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CATION FLUXES IN LYMPHOCYTE ACTIVATION

BY

TREVOR OWENS

A thesis submitted to the School of Graduate Studies and Research
of the University of Ottawa
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biology

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ABSTRACT

Conçanavalin A (Con A), at mitogenic doses, activated potassium influx in Balb/c splenic lymphocytes, from 7.35 +/- 0.6 to 12.66 +/- 0.8 fmoles/cell.hr. (1.75-fold). Influx was measured by a rapid micromethod, using $^{86}Rb^+$ as a tracer. Activation of influx was not routinely detectable until 6-8 hours after the addition of Con A. Ouabain inhibited both the activation of influx and of DNA synthesis. The ID50 for the inhibition of proliferation was 1.4 X 10^{-4}M; that for the inhibition of (Na^+,K^+)-ATPase-mediated influx, whether measured at 10 or at 45 hours of culture, was 1.8 X 10^{-4}M. These inhibitions were reversible. Con A also activated K^+ efflux, from 6.42 +/- 0.6 to 10.6 +/- 0.4 fmoles/cell.hr. (1.66-fold). This could be detected after 14 hours of culture, but not immediately after Con A addition. Intracellular potassium content increased by 2 fmoles/cell, between 5 and 10 hours after Con A addition. There was no change in (K^+)c, due to a slight increase in cell volume. The net influx was sufficient to maintain (K^+)c constant during the volume increase of blastogenesis. Furosemide (1 mM) inhibited both the activation of K^+ influx and of DNA synthesis.

Con A activated neither K^+ influx nor DNA synthesis in cells from RNC nu/nu spleen; LPS activated both K^+ influx

(1.4-fold) and DNA synthesis. Balb/c splenocytes were unresponsive to LPS; K+ influx was not activated, and DNA synthesis was activated by at most 3-fold. The activation of K+ influx was therefore both necessary for and specific to the mitogenic activation of lymphocytes. Con A binding *per se* did not activate influx.

Enrichment of Balb/c splenocytes for B- and T-cells, using wheat germ agglutinin (WGA), did not affect the 1.75-fold activation of K+ influx by Con A in any population; despite some differences in their proliferative responses, all WGA-fractionated populations showed identical influx activations. Proliferative response to Con A was never completely removed from the B-cell populations. The response to Con A of mixtures, at constant cell density, of RNC nu/+ cells (T-enriched) and RNC nu/nu cells, was assessed. The activations of both K+ influx (at 15 hours) and DNA synthesis (at 48 hours) demonstrated, through the production of excess, or unexpected response, interaction between the two populations. Provided there were more than 10% T-cells present, responses indistinguishable from those of pure T-cells were obtained. UV-irradiation of either cell type before mixing prevented this interaction, which also required co-culture. When LPS was added to such mixtures, there was no interaction; responses proportional to the number of nu/nu cells present were obtained.

In mixed cultures containing 20% T-cells (from a female mouse), and 80% nu/nu cells (from a male mouse), 40% of the cells in metaphase after 2 days incubation with Con A were identified as being from nu/nu spleen, by Hoechst 33258 staining of the chromosomes. In separate experiments, 50% of the blast cells in similar cultures stained strongly with FITC-anti-Ig. Less than 5% of the T-cells were Ig-positive. Anti-Thy 1.2 and complement treatment of nu/nu cells before mixing did not affect their activation.

Both the excess potassium uptake and ^{3}H-thymidine incorporation seen in mixed cultures were therefore due to B-cell activation, through co-culture with Con A-activated T-cells. This accounts for the constancy of K^{+} influx in WGA-fractionated populations. These results are consistent with a model whereby the flux activations are caused by T-cell soluble factors, and the ultimate level of response is regulated by macrophages.

RÉSUMÉ

La concanavaline A (Con A) favorisa l'entrée de potassium chez les splénocytes Balb/c de 7.35 +/- 0.6 à 12.66 +/- 0.8 fmoles/cell. hr. (X 1.75). L'influx est mesuré par une microméthode rapide, à l'aide du marqueur radioactif $^{86}Rb^{+}$. La stimulation de cet influx n'a été detectable qu'après 6-8 hr. d'incubation avec la Con A, et a été inhibée par l'ouabaïne, produit inhibant aussi la synthèse de l'ADN (Mesurée par l'incorporation de ^{3}H-thymidine). Mesurée après 48 hr. de culture, le ID50 de l'inhibition de la synthèse de l'ADN est de 1.4×10^{-4}M; celui de l'inhibition de l'influx du à l'ATPase(Na^{+},K^{+}), mesuré après 10 ou 45 heures de culture, a été de 1.8×10^{-4}M. Ces inhibitions furent reversibles. Con A favorisa aussi la sortie de potassium de 6.24 +/- 0.6 à 10.6 +/- 0.4 fmoles/cell.hr. (X 1.66). Cette stimulation a été mesurée après 14 heure de culture, mais n'a pas été detectable immediatement après l'addition de la Con A. Le contenu intracellulaire de potassium augmenta de 2 fmoles/cell entre 5 et 10 heures de culture. Le (K^{+})c resta constant du fait d'une faible augmentation du volume cellulaire. L'influx net fut suffisant pour maintenir constant le (K^{+})c au cours de l'augmentation du volume cellulaire de la blastogenèse. La furosemide (1 mM) inhiba à la fois l'activation de l'influx de potassium et de la synthèse de l'ADN.

La Con A n'activa ni l'influx de potassium, ni la synthèse de l'ADN dans les cellules de la souris RNC nu/nu; mais le LPS activa l'influx (X 1.4), et la synthèse de l'-ADN. Chez les cellules Balb/c, l'influx de potassium ne fut pas activé par le LPS, et la synthèse de l'ADN ne fut que triplée. Ainsi, l'activation de l'influx de potassium fut essentielle et spécifique pour l'activation mitogenique des lymphocytes. La liaison de Con A per se n'augmenta pas les flux de potassium.

L'enrichissement par lectine de grain de blé (WGA) des splénocytes Balb/c pour des cellules B et T n'affecta pas l'activation de l'influx de potassium par la Con A. Malgré des differences au niveau de leur prolifération, les populations fractionées par WGA répondent de la même manière. La réponse proliférative des cellules B par la Con A ne fut jamais enlevée. La réponse à la Con A à une mélange à densité constante de cellules de rate RNC nu/+, enrichies pour des cellules T, et RNC nu/nu fut evaluée. La mesure de l'activation de l'influx de potassium (à 15 heures) et de l'incorporation de ^{3}H-thymidine (à 48 hr.) indiqua une interaction entre les 2 populations, tel qu'on obtint une réponse identique à celle de cellules T pures si on avait eu au moins 10% de cellules T. L'interaction de ces cellules necessita une co-culture et elle fut annulée par irradiation aux UV de chaque type de cellule avant leur mélange. La réponse au LPS dans le mélange diminua proportionellement avec le nombre des cellules nu/nu.

Dans une culture de 2 jours, contenant 20% de cellules T (d'une souris femelle), et 80% de cellules nu/nu (d'une souris mâle), 40% de cellules en métaphase furent identifiées par coloration des chromosomes au Hoechst 33258 comme étant de rate nu/nu. D'autres expériences montrent que dans des cultures identiques, 50% des cellules blastiques furent marquées avec FITC-anti-Ig. Il y avait moins de 5% de cellules Ig-positif dans les cultures T-enrichies. Le traitement des cellules nu/nu par l'anti Thy 1.2 et complément avant le mélange n'affecta pas leur activation.

L'augmentation de l'influx de potassium et de la synthèse de l'ADN observée dans les cultures mixtes furent donc le résultat de l'activation des cellules B, par co-culture avec des cellules T, activées de Con A. Ceci explique la constance de l'influx de potassium dans des populations fractionées avec le WGA. Ces résultats supporteraient un modèle dont l'activation des flux serait produit par des mediateurs solubles, sécretés par des cellules T, sous l'effet de Con A, et le niveau des réponses serait déterminé par des macrophages.

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Abbreviations

Con A:	concanavalin A
FITC:	fluorescein isothiocyanate
Ig :	immunoglobulin
IL-1:	Interleukin-1
IL-2:	Interleukin-2
LAF:	Lymphocyte Activating Factor (IL-1)
LPS:	lipopolysaccharide
NAGO:	neuraminidase-galactose oxidase
PBS:	phosphate-buffered saline
PHA:	phytohemagglutinin
PNA:	peanut agglutinin
PBL:	peripheral blood lymphocyte
SD:	standard deviation
SEM:	standard error of the mean
SBA:	soybean agglutinin
TCGF:	T-cell Growth Factor (IL-2)
WGA:	wheat germ agglutinin
ID_{50}:	50% inhibitory dose

7

SECTION I

INTRODUCTION

In 1968, Quastel and Kaplan showed that ouabain, a specific inhibitor of the (Na^+, K^+)-ATPase, inhibits the activation *in vitro* of human peripheral blood lymphocytes (PBL) by phytohemagglutinin (PHA) (Quastel & Kaplan, 1968). They subsequently showed that the earliest detectable event following addition of PHA to human PBL in culture is the approximate doubling of ouabain-sensitive potassium influx (Quastel & Kaplan,1970B).

Since then it has been shown that similar flux activations are a characteristic of the in vitro mitogenic activation of many different cell types (Kaplan,1978).In the intervening years, our understanding of the interactions involved in the activation of lymphocytes has increased enormously, and the roles these activated cells play in the immune response are now much better appreciated. Since all work to date has been with unfractionated, heterogenous lymphocyte populations, it is appropriate that the activation by mitogens of monovalent cation fluxes in lymphocytes be reassessed, to confirm that this activation is a specific consequence of the stimulation to proliferate of cells in culture. These fluxes, as early events of the activation

process, may enable us to dissect the cellular interactions which are so much a characteristic of the immune response.

This introduction will review the immune system, the cells which mediate its response and the role of monovalent cations in the functioning of these and other cells, emphasising their role in the regulation of cell proliferation, which is essential to the immune response.

1.1 THE IMMUNE SYSTEM

All vertebrates and some higher invertebrates possess an immune system, which responds to foreign or antigenic substances (Stites & Caldwell, 1976). The immune systems of the higher vertebrates, especially mammals, are the best developed. This review will concentrate on those of the human and the mouse.

The immune system responds to bacterial and viral infections, and is also important for the control of malignancies (Burnet, 1970). Its importance can be appreciated by consideration of some of the consequences of its deregulation, such as aging and autoimmune diseases (Lance *et al*, 1977).

To understand the regulation of a system, it helps to understand the system itself. In the case of the immune system, one needs to understand the effector cells.

1.1.1 Lymphocytes

Lymphocytes, with macrophages, are the principal effector cells of the immune response. Morphologically, resting lymphocytes are unprepossessing. They are small, round cells, about 6 µm in diameter, with a very high nuclear to cytoplasmic volume ratio, and chromatin in a highly condensed state. Stimulated lymphocytes are more diverse in morphology; they are larger than resting cells (up to 20 µm in diameter), and have a much lower nuclear/cytoplasmic volume ratio. Their decondensed chromatin and highly organized cytoplasm (especially in the case of plasma cells) bespeak active cells.

There are two main classes of lymphocytes. Those that mediate the humoral immune response i.e. make antibody, and those that mediate cellular immune responses. Antibody-producing cells were shown (Warner & Szenberg,1964) to arise from the bursa of Fabricius in chickens, and were designated B-cells. Fagraeus showed that B-cells develop into plasma cells, which actually make the antibody; she has recently reviewed this work (Fagraeus, 1981). In mammals, there is no bursa,and B-lymphocytes are considered to derive from the bone marrow, although it is not clear whether this is properly a bursal- equivalent (Cooper & Lawton,1972). The mediation of cellular responses by cells derived from the thymus (T-cells) was shown by Miller, and by Good (Miller & Osoba,1967;Good & Gabrielson,1965).

