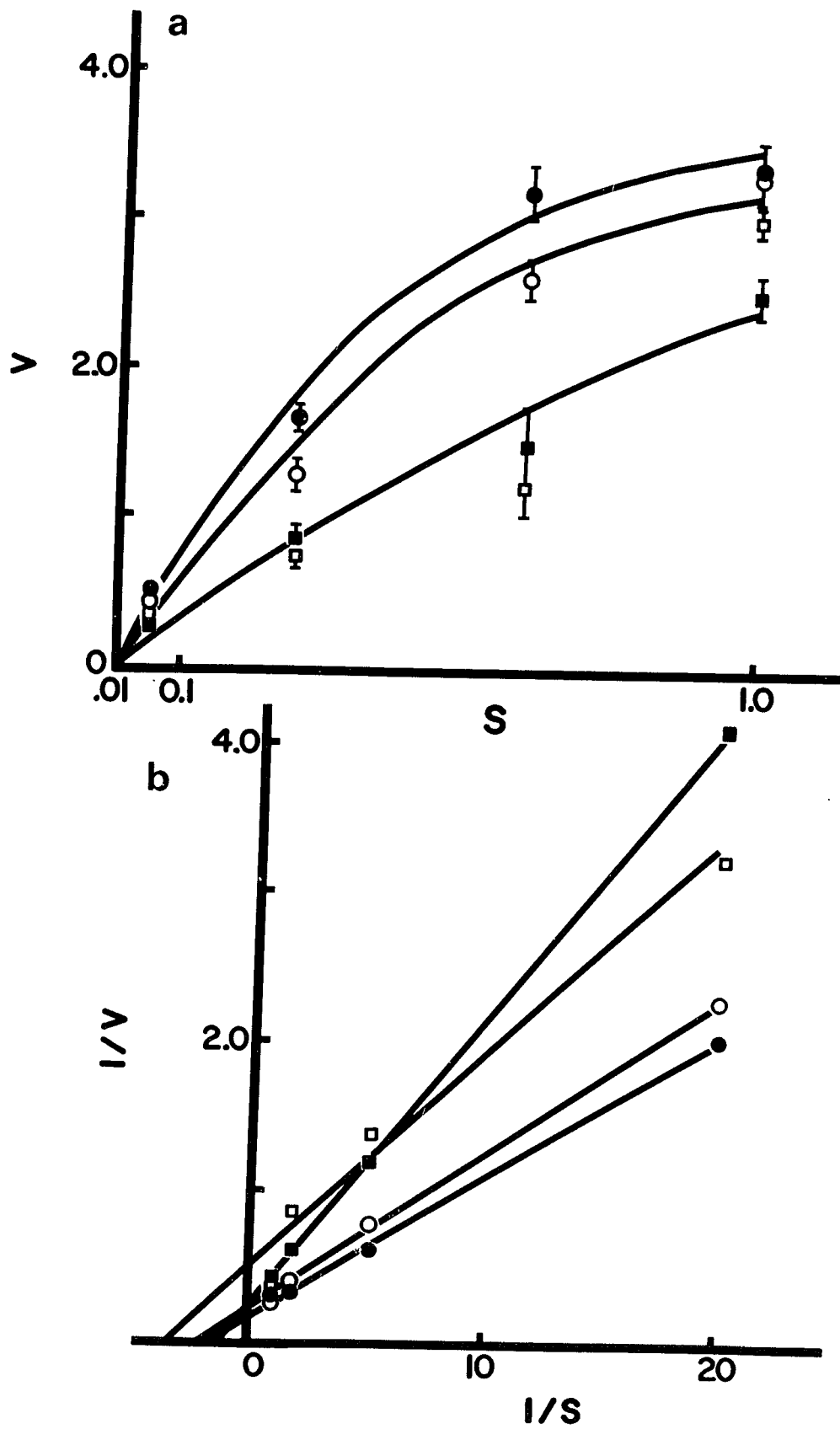
TABLE 16

Effect of Cyclic AMP, Dibutyryl Cyclic AMP,
Adenosine Triphosphate and Theophylline on
AIB Uptake by Lymphocytes[1]

Agents	Concentration (M)	AIB Uptake[3] (CPM ± SEM)	
		PHA	Control
none	———	278 ± 18	153 ± 2
cAMP	5×10^{-3}	141 ± 11	137 ± 12
DB-cAMP	1×10^{-3}	147 ± 3	158 ± 15
theophylline	5×10^{-3}	187 ± 12	145 ± 7
ATP	5×10^{-3}	131 ± 6	140 ± 9

[1]Lymphocytes were incubated with or without PHA for 2 hours. ^{3}H-AIB uptake was then determined with a 15 minute pulse.

[2]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.

[3]Each value is the mean uptake in CPM ± SEM of 3 or 4 replicate cultures.

Most of the above agents were found to alter lymph-
ocyte transport properties. Cyclic AMP, DB-cAMP, theo-
phylline 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 inhib-
ition 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 import-
ance of Ca^{++} in the process of lymphocyte transformation
induced by PHA. Also they have demonstrated that exog-
enous 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 transforma-
tion 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

Figure 30.

Time-course studies of the mixed lympho-
cyte reaction. a. RNA synthesis was
determined by incubation with ^{3}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}H-
thymidine during the final 4 hours of
each 24 hour interval. Each point was
the mean CPM $\pm$ SEM of 3 replicate cul-
tures 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 du-
plicate cultures.
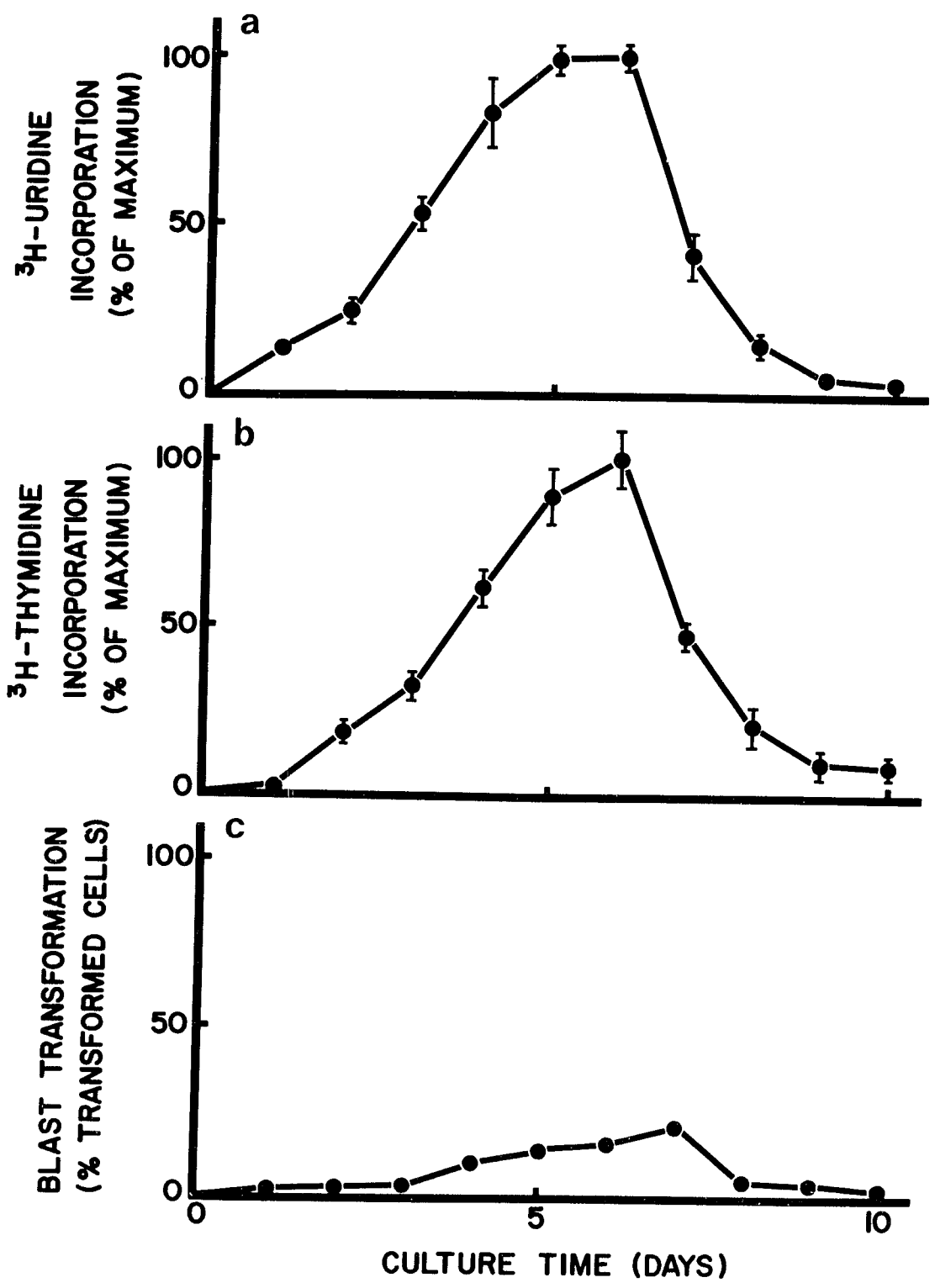
Figure 31.