1.1.2 Ontogeny of Lymphocytes

Lymphocytes derive from the same pluripotent stem cells which give rise to erythroid cells(Hume & Weidemann,1980). In mammals, these stem cells can first be identified in fetal liver, from where they migrate to the bone marrow in the adult.

On leaving the bone marrow, T-cells migrate to the thymus, where they acquire (in mouse) Thy-1 antigen (Cantor & Boyse,1978). They mature in the thymus (this may involve proliferation), and then migrate to peripheral lymphoid organs such as the spleen and the lymph nodes. They have by then acquired alloantigens of the Lyt-series (Cantor & Boyse,1978). It is currently felt that immature T-cells bear the Lyt 1+,2+,3+ antigens, and that their commitment to a differentiated function involves the loss of one or some of these antigens (see below). In the absence of mature T-cells, or factors from them, immature T-cells such as prothymocytes are incapable of T-cell function (Smith et al, 1979). Antigens of the Qa-series, as yet incompletely characterized, are also acquired in T-cell maturation (Stanton et al, 1978). The site of B-cell differentiation and maturation in mammals (the equivalent of the bursa in birds) has not been definitively established. Likely sites are fetal liver, adult bone marrow and/or gut associated lymphoepithelial tissue (Owen et al, 1974;Cooper & Lawton,1972;Melchers,1977). While B-cell differentiation is be-

lieved to be antigen- independent, B-cell maturation (to a plasma cell) is antigen-driven. Differentiated B-cells express the Ly-b antigen . This can be detected prior to expression of surface Ig (Sato & Boyse,1976; Silverstone et al, 1978).

1.1.3 The Nude Mouse

In 1968, mice of a strain which had hithertofore been noted only for hairlessness and susceptibility to infection were shown to be athymic (Pantelouris,1968).These animals are devoid of all T-cell function. Despite some difficulty in establishing and maintaining colonies, they have been extensively bred and studied.Kindred (1979) has reviewed the contribution these mice have made to immunology. For some time they were thought of as "B-cell mice", a description which although describing their function in immune response quite accurately, suggests an inaccurate picture of their complement of lymphocytes.

Embryos up to 12-15 days old from nude mice are indistinguishable from those of normal mice. Thereafter, they suffer almost complete thymic degeneration. There is however no defect in the bone marrow of these mice, and in fact cells of the T-cell lineage, some bearing detectable T-cell antigens, such as Thy-1, are produced . Since these are not processed by the thymus, they remain non-functional. T-cell function can be reconstituted in these mice by injec-

tion or implantation of mature T-cells, activating factors from mature T-cells, or mature thymus tissue (Kindred,1979).

1.1.4 Lymphoid Tissues

The primary lymphoid tissues are the bone marrow, thymus, gut-associated lymphoepithelial tissue, and fetal liver. Secondary lymphoid tissues include the spleen, lymph nodes and peripheral blood . These latter are the sites wherein lymphocytes interact with antigen, and with each other; this cellular activation is essential for the immune response. The secondary lymphoid tissues, and the thymus, are the principal sources of cells used for in vitro studies.

1.1.5 The Humoral or B-cell Response

The humoral immune response is mediated chiefly by B-cells. Resting B-lymphocytes carry on their surface immunoglobulin molecules. On each antigen molecule are one or more epitopes or antibody-recognizing sites. For each epitope there is a corresponding antibody idiotype; this is a variable region on the antibody, formed by both the heavy and light chains, which is the combining site specific for that epitope (Taussig,1975).

These clones, each of which contains 0.01-0.001% of all B-cells, are expanded through mitosis in response to antigen (McConnell,1975). The cells enlarge to become blast cells

(lymphoblasts), then as they develop more endoplasmic reticulum, they can be identified as plasmablasts, from which they develop into smaller (about 9-12 um in diameter) plasma cells; these are the body's secretory cells par excellence, being readily identifiable by the distended cisternae of their endoplasmic reticulum. It is these cells which make and secrete antibody.

1.1.6 Cellular or T-cell Responses

T-cell responses are of three classes.

1.1.6.1 T-cell help

T-cell help includes both T-T and T-B help. T-T help will be dealt with in a later section. T-B help refers to the involvement of T-cells in the recognition of antigen and the production of antibody by B-cells. Triggering of B-cells in the absence of T-cell help only occurs in response to some antigens such as bacterial lipopolysaccharide (LPS) (Andersson et al, 1972). In all other cases, T-cells are required for both recognition and activation. There are idiotype-specific T-cells, just as there are idiotype-specific B-cells. These T-helper cells release soluble factors, which, in concert with macrophage- presented antigen, activate B-cell antibody production . Some T-cell factors bear Ia determinants (see below), and can be absorbed by antigen; they therefore correspond to the antibody idiotype, and to the

T-cell antigen receptor (Tada & Okumura,1980). Helper T-cells are of Lyt-1+ phenotype, having lost the Lyt-2+,3+ antigens (Hirst et al, 1975).

1.1.6.2 T-cell Suppression

Suppressor cells are the down-regulators of the immune response. They serve as the terminators of the immune response, shutting off effector T-cells and B-cells (Gershon,1974; Janeway,1978). Like helper cells, they produce soluble factors, which can also be idiotype-specific (Tada & Okumura,1980). They are responsible, with Interleukin-2 depletion, for the cessation of mitogen-activation after one or a few rounds of division (Hume & Weidemann,1980). While suppressor cells are activated by mitogens such as Con A, it is not clear whether they need to proliferate to exert their effects (Dutton,1973). T-suppressors are of Ly-2+,3+ phenotype. Whether a mitogen induces helper cells, suppressor cells, or both, depends, amongst other things, on the mitogen dose (Persson et al, 1978A).

1.1.6.3 T-cell cytotoxicity

Cytotoxic T-cells (CTL) are generated by the mixed lymphocyte reaction (MLR - see below).They arise, through proliferation, in response to the recognition of alloantigen, in the presence of Ia-bearing cells (Hayry & Defendi,1970;Nedrud et al, 1975). Mitogens can induce nonspecif-

ic cytotoxic T-cells, which produce lymphotoxins, and can also facilitate the emergence of specific CTL from primed populations (David & David,1972; Wagner & Pollinghoff,1978). CTL function in immune surveillance, having the potential to lyse specifically tumor cells. The mechanism of this lysis is not fully understood; it requires cell-cell contact (Berke,1980). CTL are also of Ly-2+,3+ allotype(Bach et al, 1979).

1.1.7 Macrophages

Macrophages have two principal functions in the immune response; these are phagocytosis, and antigen-presentation. Phagocytes engulf foreign particles (cells and/or cellular constituents) and destroy them by lysosomal digestion. Phagocytosis is enhanced by opsonization (the binding of antibodies to the antigenic particle): macrophages have Fc and C3b receptors on their surface and can thus bind the opsonized particle (McConnell, 1975; Sell, 1980).

All interactions between lymphocytes and antigen are mediated by macrophages. This is the antigen-presenting function of macrophages, whereby antigens are ingested by the macrophage, and then presented on its surface for recognition by either T- or B-cells. This includes interaction with mitogens (Rosenstreich & Mizel,1978). Macrophages also secrete soluble factors which regulate the immune response (see below).B-cell response to T-independent mitogens such

as LPS does not have the same absolute requirement for macrophages (Persson et al, 1978B).

Both T-cells and macrophages can be replaced entirely in the antibody response by soluble factors derived from these cells (Watson et al, 1979; Shiozawa et al, 1980). These factors result from the prior interaction of both cell types with antigen. Needless to say, B-cells cannot be replaced by soluble factors.

1.1.8 The Mixed Lymphocyte Reaction

When allogeneic lymphocytes are mixed in culture, cells from both populations are activated. This is the MLR (Bain et al, 1964). The reaction can be divided into two components, stimulus and response. The cells which stimulate are usually B-cells or macrophages. These cells bear Ia antigens. T-cells are the responders; all responses involve an activation to proliferate, although there may also be recognition which does not in itself lead to proliferation. The stimulus to proliferate is determined by I region differences in the mouse (McDevitt, 1978). If K and/or D region differences are also recognized, then the activated T-cells differentiate to become cytotoxic cells (Bach et al, 1979). Cytotoxic T-cells generated through MLR mediate graft-versus- host reactions during transplantation, and also mediate cytolysis of tumor tissues (immune surveillance) (Burnet, 1970; Lance et al, 1977).

1.1.9 Genetics of the Immune Response

The immune response is under the control of a number of genetic loci. Chief among these is the Major Histocompatibility Complex, which is designated HLA in man, and H-2 in mouse, located on chromosomes 6 and 17 respectively (Klein, 1975). Contained within this complex is the I subregion, which controls cellular interactions and therefore the induction and the magnitude of response (McDevitt, 1978). The Ia (I-subregion associated) antigens are encoded by these loci; these antigens are expressed chiefly on B-cells and macrophages, although there is a low level of Ia expression on T-cells (this is elevated on activated T cells). The I-subregion also determines the nature, or direction of the response. For instance, T-help is an I-A/E associated activity, while I-J is associated with suppression (Sell, 1980). The K and D subregions control the induction of cytotoxicity. The Ss region determines serum protein levels (Sell, 1980).

1.1.10 Requirement for Blast Transformation

All the activations described so far have in common their requirement for blast transformation and proliferation. That is to say, none of the effector cells described (with the possible exception of the suppressor cell) can be derived from their progenitor cells without first becoming an enlarged blast cell, followed by DNA synthesis and cell division.

Clonal expression requires clonal expansion, and there can be no clonal expansion without prior cellular expansion.

1.2 IN VITRO ACTIVATION OF LYMPHOCYTES

1.2.1 Polyclonal Activators

As mentioned above, each individual clone of lymphocytes, whether B or T, constitutes less than 0.01% of the total population. Activation by antigen may raise this, through clonal expansion, to 1-3% (McConnell, 1975). Therefore, were lymphocytes to be activated *in vitro* by a monoclonal or even an oligoclonal activator (eg. antigens such as sheep red cells), there would ensue a low-level response. Nowell's discovery of the mitogenic potential of Phytohemagglutinin (Nowell, 1960) introduced polyclonal activators. Many other plant lectins were found to be mitogens, as were a diverse array of other materials.

These agents all have in common the ability to interact with the cell surface and thereby cause the cell to undergo blast transformation and subsequent DNA synthesis and cell division. They can activate as many as 70-80% of all lymphocytes in peripheral blood (Ling & Kay, 1975). Since these processes are crucial to any immune response, these *in vitro* activators have become very widely studied, to the extent that *in vitro* leucocyte culture has become a discipline of its own (Kaplan,1979A). The fact that the activation produced is polyclonal, i.e. non-specific, does not detract

from their usefulness, for it is appreciated that all cell activations involve much the same series of events, and it is through the understanding of these general events that we will come to understand their specific characteristics.

1.2.1.1 Lectins

Lectins are hemagglutinating proteins (see TIBS march 1981, for definition). Some plant lectins are mitogenic for lymphocytes. The two mitogenic lectins most commonly used are concanavalin A (Con A), which is isolated from the jack bean , and PHA, from the kidney bean (Ling & Kay, 1975). All lectins bind to lymphocytes. The receptors on the lymphocyte surface for lectins are carbohydrate residues, covalently attached to membrane proteins (Sharon & Lis, 1972). Lectins are polyvalent; they crosslink receptors on the cell surface. For instance. Con A is a tetrameric protein, with 4 binding sites (Edelman et al, 1972). Lectins can cause their receptors to both patch and cap. However, capping is neither necessary nor sufficient for mitogenic activation. For instance, succinyl-Con A, a derivatized form of Con A that is divalent and does not cap, is mitogenic, while both peanut agglutinin (PNA) and soybean agglutinin (SBA) can cap, but are not mitogenic (McClain & Edelman, 1976). There have been reports that wheat germ agglutinin (WGA) is mitogenic for human lymphocytes, but the degree and persistence of the activation are low, and it is difficult to demonstrate (Udey

& Parker,1980). Interestingly, PNA and SBA are mitogenic for lymphocytes which have been pretreated with neuraminidase, an activation protocol reminiscent of activation by sequential treatment with neuraminidase and galactose oxidase (NAGO) (Novogrodsky et al, 1977) (see below).