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 $\pm$ SEM

of 4 replicate cultures expressed as a

percentage of the mean CPM of mixed cul-

tures not treated with EDTA (2,500 CPM).
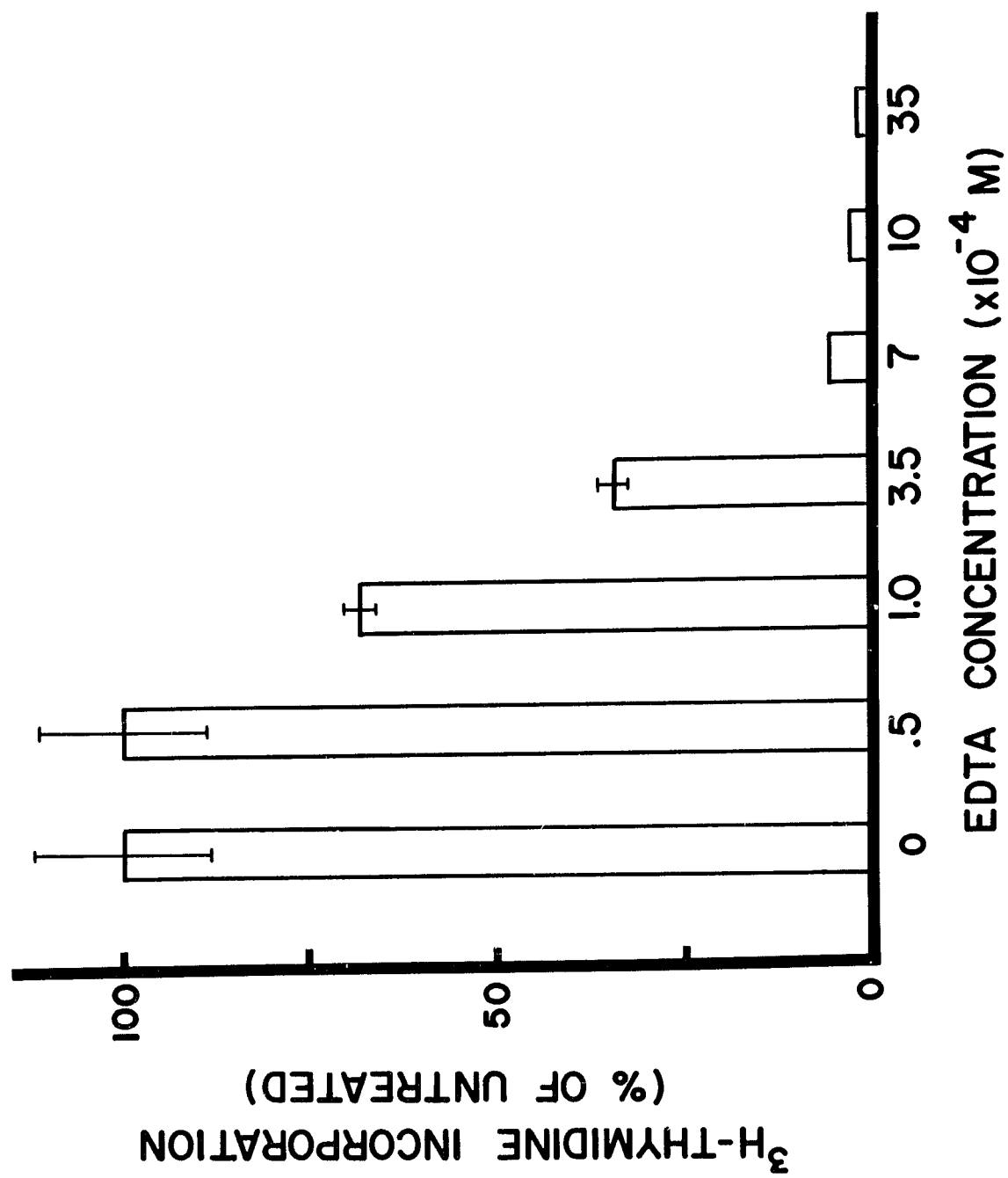
Figure 32.

Effect of EGTA on DNA synthesis in the

mixed lymphocyte response compared to the

PHA response. $\boxtimes$, mixed lymphocyte

cultures; $\square$, 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-thy-

midine incorporation was determined at

72 hours with the isotope present for the

final 4 hours. Each bar is the mean CPM

$\pm$ 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).
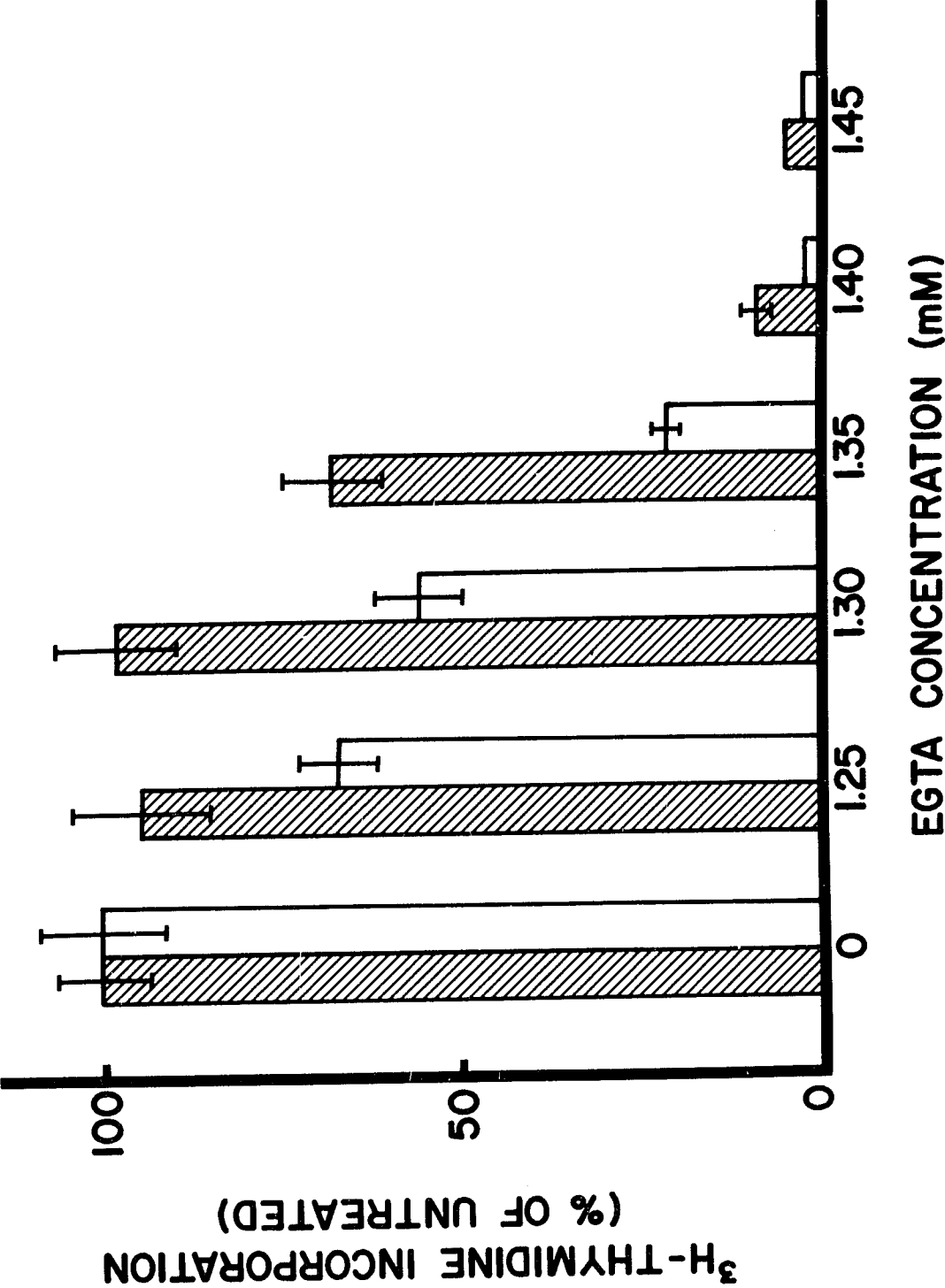
total inhibition was observed for both at 1.4 mM.