Other mitogenic lectins are lentil lectin,and pokeweed mitogen (PWM): the latter is in some species a B-cell mitogen, although its action is T-dependent (Greaves & Janossy,1972).

1.2.1.2 Other Activators

The interaction of lectins with the cell membrane has two aspects; an interaction with glycosylated proteins, and their crosslinking. In the search for the "activating principle", researchers have attacked both these angles.

There are two activating treatments involving direct chemical modification of membrane sugars; periodate oxidation, and activation by NAGO (Novogrodsky & Katchalski, 1972; 1973). It was recently shown that the sugar residue which is the site of action for NAGO action can be protected by previous binding of Con A, suggesting that both mitogens share a "receptor"(Gordon et al, 1980). Both NAGO and periodate oxidation cause proliferation of T-cells only, in both mouse and man (Novogrodsky & Katchalski, 1973; O'Brien et al, 1979). It has also been shown that macrophages are essential for NAGO activation (Phillips et al, 1980B) (as

they are for all *in vitro* activations. The macrophage may act by presentation of the agent to the responding cell, and/or by production of soluble factors required by the responding cell (see below)).Neuraminidase treatment of macrophages enhances their ability to interact with T-cells; neuraminidase-treated macrophages are more potent accessory cells (Sakano,1980), and NAGO-treated macrophages can themselves induce lymphocyte activation (Greineder & Rosenthal,1975). NAGO is mitogenic for Lyt $1^+,2^+$ T-cells (Phillips *et al*, 1980A).

Agents other than lectins which function by crosslinking membrane receptors include anti-immunoglobulin antibody and LPS, both of which are B-cell mitogens (Parker, 1980). LPS activation is specific for mouse cells, and is a T-independent activation. It is interesting that bacterial antigens are recognized in a T-independent manner, although there is one (spA, staphylococcal protein A) which has been variously reported to be mitogenic for T- and B-cells (Kasahara *et al*, 1979; Sakane & Green,1978).

1.2.2 Mitogen Specificity

A mitogen specific for any one cell type is defined here as an agent which, when added to a culture which does not contain that cell type, does not induce mitosis. This is a necessarily careful definition, because interactions between different cell types which follow specific activation, can

cloud the interpretation. Most polyclonal activators are T-cell mitogens, including all the mitogenic lectins.

Determination of the specificity of the action of a mitogen requires well-defined systems i.e. cleanly fractionated cultures, whose 'B-ness' or 'T-ness' is assured. Assessment of the specificity of a mitogen therefore depends on the method used to prepare the "purified" culture.

1.2.2.1 Cell Fractionation

Fractionation of lymphocytes is either on the basis of selection for or against some surface marker or antigen. Selection for a marker usually leads to more homogeneous end-populations than does selection against markers.

The usual method for B- and T-cell separation is the nylon wool column (Julius et al, 1973; Greaves & Brown, 1974). Removal of T-cells from B-cells and macrophages is best acheived using anti-thy 1 or anti-theta antibody and complement. Anti-Thy 1/complement lysis is selective, in that different T-cell populations express differing amounts or densities of Thy 1 (Cantor et al, 1975). B-cells can be purified using absorption chromatography, binding the B-cells to anti Ig-coupled matrices.

A useful property of the non-mitogenic lectins SBA and WGA is their specific agglutination of B-lymphocytes (Reisner et al, 1976; Bourguignon et al, 1978). Agglutinated B-cells can be separated by gravity sedimentation from the T-cells. The resultant T-cell populations are 95% Ig-negative.

It is noteworthy that both insolubilized Con A and PHA are mitogenic for B-cells (Greaves & Janossy, 1972; Andersson et al, 1972). This fact, taken together with the fact that LPS is a highly cross-linked polymer, has led to the idea that B-cell mitogenicity is somehow related to such multivalency; the concept of "antigen focussing", by which macrophages are considered to present B-cells with a highly ordered and repetitive array of antigen/mitogen is one manifestation of this line of thought (Basten & Mitchell, 1976).

1.2.3 Interleukins

The term Interleukin refers to soluble factors derived from T-mitogen -activated cultures. In 1979 , Watson et al, showed that a variety of soluble factors, obtained from Con A-activated spleen cell cultures, were in fact the same factor, in terms of their activity and their biochemical behaviour . This factor was named Interleukin-2 (IL-2) in 1979 (Mizel & Farrar, 1979). It was previously known as T-cell Growth Factor (TCGF), T-replacing factor (TRF) or co-stimulator. Also re-named at the same workshop was a macrophage-produced factor which had been known as Lymphocyte activating Factor (LAF); this is now called Interleukin-1 (IL-1).IL-2 is produced by T-cells; both IL-1 and IL-2 act on mitogen primed T-cells.

1.2.3.1 Interleukins in T-cell Activation

The sequence of events which follows addition of a T-mitogen to a culture containing mature T-cells (usually spleen cells) is now considered to be as follows: the mitogen binds to both T-cells and to macrophages. There is an interaction between macrophages and Ly-1+ T-cells which results in IL-1 being produced by the macrophages. This IL-1 binds to other Ly-1+ T-cells, and induces the production of IL-2. T-cells which have bound mitogen are induced to express IL-2 receptors; this process requires protein synthesis, and is independent of macrophages. These cells bind or absorb IL-2, and are thus activated to begin blastogenesis. This whole sequence takes between 6-20 hours from the addition of mitogen (Smith,1980; Gronvik & Andersson, 1980; Larsson et al, 1980). Since production of IL-2 is of short duration, probably due to suppression, there is ultimately a depletion of IL-2 in the culture supernatant, and the activation process stops. If fresh IL-2 is added, the process of DNA synthesis and cell division can be maintained indefinitely - in fact, clones of CTL have been maintained for periods of years by these means (Smith et al, 1979).

Some interleukins have cross-specific activity; rat and human IL-2 activate mouse cells, for instance. These activities are not histocompatability restricted . Neither factor has yet been purified to homogeneity, and the active principle(s) of each have not been identified.They are both prote-

ins, their molecular weights are considered to be in the range 10-20 K for IL-1, and 30-40 K for IL-2 (Smith et al. 1979)..

The specificity of the response is therefore contained within the cell subtypes, rather than being determined by the mitogen. Con A binds equally to all T- cells, as well as to B-cells, but only those expressing a particular antigen can either participate in IL-1/2 production, or respond to IL-1. Thymocytes respond poorly to Con A or to PHA. However, in the presence of exogenous IL-2, they generate CTL normally (Kruisbeek et al, 1980). Their immaturity is therefore related to their inability to produce IL-2. IL-1 production by a macrophage can be induced directly by its stimulation with either LPS or phorbol myristic acetate (PMA) - it will however have no effect on T-cells unless some T-cell mitogen is present (Smith et al, 1979).

Macrophages from the spleens of nude mice produce IL-1 normally (Gronvik & Andersson, 1980). However, there is no T- cell activity in these spleens. There are cells which are of the T-lineage in nude spleen (Kindred 1979). They can be induced to express T-cell function (for instance, cytotoxicity) by addition of IL-2, either in vivo or in vitro (Dennert & Hyman, 1980; Wagner et al, 1980).This indicates that thymic maturation of T-cells involves their being conferred either with the ability to produce IL-2 or to respond to IL-1.

Activation is therefore a two-signal process, with Con A as the first ,and specificity-determining signal, and IL-2 as the second, or amplifying signal (Larsson et al, 1980).

1.2.4 Secondary B-cell Activation

Not only are T-cells secondarily activated by other, mitogen-activated T-cells, but B-cells can also be activated by activated T-cells. These activations can be mediated by soluble factors. Both proliferation and production of polyclonal antibody can be demonstrated (Andersson et al, 1972). T-cell factors can induce proliferation, antibody synthesis, or both, depending on the factor (Potash, 1981).

So it is clear that while Con A is a T-cell mitogen, the T-cells which it activates can themselves activate both other T-cells, and B-cells. Reports of Ig-bearing blast cells in Con A-activated cultures, which were at one time regarded as controversial, may now be regarded more favourably (Phillips & Roitt, 1973).

These responses and interactions are complex. However, they are all mediated by lymphocytes, and these lymphocytes have all undergone a similar biochemical activation. In particular, they have all engaged in both blast transformation and DNA synthesis. To understand the interactions described above ,then, one must concern oneself with the molecular biology of lymphocyte activation.

1.2.5 Biochemical Parameters of in vitro Activation of Lymphocytes

The sequence of biochemical events which follows the addition of a mitogen to lymphocytes in culture is as follows.

1. There is no requirement for entry of the mitogen into the cell. Therefore, the events described below all result from the interaction of the mitogen with the cell membrane.

2. Within an hour of the addition of mitogen, many groups have detected increased calcium influx into lymphocytes (Allwood et al, 1971; Whitney & Sutherland, 1974; Freedman et al, 1975; Hesketh, 1979). While it seems likely that Calcium exerts a controlling role in lymphocyte activation (Bard et al, 1978), as it has been shown to do in the activation of other cell types viz. echinoderm eggs (Steinhardt & Winkler, 1979), the fact that any change in cytoplasmic free calcium must be transient, due to its rapid sequestration by organelles, makes measurements difficult . Interpretation is also hindered by the heterogeneity of the cells studied , and the multiplicity of Ca^{++}-binding sites on the cell surface, and Ca^{++}-exchangeable pools within the cell (see Hume & Weidemann, 1980).

3. In human lymphocytes, early (within 15 minutes) increases in monovalent cation fluxes have been conclusively demonstrated; some of these increases are ouabain-sensitive, as is the whole activation sequence (Kaplan, 1978). This will be discussed in more detail in a later section.

4. There are early changes in membrane phospholipid metabolism. These include an activation of the enzyme lysolecithin acyl transferase.The net result of this activation is the replacement through turnover of saturated by unsaturated fatty acids in position 2 of membrane phosphatidyl choline and phosphatidyl ethanolamine (Ferber & Resch, 1977). This results in an increased membrane fluidity; this may contribute to the increased transmembrane ion movements . There is also increased phosphatidyl inositol turnover, which may be due to increased activity of polyphosphoinositide phosphodiesterase (Schellenberg & Gillespie, 1977).

5. Many workers have detected a transient increase in cAMP concentration immediately following the addition of mitogen (Parker,1978). There is some disagreement over this finding, as cAMP has often been associated with negative regulation of cellular activity (Pardee <u>et al</u>, 1978). In the case of stimulated lymphocytes, there is a later increase in (cAMP), which corresponds to the G1-S boundary, followed by a fall in mid-S phase (Foker <u>et al</u>, 1979). The "Yin-Yang" hypothesis holds that cellular activity is regulated by the opposing activities of cAMP and cGMP (Goldberg <u>et al</u>, 1975). There have been detected changes in cGMP levels in activated lymphocytes which variously support and contradict this hypothesis (see Hume & Weidemann, 1980).