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

Increased Uptake of Calcium In Mixed Lymphocyte Cultures[1]

Experiment	Calcium Uptake[2] (n moles ± SEM)			Statistical Significance (t-test)
	A	B	A + B	
1	31.3 ± 2.2	33.3 ± 2.0	44.0 ± 1.6	p < 0.01
2	45.3 ± 1.2	37.7 ± 1.3	54.5 ± 1.0	p < 0.01

[1]Lymphocytes from 2 donors (A and B) were cultured individually (10^6 cells) or in mixed cultures (0.5×10^6 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.

Figure 33.

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 $\pm$
SEM of 3 replicate cultures expressed as
a percentage of the mean CPM of mixed
cultures without cAMP (2230 CPM).
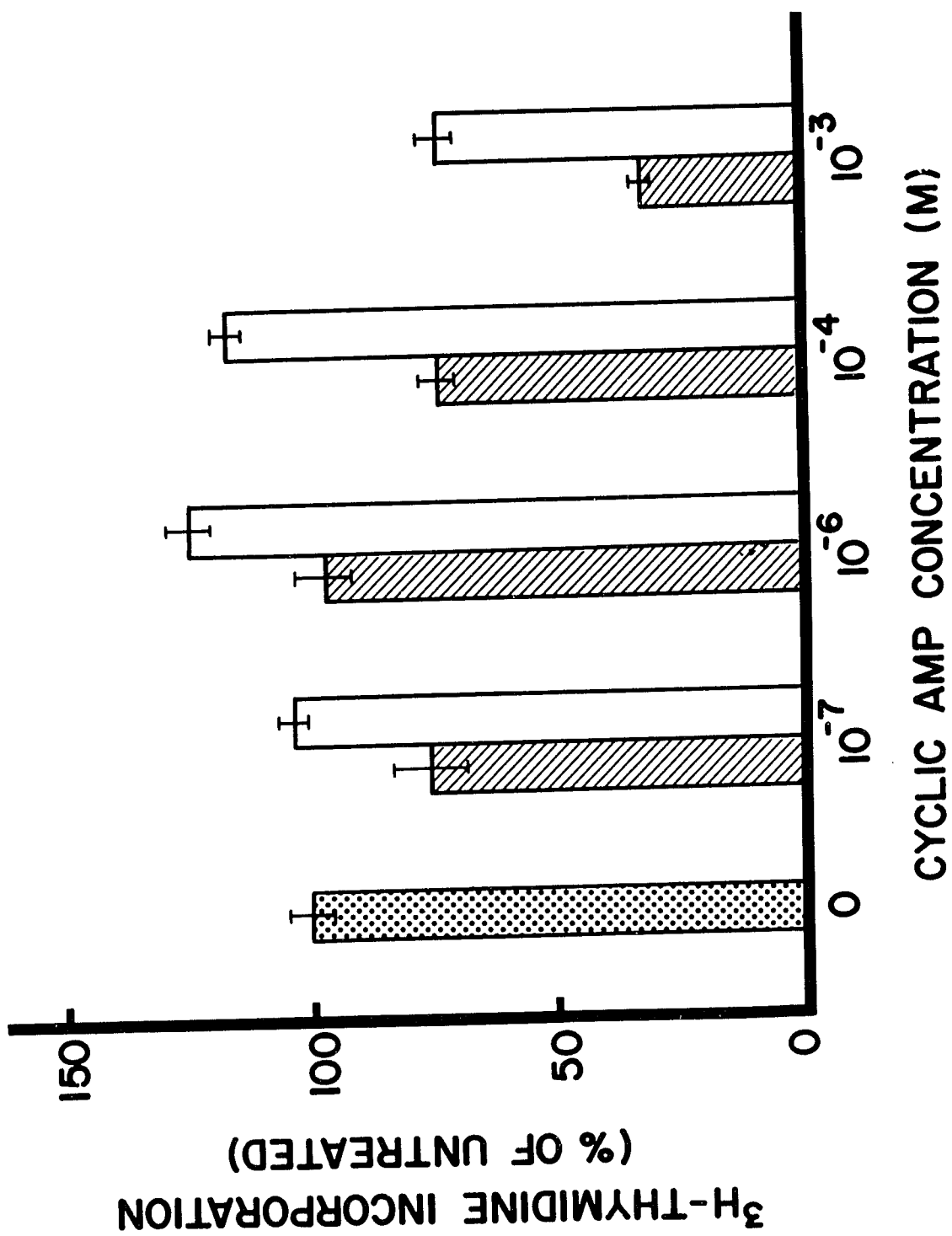
previously (167). Maximum levels of synthesis are ob-
served after about 6 days in contrast to the 3 days re-
quired to attain maximum levels in PHA-stimulated cul-
tures. The present results verified that maximum DNA
synthesis occurred at day 6, and also that maximum RNA
synthesis occurred at day 5 and maximum morphological
transformation at day 7.