6. In the first 6 hours, there are increased uptakes of all metabolites, including amino acids, sugars, and uridine

(Mendelsohn et al, 1970; Peters & Hausen, 1971 A/B; Hume & Weidemann, 1978). The amino acid uptakes are those mediated by the 'A' or Na+-dependent pathway (van den Berg,1974). Uptake of sugars in many cell types is known to be Na+-dependent (Crane & Dorando, 1980),although similar Na+-dependent uptake has not been shown in stimulated lymphocytes. Early uridine uptake reflects an increased pool size, rather than a net synthesis, although net rRNA synthesis begins as early as 6 hours (Rubin & Cooper, 1965). Early uridine uptake is not essential for proliferation. The other uptakes correlate with net synthesis (Peters & Hausen, 1971 A).

7. Protein synthesis is activated within the first 4 hours (Levy & Rosenberg, 1973). This represents translation from preformed mRNA - there is no new transcription until about 11 hours.However, there is processing of preformed mRNA in the first 10 hours, and export to the cytoplasm (Hauser et al, 1978; Mitchell et al, 1978). The early control of activation is therefore post- transcriptional. Activation of protein synthesis is required for blast transformation (Schafer & Mitchell, 1979).

8. In mouse splenocytes, there has been shown an early (within 2-4 hours) depolarization of the membrane potential, which is followed by repolarization and then hyperpolarization over the next 48 hours; the depolarization is due to binding of the mitogen, and is specific to those cells acti-

vated by the mitogen. The repolarization seems to correlate with a secondary activation, and is possibly due to IL-2 (Kiefer et al, 1980).

9. After about 12-14 hours, the cell volume increases. This blast transformation is dependent on previous protein synthesis. The nuclear/cytoplasmic volume ratio also decreases (Ling & Kay, 1975).

10. At about the same time, nuclear decondensation begins, with concomitant transcriptional activation. The cell has by now left G0 and entered G1 - it becomes committed to entering S phase some time towards the end of G1 (Riddle et al, 1979). The exact time of commitment is not easily defined: it may vary for each individual cell (the usual measurement is of "population commitment", which is a consensus measurement). Cells which are directly activated may be committed as early as 5 hours after addition of mitogen, while those which are activated through secondary interactions will be committed later: activation is therefore asynchronous.

11. After about 30 hours, DNA synthesis can be detected, and peaks between 48 and 60 hours. Cell division follows. Activity of the enzyme thymidine kinase is stimulated by as much as 200-fold, concomitant with the activation of DNA synthesis (Munch- Peterson & Tyrsted, 1977; Strauss et al, 1977).

Chief among the earliest events in this activation sequence are those involving transmembrane cation fluxes: sodium and potassium influx and efflux, membrane potential changes, Na-dependent metabolite uptakes. These are essential for all subsequent activation. They are also the earliest events to have been established with certainty in the activation of human lymphocytes. Similar observations have been made with other cell types; these will be reviewed in the following section.

1.3 REGULATION OF CELL ACTIVATION BY MONOVALENT CATIONS

The monovalent cations which will be discussed are sodium and potassium. Sodium and potassium cross the membranes of mammalian cells by passive diffusion and by two other routes. These are defined by inhibitors, and are the sodium pump, which is ouabain-sensitive, and ouabain-insensitive movements, which in some systems include a furosemide-sensitive component (Gargus & Slayman,1980).

1.3.1 The Sodium Pump

This enzyme,(described by Skou (1957)), is a Na^+ and K^+ -dependent ATPase (EC 3.6.1.3.). It translocates Na^+ and K^+ in opposite directions across most eucaryotic membranes, against considerable electrochemical gradients, at the expense of ATP (Post *et al*, 1960). Excitable membranes (eg. nerve) are richest in this enzyme . The enzyme consists of

two catalytic subunits, of M.W. 95,000 and four glycoproteins of M.W. 55,000 daltons; these all span the membrane (Robinson & Flashner, 1979). Phospholipids, constituting about one-third of the enzyme by weight, are also required for its activity (Skou, 1975). A 12,000 dalton M.W. protein has also been implicated in its activity (Forbush <u>et al</u>, 1978). There are binding sites for Na^+ and K^+, both on the large subunit; that for Na^+ is internal, that for K^+ is external. Intracellular Mg^{++} is required for pump operation (Glynn & Karlish, 1975). The enzyme translocates 3 Na^+ ions for every 2 K^+ ions. Since the direction of translocation is Na^+-out and K^+-in, it is electrogenic, and so contributes significantly to maintenance of the transmembrane electrical potential (Hoffman <u>et al</u>, 1979). It is therefore central to all cell function. Due to the importance of the transmembrane potential and ion gradients, and of the intracellular ionic environment, to all cellular metabolism, any inhibition or activation of this enzyme will have pleiotropic effects (Kaplan, 1978). The operational definitions of sodium pump activity are Na^+,K^+-dependent ATPase activity, and ATP-driven Na^+,K^+ translocation, both ouabain- sensitive. The requirement for Na^+ is almost absolute, while Li^+, Rb^+,Cs^+, Te^+ and NH_4^+ can all substitute for K^+ (Robinson & Flashner, 1979).

1.3.1.1 Ouabain

Ouabain is a cardioactive steroid. It binds reversibly to the large subunit of the (Na^+,K^+)-ATPase, competing with potassium binding, although they do not actually share a binding site (Robinson & Flashner, 1979). The only known site of action or of binding of ouabain is the (Na^+,K^+)-ATPase. Ouabain does not bind to either the small subunits, or to the inner face of the membrane. Its binding is inhibited by high (K^+). Sodium pumps isolated from membranes of most species are essentially biochemically identical (Skou, 1975). However, cells from various species are not equally sensitive to ouabain; this appears to be due to alterations in the interaction between ouabain and the pump, rather than to any change in the response of the ATPase itself (Lelievre et al, 1979). Rodent cells are about 4 orders of magnitude less sensitive to ouabain than are human cells (Robinson & Flashner, 1979).The sensitivity of mouse (Na^+,K^+)-ATPase to ouabain can be increased 300-1000 fold by removal of a Ca^{++}-binding protein from the inner membrane face (Charlemagne et al, 1980).

1.3.1.2 Regulation of the Sodium Pump

The sodium pump shows a third-power dependence on internal sodium concentration; this reflects its primary activity, which is to maintain a low intracellular (Na^+) (Glynn & Karlish, 1975). The sodium pump in rat submandibular gland can be regulated by cGMP and by Ca^{++} (Shi et al, 1980).

1.3.2 Furosemide-Sensitive Fluxes

Furosemide, a sulfonamide diuretic, inhibits electrically-silent ouabain- insensitive K^+ fluxes in some cells. These have been identified in mouse L cells and avian erythrocytes as a linked Na^+-K^+ co-transport (McManus & Schmidt, 1978), and as a linked K^+-Cl^- co-transport in ascites cells (Bakker- Grunwald et al, 1980). Unlike ouabain-sensitive K^+ movements, both K^+ influx and efflux, mediated by the furosemide channel, are inhibited by the drug (Gargus & Slayman, 1980). These fluxes have been implicated in the control of cell volume (Kregenow, 1971).

1.3.3 Monovalent Cations in Lymphocyte Activation

In 1968, Quastel and Kaplan showed that ouabain inhibits reversibly all parameters of the activation by PHA of human PBL (Quastel & Kaplan, 1968). It was shown that within 30 minutes of the addition of PHA to these cells, potassium influx is approximately doubled; this doubling was characterized by an increased Vmax, with no change in Km. Neither actinomycin D nor cycloheximide could inhibit the flux activation (Quastel & Kaplan, 1970B; Quastel et al, 1970). Potassium efflux was also doubled (Segal et al, 1976; Hamilton & Kaplan, 1977). These results have been confirmed by other labs (Averdunk, 1976; Segal & Lichtman, 1976). It was also shown that mitogen activation causes the lymphocyte membrane to become leaky (which could explain the increased

K^+ efflux) (Segel *et al*, 1975). There are also increases in Na^+ influx and efflux, the latter being ouabain-sensitive (Averdunk, 1976). It was shown that potassium concentration does not change appreciably in the first 20 hours after stimulation (Segel & Lichtman, 1978). While the activation of human lymphocytes was totally inhibited by 10^{-7} M ouabain, rat lymphocytes required 10^{-3} M for total inhibition (Quastel & Vogelfanger, 1971). For a review of these findings, see Kaplan (1978).

The increases in influx and efflux produced by stimulation are equal in magnitude. This means that there should be no net change in intracellular (K^+). Potassium concentration has been reported to both decrease following stimulation (Negendank & Collier, 1976; Averdunk & Gunther, 1980A), or to remain constant (Segel & Lichtman, 1978). The lymphocyte increases dramatically in volume after 16-20 hours; to maintain $(K^+)c$ within the range of measured values, there must therefore be a net increase in potassium content. This should be reflected by an imbalance between influx and efflux (i.e. by a net influx). But such has not yet been found; this paradox has been discussed by Kaplan (1978,1979B).

The increased leakiness of the membrane following stimulation suggested a possible mechanism for the sodium pump activation. This was that an increased intracellular (Na^+) led, through the known third-power dependence of pump activ-

ity on (Na+)c, to an elevation of ATPase-mediated pumping (Kaplan & Owens, 1980). Indeed, Segel & Lichtman (1979)/Segel <u>et al</u> (1979) and Averdunk & Gunther (1980A) have shown an increase in intracellular sodium concentration following PHA addition to human PBL. There have been similar findings in other systems (see below).

There is evidence that new, previously cryptic (Na^+,K^+)-ATPases are exposed following mitogen binding, through some membrane reorganization. Thus, there has been detected an increased ^{3}H-ouabain binding within 30 minutes of addition of PHA to human PBL (Averdunk & Lauf, 1975; Quastel & Kaplan, 1975).

Quastel has recently shown that EGTA, a Ca^{++} chelator, induces uptake of $^{86}Rb^+$ by human lymphocytes (Quastel <u>et al</u>, 1980). This is an intriguing finding, as it suggests that Ca^{++} may somehow control sodium and potassium uptakes, perhaps through an effect on the membrane permeability. Intracellular Ca^{++} is known to control K^+ fluxes in red cells (Gardos <u>et al</u>, (1975)). Ca^{++} also regulates polyphosphoinositide phosphodiesterase activity (Michell, 1975).

1.3.4 Activation of Other Systems

Kaplan (1978) has reviewed other systems whose stimulation to proliferate is accompanied by an increase in monovalent cation fluxes. These include fibroblasts, stimulated by both serum and growth factors, regenerating hepato-

cytes, fertilized echinoderm eggs and virally-transformed cells. In all of these systems, stimulation of cells to proliferate is accompanied by an elevation of these fluxes, and these activations are inhibited by ouabain (Kaplan, 1978; Leffert, 1980).

As regards the mechanism of these activations, there has been some support for the idea that they are mediated via an increased permeability to sodium, thus activating the pump. Toback (1980) has shown that growth of cultured kidney cells can be induced by an increase in the extracellular (Na^+). Smith & Rozengurt (1978) found that monensin, an agent which facilitates sodium entry into cells, can induce the activation of ouabain- sensitive potassium influx into fibroblasts. Increased Na^+ influx and intracellular (Na^+) have been demonstrated in these cells following serum stimulation (Mendoza et al, 1980). Monensin has been shown to stimulate Na^+- dependent amino acid uptake by mouse fibroblasts (Smith & Austic, 1980). In MSA-stimulated chick embryo fibroblasts, there is also activation of Na^+-dependent amino acid uptake (Derr & Smith, 1980). Koch and Leffert (1979) found that amiloride, an inhibitor of passive sodium entry into cells, can inhibit the proliferation of liver cells which follows partial hepatectomy. A hyperpolarization of the membrane potential has been demonstrated during rat liver regeneration, which could result from an activated Na^+ pump (Wondergem & Harder, 1980). However, Moolenaar et al (1981) find that am-

iloride, while inhibiting Na^+ influx and sodium pump activation in serum-stimulated neuroblastoma cells, does not affect an early depolarization induced by serum addition; this suggests that not all electrical events can be attributed to activity of either the (Na^+,K^+)-ATPase, or of Na^+ channels. While Lubin (personal comm.) has recommended caution in the interpretation of results obtained using amiloride, these results, together with those of Segel & Lichtman (1979) suggest that there may in fact be a common mechanism for the activation of pump activity in these systems , involving an increased membrane permeability to sodium.

The possibility that new pump sites are exposed during activation of cells other than lymphocytes is suggested by the finding that ouabain-binding is elevated in growing and virally-transformed fibroblasts, and that stimulation of these cells with serum leads to an increased binding of ^3H-ouabain (Johnson & Weber, 1979, 1980).

1.3.5 Cation Fluxes and Differentiation

Many inhibitors of lymphocyte proliferation, including ouabain, induce differentiation in other systems, most notably in the Friend cell (Bernstein et al, (1976)). Ouabain can also induce differentiation in the chick embryo lens cell (Piatagorsky et al, 1980). It has been suggested that in all these cells proliferation is accompanied by an activation, and differentiation by a depression, of monovalent cation

fluxes (Kaplan & Owens, 1980). Indeed, such a depression has been shown for both the induced Friend cell and the differentiating lens cell (Mager & Bernstein, 1978; Piatagorsky et al, 1980). This would predict that a differentiating lymphocyte would show a depression of potassium uptake. While this has not been shown, there is an observation which may be related, which is that human lymphocytes, pretreated for 48 hours with a low ouabain concentration, produce an elevated level of stimulation in the MLR (Christen et al, 1975). It is possible that this pretreatment has induced a differentiation which causes "superstimulation".

1.3.6 Control of Cellular Metabolism by Monovalent Cations

1.3.6.1 Na^+/K^+ ratio

Lubin has shown that protein synthesis in E. coli and in mammalian cells in culture is inhibited if intracellular (K^+) falls below a critical concentration (Lubin, 1964, 1967; Ledbetter & Lubin, 1977; Cahn & Lubin, 1978). The effect was shown to be on the protein synthesis apparatus itself, and not on precursor transport (Ledbetter & Lubin, 1977). However, since then, Lubin has shown that a degree of inhibition of the sodium pump which is by itself not sufficient to lower (K^+)c enough to affect protein synthesis can inhibit proliferation (Lubin, 1980), which suggests that it is not the effect of intracellular (K^+) on protein synthesis per se which is the controller. Lubin has pointed out that

any effect on either Na^+ or K^+ cannot with certainty be distinguished from an effect on the Na^+/K^+ ratio. He has measured this ratio in mouse 3T3 cells, and shown that when it is raised, proliferation can be halted. Interestingly, control of a similar nature is not evident in transformed SV3T3 cells (Lubin, 1980). In contrast, Moscatelli et al (1979) found that either lowering $(K^+)c$ to 22%, or raising $(Na^+)c$ to 820% of normal levels, had no effect on DNA synthesis in chick embryo fibroblasts for 16 hours following the change. Piatigorsky has shown that the differentiating effect of ouabain on lens cells correlates with an alteration in the Na^+/K^+ ratio, and that this in fact controls the selectivity of translation (Shinohara & Piatagorsky, 1980). Other demonstrations of the effects of the intracellular ionic environment on translation include those of Cameron (Cameron et al, 1980).

1.3.6.2 pH

Changes in intracellular pH have been implicated in the control of the mitotic cycle (Gerson, 1978; Gillies & Deamer, 1979). It has been suggested that the increases in Na^+ influx in many cell types are mediated by Na^+/H^+ exchange - this is blocked by amiloride (Steinhardt & Winkler, 1979). There has been a preliminary report that the intracellular pH of lymphocytes increases following stimulation (Gerson, 1981). The intracellular pH of echinoderm eggs also increases upon fertilization (Steinhardt & Winkler, 1979).

Any change in activity of the (Na^+, K^+)-ATPase will be reflected both by changes in the membrane potential, and by changes in the total intracellular ionic environment. Changes in both membrane potential and sodium fluxes will affect both amino acid and sugar uptakes, and may also exert a selectivity on these uptakes (Johnstone & Laris, 1980).

It is clear that the cell membrane, and monovalent cation fluxes in particular, are intimately involved in the control of cell proliferation. It is also clear that cellular proliferation is an essential component of the immune response. In fact, it was with cells of the immune system that this control of proliferation by monovalent cations was first demonstrated. It is generally accepted, as a result, that activation of monovalent cation fluxes is specific to, and necessary for the activation of a cell to proliferate (Kaplan, 1978).

However, all work to date on ion fluxes in lymphocytes has been with unfractionated populations. For this reason, it is impossible to be sure that the ion fluxes generated by the addition of a mitogen are in fact confined to those cells which are activated to proliferate, and are not produced in all cells which bind the mitogen. Neither has elevation of cation fluxes been shown for cells which are secondarily activated, through interactions subsequent to mitogen addition.

1.4 PURPOSE OF THIS THESIS

The purpose of this thesis is to examine the specificity of the activation of potassium influx which accompanies the mitogenic activation of lymphocytes in vitro, and then to use this flux activation in an analysis of the interactive activations which are known to follow the primary activation by mitogen.

1.4.1 Approach

The system chosen for this study was the mouse splenic lymphocyte. This was for the following reasons:

1. Although activation of rodent lymphocytes has been shown to be inhibited by ouabain (Quastel & Vogelfanger, 1971), the activation of cation fluxes has not been shown for this system. Since the mouse is the most used animal in immunology, it is important that this be established.
2. Mouse cells are available in larger numbers, from inbred animals, and are more amenable to fractionation techniques than are human lymphocytes.
3. Mice, as rodents, are orders of magnitude less sensitive to ouabain than are humans -this is an interesting difference, worthy of study for its own sake (even though it is not the primary focus of the project).

The approach to be taken is to confirm that activation of mouse splenic lymphocytes is accompanied by activated cation fluxes, as in other systems. These fluxes will be characterized in terms of their kinetics and mode of action. Their specificity will then be assessed, using cell-specific mitogens and either fractionated cell populations, or inbred nude mice as a source of T-mitogen unresponsive cells. The involvement of these flux activations in cellular interactions (and vice versa) can be examined by mixing populations of cells of various response capabilities.

SECTION II

MATERIALS AND METHODS

2.1 MICE

Balb/c mice were obtained from Bio-Breeding Labs., Ottawa, and from Charles River Labs. Animals between 8-10 weeks old were used in all experiments. Most of the animals used were male. In later experiments, females were also used.

BNC nu/nu and nu/+ mice were obtained from the Department of Clinical Studies, University of Guelph, Guelph, Ontario. Mice between 6-10 weeks old were used in all experiments. Nude mice were maintained in a draught-free environment, at a fairly constant temperature (23 - 27°C). Bedding and food were autoclaved, the water was chlorinated, pH 3.5.

2.2 CELL PREPARATION

Mice were killed by cervical dislocation. Their spleens were removed and disrupted on a wire screen, into a drop of calf serum (Flow; Canadian Veterinary Biologics Ltd., Smiths Falls, Ontario). Red blood cells were removed from the resultant cell suspension by lysis with NH_4Cl (0.83%, 5-7 minutes at room temperature). Resultant suspensions (95% lymphocytes) were washed once with RPMI 1640 (Flow) before use.

2.3 CELL FRACTIONATION

B- and T-cell subpopulations were separated by agglutination of the B-cells with wheat germ agglutinin (WGA), by the method of Bourguignon et al (1979). This was carried out before removal of the red cells. Briefly, between 3 and 4 X 10^8 cells were suspended in 1 ml. of WGA (Calbiochem), (100 ug/ml., in RPMI 1640) for 5 minutes at room temperature, then layered onto a 50% calf serum/RPMI 1640 gradient. After 30 minutes at room temperature, the T (top) and B (bottom) fractions were collected and washed, once with 200 mM N-acetyl D-glucosamine (Calbiochem), and once with RPMI 1640. In earlier experiments they were then recycled, but for enrichment of cells for the T-cell/ nude-cell mixtures, only one cycle was used.

2.4 ANTI-THY 1.2 AND COMPLEMENT LYSIS

Monoclonal anti-Thy 1.2 (New England Nuclear, AKR anti- C3H) was added to 1 ml. of splenocytes (about 2 X 10^8 cells), at a final titer of 1/800. Cells were incubated with antibody on ice for 1 hour, washed once, then incubated at 37°C for 1 hour in 1/20 guinea pig complement (kindly provided by Dr. J. Laing, Agriculture Canada), in RPMI 1640. They were then washed twice, and recycled once through the entire procedure.

2.5 ULTRAVIOLET LIGHT IRRADIATION

Washed cells were suspended at 2 X 10^6 /ml in phosphate-buffered-saline (PBS), and 5 ml aliquots placed in siliconized glass petri dishes. These were exposed to a 15W germicidal UV lamp, the petri dish uncovered, for 10 seconds - this corresponded to a dose of 84 ergs/mm2 (Castellanos, 1980). Cells were then washed twice before culturing.

2.6 CELL CULTURE

Cells were cultured in RPMI 1640, supplemented with 6% fetal calf serum (Flow), penicillin (100 units/mL) - streptomycin (100 µg/mL) (Difco), and 2 mM L-glutamine, buffered (20 mM Hepes) to pH 7. All cultures were at a density of 2 X 10^6 cells/mL, unless otherwise indicated. Cells were cultured either in microplates (Linbro), at 200 µL/well (flat- and round-bottomed wells were used interchangeably); in plastic tubes (Falcon), at 0.5-1.0 mL/tube (depending on the experiment); or in plastic flasks (Corning), at 10 mL/flask. Containers were kept sealed, and cells were cultured at 37°C. In later experiments I found that cell survival in culture was improved by supplementing the cultures every 24 hours with complete culture medium (10% volume).

2.7 MITOGENS

Con A (Calbiochem, Sigma) was stored at -20°C, (1 mg/mL in isotonic saline) and was added to cultures at from 2-4 µg/mL, depending on the batch; all batches of mitogen were assayed for optimal dose on preparation. LPS (E.coli 055:B5, (Difco), and E.coli 011:B4, a gift from Dr. Phyllis Kind, George Washington university, Washington, D.C.) was stored at -20°C, at 1 mg/mL., in saline, and was added to cultures at 25-40 µg/mL; the dose optimum for LPS showed a broad peak.

2.8 INHIBITORS

Ouabain (Sigma) was stored at 4°C, 2 X 10^{-2} M in saline. Furosemide (Hoechst) was stored at 4°C, 2 X 10^{-2} M, in RPMI 1640. Amiloride (Merck-Frosst) was stored at 4°C, 10^{-2}M in water. Inhibitors, when used, were added within 5 minutes of the mitogen(s).

2.9 ACTIVATION ASSESSMENT

Cells were pulsed for 1-2 hours with 2 µCi (1 Ci = 37 GBq) ^{3}H-thymidine per mL (Amersham, New England Nuclear). Cells were harvested (between 48 and 72 hours after initiation of culture) either from microplates using a multi-well cell harvester (Titertek), or from tubes using a manifold (Millipore), onto glass fiber filters (Whatman). When using a manifold, cells were washed once with 5% trichloroacetic acid,

4°C. Filters were dried and radioactivity was counted in a scintillation counter (Beckman). When activation was expressed in terms of stimulation index (SI), this was calculated as follows:

SI = ((cpm in mitogen-treated cells) - (background))/((cpm in resting cells) - (background)).

To determine the time of activation of protein synthesis, 10 µCi ^{3}H-leucine (Amersham) was added per mL. culture, and cells were sampled on a manifold at various times afterwards.