It was found that transformation in mixed lympho-
cyte cultures was inhibited by both EGTA and EDTA over
approximately the same concentration range that was in-
hibitory 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^{++} 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^{++} requirements of transforming lymphocytes
were studied by using EGTA or by adding Ca^{++} to low Ca^{++}
medium.   EGTA progressively inhibited PHA-induced stim-
ulation with increasing concentrations above the normal
medium Ca^{++} 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 non-
toxic 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^{++} alone or in combination with other
divalent cations (136), no conclusions regarding Ca^{++}
requirements could be drawn.   However, the EGTA effect
could be totally reversed by adding excess Ca^{++} while
other divalent cations were ineffective.

It is important to note again the specificity of
the Ca^{++} 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^{++} were still present to permit growth so Ca^{++} had more than a non-specific growth promoting role. It is important to note that both EDTA and ouabain, which alters Na^+ and K^+ 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^+ are necessary to maintain optimum levels of protein synthesis (170), and the influence of Mg^{++} 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^{++} 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 permit 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 related 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 (recall 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 present 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 expend-
iture of metabolic energy (151). Also the metabolic in-
hibitors 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 inhibit-
ed 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 meta-
bolic inhibitor sodium fluoride, has been observed in
muscle granules (173) and probably depends on co-trans-
port of the fluoride anion. This would result in pre-
cipitation 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 coupl-
ed 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 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 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 Ca^{++} uptake had increased further probably as a result of a small increase in V_{max} in addition to the decreased K_m.

These observations are interpreted to mean that PHA enhances Ca^{++} uptake mainly by increasing the affinity of the hypothetical 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_{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_{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 require-
ment for exogenous Ca^{++} in the initiation of cell pro-
liferation in normally resting lymphocytes which have
been treated with the mitogen, phytohemagglutinin.
However, it is crucial to note that the actual prolifer-
ation of the cells after DNA synthesis has begun is in-
dependent of the Ca^{++} levels which are necessary during
the initiation phase of the response. The additional
observation that the mixed lymphocyte reaction, a pro-
cess 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^{++}. It was demonstrated that Ca^{++} 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^{++} (110). Whitfield and his colleagues found that in the rat thymocyte system Ca^{++} 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^{++}-mediated regulation.

However, the fact that PHA produces a rapid, marked enhancement of Ca^{++} accumulation argues that Ca^{++} 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^{++} in other systems.

Inhibition of the cAMP-degrading phosphodiesterase from rat thymocytes by Ca^{++} would provide a means of subsequent regulation of cAMP production (161). The ability of Ca^{++} 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^{++} 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^{++} 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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