2.10 POTASSIUM INFLUX

2.10.1 Influx Measurement

Preliminary experiments established that $^{86}Rb^{+}$ was transported identically to ^{42}K. Since the latter is a short-lived isotope, $^{86}Rb^{+}$ was used as a tracer for potassium in all experiments. At specified times after mitogen addition, approximately 40 µCi (in 25 µL) of $^{86}RbCl$ (New England Nuclear) was added to each mL of culture, in plastic tubes. Cells were sampled by centrifugation of 200 µL aliquots through 100 µL of either corn-oil (Mazola) and n,n-dibutylphthalate (10:3), or silicon oil (Dow Corning 550 and 560 (12:13)), using a Beckman microfuge (Oliver & Paterson, 1971; Strauss et al, 1976). The tips of the tubes were cut off and the radioactivity in the pellets counted in a gamma counter. Counts were converted to potassium uptake according to equations 1. and 2.

1) SRM (counts/fmol K^+) = (counts/mL)/(Z (fmol/mL)),

where SRM was the specific radioactivity of the medium, and Z the concentration of K^+ in the supernatant (5.4×10^9 fmol/mL).

2) K^+ uptake (fmol/cell) = ((counts/pellet) - (trapped counts))/ (SRM X (viable cells/pellet)).

2.10.2 <u>Trapped Space</u>

Invariably, some medium remained associated with the cells. It was necessary to correct for the counts contained within this trapped medium, to accurately determine uptake. Two methods were used for this correction.

The first involved use of the gamma-emitter $^{99}Tc(m)$, which was kindly supplied by Jack McLean, Radiation Protection Branch, Health & Welfare Canada. This isotope does not bind to cells in the absence of a reducing agent (Gillespie <u>et al</u>, 1973).Cell suspensions were labelled with $^{99}Tc(m)$ and aliquots taken immediately and centrifuged. Radioactivity in the pellet was counted on a gamma counter, and the volume of entrapped medium calculated from the radioactivity per mL of the supernatant. Figure 1 shows the volume in the pellet as determined by this method, plotted against the number of cells in the pellet. The relationship between the two is linear, the volumes determined are similar to those determined by Strauss <u>et al</u>, (1976), who used ^{14}C-sucrose as a marker. Knowing the number of cells per pellet in any exper-

iment, it was possible, by reference to this figure, to calculate the volume of trapped medium, and hence the counts to be subtracted.

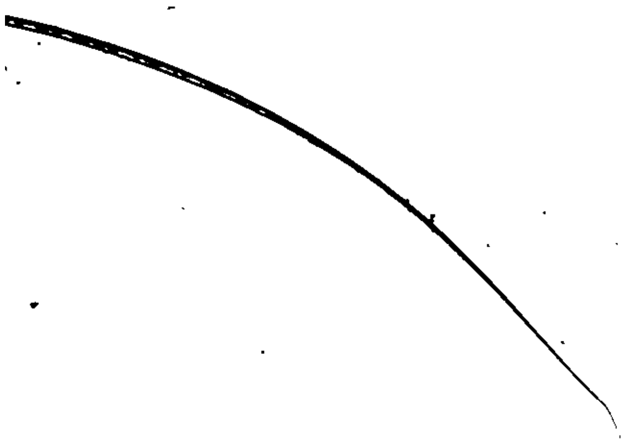
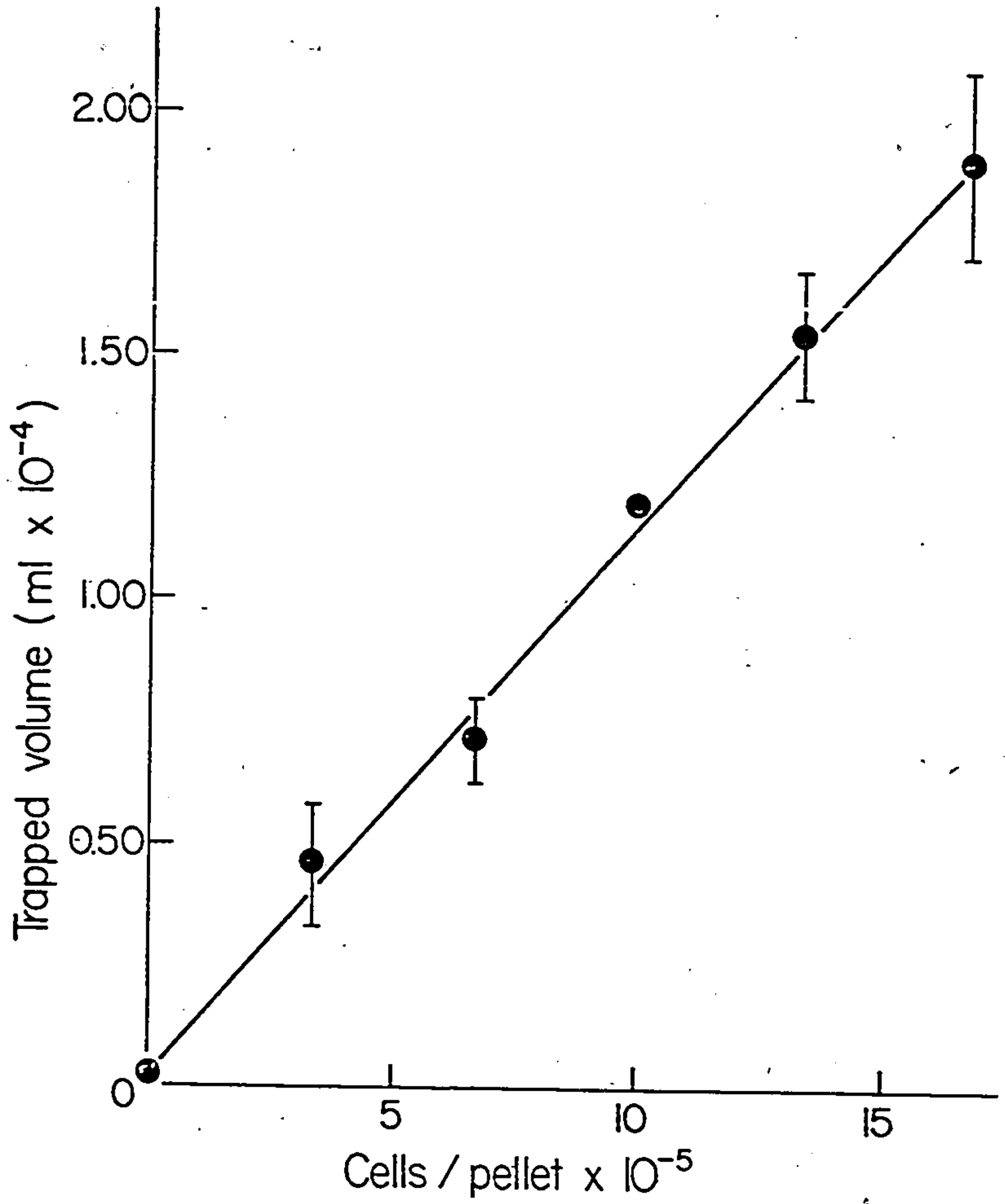


Figure 1. Volume of medium trapped in a pellet (as measured from the content of $^{99}Tc(m)$), vs. the number of cells which had been centrifuged through oil. Points are the means of 6 replicates, bars are standard deviations.



Trapped volume (ml x 10^{-4})
2.00
1.50
1.00
0.50
0
5
10
15
Cells / pellet x 10^{-5}

A more convenient method, which did not involve the use of a short-lived isotope, and which therefore could be carried out concurrently with uptake experiments, was the zero time uptake method. $^{86}Rb^{+}$ was added to cells which had been cooled to 4°C. They were immediately (within 30 seconds) s ampled by centrifugation. The 30 second, 4°C uptake into the pellet represents trapped medium; under these conditions, transport is insignificant. Figure 2 shows the results of one experiment which illustrate the use of this method.

Both methods gave comparable results in the range of cell densities used. In fact, the subtracted counts never constituted more than 20% of the total uptake , and this only for the earlier time points.

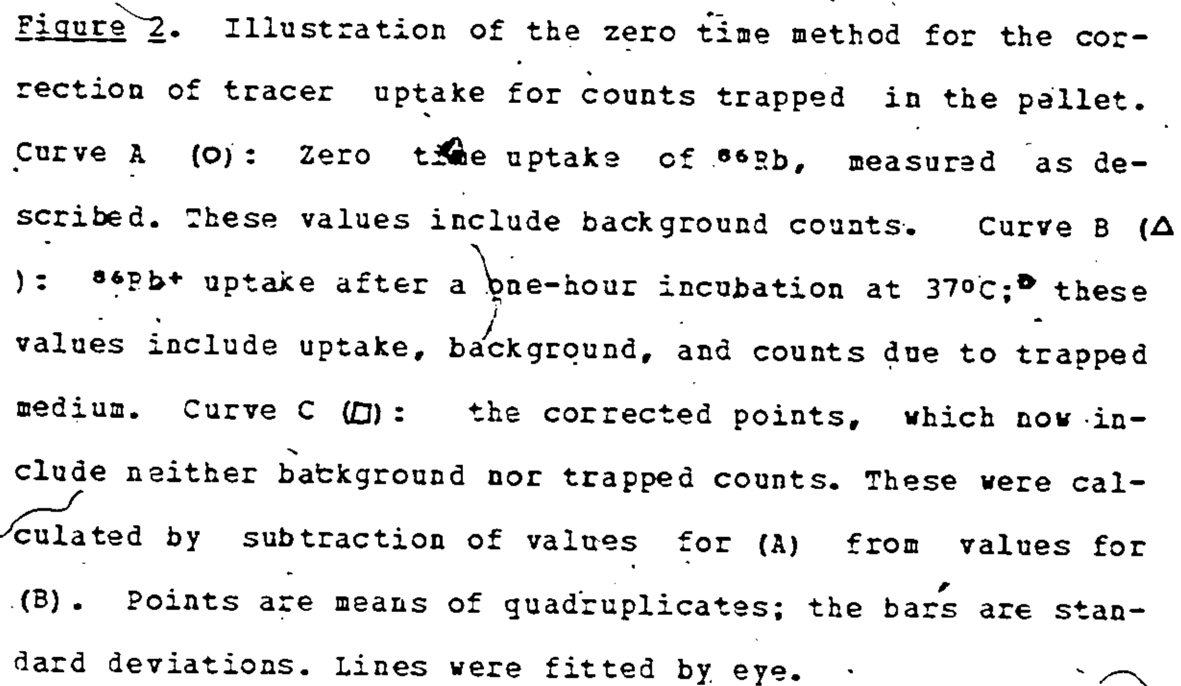
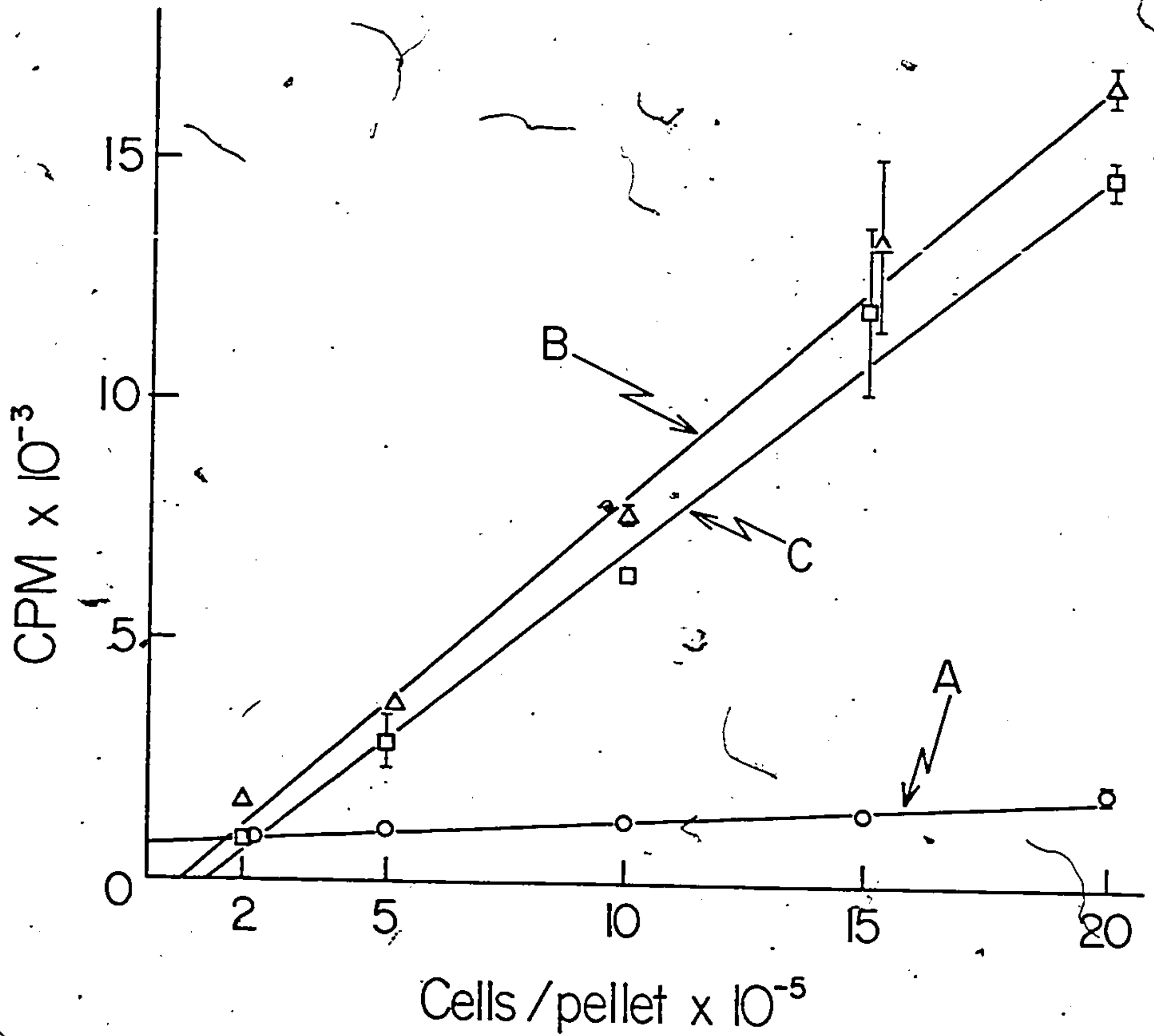


Figure 2. Illustration of the zero time method for the correction of tracer uptake for counts trapped in the pellet. Curve A (O): Zero time uptake of ^{86}Rb, measured as described. These values include background counts. Curve B (Δ): $^{86}Rb^{+}$ uptake after a one-hour incubation at 37°C; these values include uptake, background, and counts due to trapped medium. Curve C (□): the corrected points, which now include neither background nor trapped counts. These were calculated by subtraction of values for (A) from values for (B). Points are means of quadruplicates; the bars are standard deviations. Lines were fitted by eye.



CPM x 10^{-3}
15
10
5
0
2
5
10
15
20
Cells / pellet x 10^{-5}
A
B
C

2.11 POTASSIUM EFFLUX

Efflux of $^{86}Rb^{+}$ was measured over a period of 2.5 hours using cells that had been preloaded with the isotope for 14 hours. When efflux was measured from 0 -2.5 hours, cells had been preloaded for 14 hours, then washed once with ice-cold PBS and resuspended in Con A-containing medium. For measurement at 14 hours, cells were preloaded in the presence of Con A, then washed and resuspended as above. No significant loss of isotope from the cells occurred during the PBS wash; the radioactivity washed from the tubes did not exceed that expected from residual medium. Samples were taken exactly as for influx. Efflux was calculated as the regression coefficient for plots of -ln (Pt/P0) vs. time in unlabelled medium, where Pt and P0 are cell tracer contents at times t and zero, respectively. These curves represent fractional loss from the cells. Efflux was calculated from these slopes as described for influx in section 3.16.2

2.12 POTASSIUM CONTENTS

Cells were cultured at 3 X 10^6/mL, 20 mL in flasks. At specified times, cells were collected by centrifugation in acid-washed glass tubes. The supernatants were poured off, and the insides of the tubes wiped dry. The cells were then recentrifuged. The pellets were then washed three times, taking care not to resuspend any cells, with choline chloride (125 mM), buffered with Hepes (20 mM, pH 7). They were

then suspended in 2.5 mL distilled water, and sonicated for 2 minutes at 50/60 Hz, 80 W (Branson). The suspensions were then made up to 5 mL., and the potassium concentrations measured using an EEL flame photometer. Potassium content per cell was determined by calculation. These values were corrected for the potassium content of the medium trapped in the pellets by measuring the volume of this medium using ^{99}Tc(m), in separate experiments. This volume was 4.4 +/- 0.5 (SD) µL.

2.13. METAPHASE SPREADS

Cells were cultured in flasks. After 2 days, colcemid (Difco) was added (0.1 µg/mL) for 3-4 hours. Cells were collected by centrifugation, washed, and gently resuspended in 0.075 M KCl for 20 minutes. They were then fixed in methanol: acetic acid (3:1), and spread on glass slides. Spreading was accomplished by gently blowing on the slide as it dried under an incandescent light bulb. Best results were obtained by resuspending the cells in fresh fixative before spreading.

2.13.1 Hoechst 33258 Staining

Hoechst dye 33258 (Calbiochem) was stored at 500 µg/mL, at 4°C, wrapped in foil, in 0.15 M NaCl, 0.03 M KCl, 0.1 M Phosphate, pH 7. For staining, this was diluted to 0.5 µg/mL. Slides were immersed in the stain in a coplin jar for 90

seconds at room temperature, then washed twice in PBS and once in water. Spreads were mounted in immersion oil under coverslips for scoring and microscopy.

2.13.2 Scoring

To eliminate bias in the counting of male and female spreads, slides from 2 experiments were mixed and randomly number-coded (twice) by two people not associated with the experiment, and then scored blind.

2.14 IMMUNOFLUORESCENCE

2.14.1 Routine Assessment of Fractionated Populations

The method of Lamelin et al (1972) was used. Cells were washed, then cultured in serum-free medium for one hour. They were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse heavy and light chain immunoglobulin (Cappell Labs.) for 30 min. at 4°C. The antiserum had previously been adsorbed with mouse liver- acetone powder for one hour at 4°C - it was subsequently used at a titer of 1/8. After incubation with antibody, the cells were washed with PBS, then resuspended and air-dried onto glass slides. They were then fixed in methanol:acetic acid (1:3), washed, and viewed under UV. All microscopy was carried out using a Zeiss photomicroscope.

2.14.2 **Determination of the Nature of Blast Cells**

The above method , while adequate for the assessment of the number of cells bearing a given marker, was rather harsh. There also tended to be high background fluorescence. A more careful method was used for the identification of the nature of responding cells in mixtures.

About 5 X 10^5 cells were collected by centrifugation and washed once. They were incubated in a volume of 100 µL at 4°C, with FITC-goat anti-mouse Ig, at a titer of 1/40 (this antiserum had not been adsorbed). After a 15 minute incubation, cells were washed once with serum-containing medium, once with PBS, then fixed (1 min.) with 3% paraformaldehyde in PBS (pH 7). They were again washed, suspended in PBS and allowed to settle onto poly-L-lysine- coated coverslips. These were then mounted in 50% glycerol in PBS (pH 7.2) for scoring and photography. Cells were sized using a calibrated eyepiece; cells with a diameter greater than 8 um were considered to be blast cells. Only cells which showed strong fluorescence were scored as Ig-positive.

The significance of differences between treatments in all experiments was assessed using Student's t test.

SECTION III

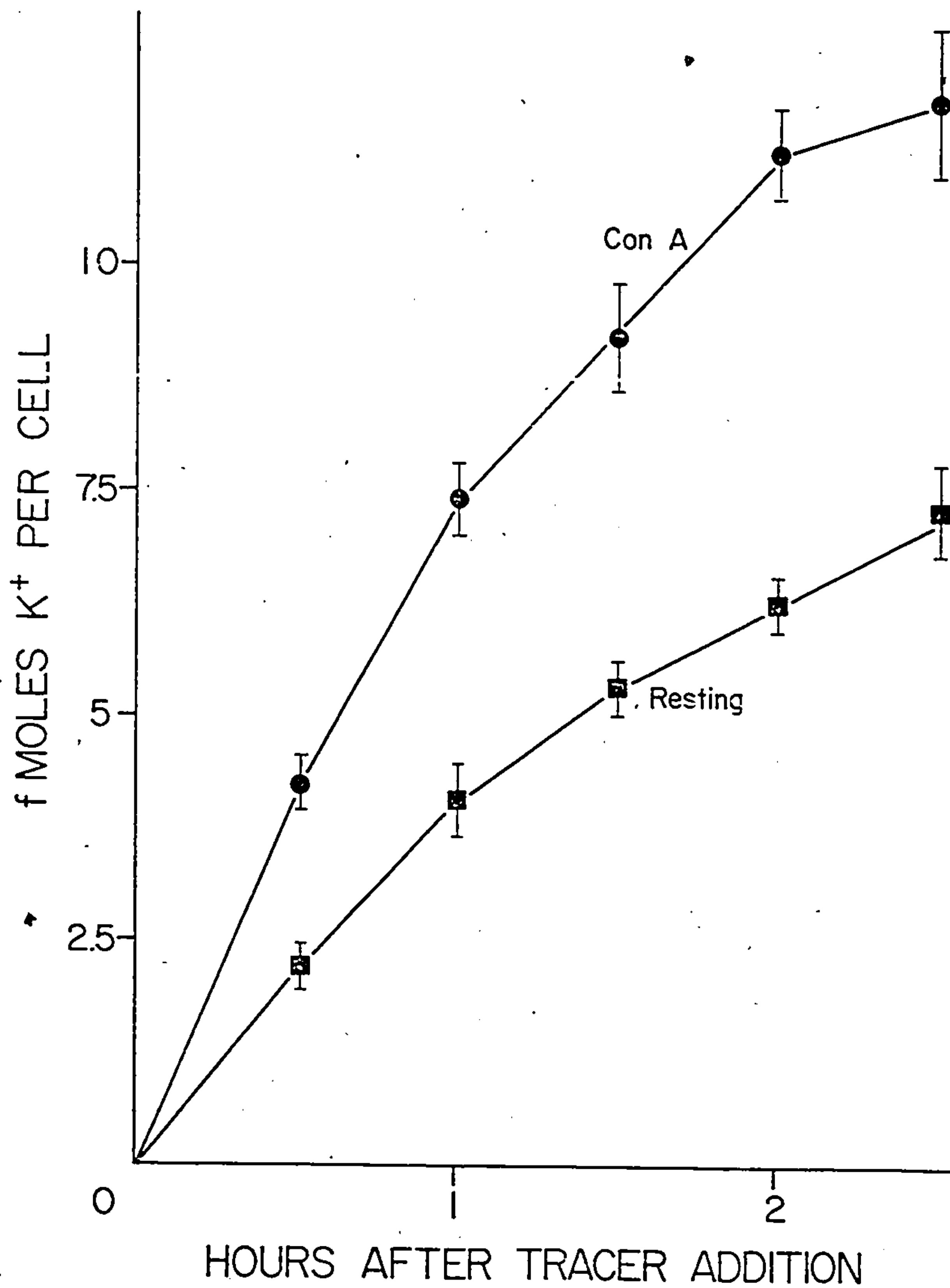
RESULTS

Con A is mitogenic for mouse splenocytes in cultures, inducing a 20-50 fold activation of ^{3}H-thymidine incorporation, measured 48-60 hours after the initiation of culture. This activation is known to be inhibited by ouabain (Quastel & Kaplan, 1970A; Quastel & Vogelfanger, 1971); therefore it requires sodium pump activity. To determine whether Con A in fact stimulates the activity of the (Na^{+},K^{+})- ATPase, potassium influx into cells was measured, and its ouabain sensitivity determined.

3.1 ACTIVATION OF POTASSIUM INFLUX

Figure 3 shows the effect of Con A on total potassium influx into Balb/c splenic lymphocytes, measured 14-18 hours after the addition of Con A. In this and subsequent figures showing influx, tracer was added to the cells at time zero (on the abscissa), which was between 8 and 15 hours after the initiation of culture. $^{86}Rb^{+}$ was used as a tracer for potassium. Con A caused an approximate doubling of the net rate of uptake of potassium.

Figure 3. Total uptake of potassium into Balb/c splenocytes, both resting and stimulated with Con A for 14-18 hours. Tracer ($^{86}Rb^+$) was added at time zero. Results from 9 experiments are combined, the bars represent standard error of the mean. In this and all subsequent figures showing combined data from a number of experiments, each individual experiment was performed in triplicate.



f MOLES K+ PER CELL
10
7.5
5
2.5
0
Con A
Resting
1
2
HOURS AFTER TRACER ADDITION

For all assays described hereunder, parallel cultures were maintained, and their activation to proliferate assessed at 48 hours, by ^{3}H-thymidine incorporation. Only the results from cultures which were shown by this assay to proliferate will be presented in this thesis.

3.1.1 Time of Flux Activation

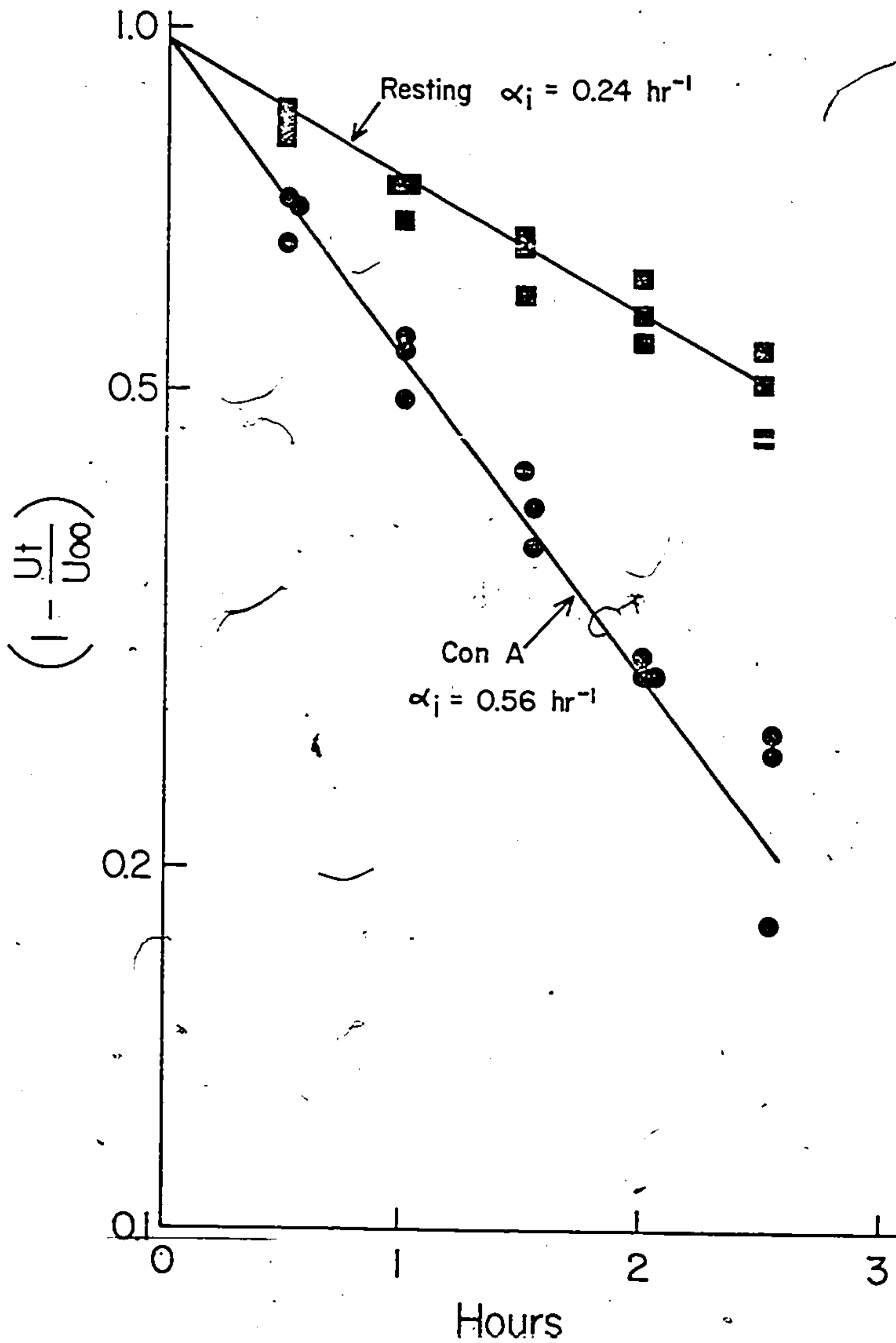
The activation of K^+ influx was not detectable until 6-8 hours after addition of Con A. The precise time of activation was not determined. Activated influx was not routinely detectable at 4 hours, was apparent at 6 hours and was maximal at 8 hours. There was some variability in the time of influx activation, which was probably a consequence of the asynchrony of these cultures. The stimulated rate was then maintained for a further 10 hours; measurements were not made after that time. Activation of protein synthesis was detectable in some experiments earlier than activation of potassium influx.

3.1.2 Rates

These data were converted from cell potassium content to fractional uptake $(1 - U_t/U_\infty)$, where U_t is the uptake at time t, and U_∞ is the maximal uptake (estimated from hyperbolic plots such as Figure 3), and the log of this value plotted against time, as shown in Figure 4. This gave a linear plot, which allowed accurate calculation of the mean net

influx over the period of the assay (2.5 hours). Slopes of these lines were calculated by linear regression analysis.

Figure 4. Data for potassium uptake from Figure 3, expressed as the log of the fractional uptake, as described in the text, and plotted against time of uptake. This is total uptake, as shown in Figures 3, 11, and 12. Each point represents the combined results from a group of three experiments. Lines were fitted using linear regression analysis.


1.0
0.5
0.2
0.1
Resting α_i = 0.24 hr^{-1}
Con A
α_i = 0.56 hr^{-1}
$\left(1 - \frac{U_t}{U_\infty}\right)$
0
1
2
3
Hours

Con A caused an increase in the rate constant from 0.24 $hr.^{-1}$ in resting cells to 0.55 $hr.^{-1}$ in stimulated cells. This represents a 2.3-fold activation of net total K^+ influx by Con A. If the 30 minute time point was used as an estimator of the initial rate of uptake (Figure 3), rates of 4.6 +/- 0.2 and 8.5 +/- 0.4 fmoles/cell.hr. were obtained for resting and Con A-activated cells respectively. This shows a 1.85-fold activation of influx. That the degree of flux activation was less using this rate determination may reflect different saturation kinetics of the resting and stimulated influx; since the fractional uptake line included data from all points assayed (including those nearer saturation), it would show a degree of activation that reflects this. The 30 minute estimator, on the other hand, was a better measure of the initial rate of uptake.

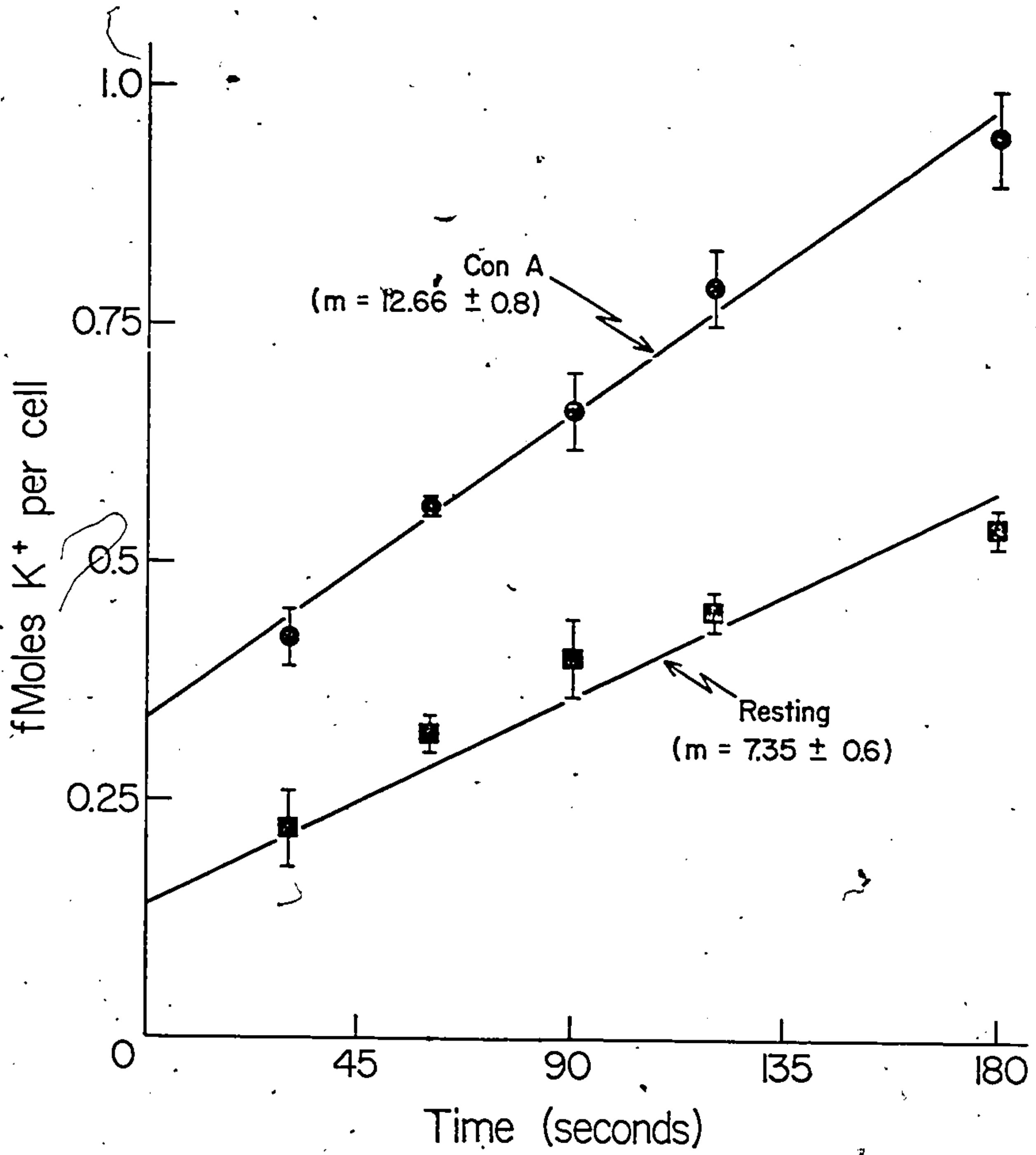
To convert the slopes of the fractional uptake line to influx, (for comparison with the 30 minute rates), the method of Segel & Lichtman (1976) was used. Essentially, multiplication of the slope (hr^{-1}) by the intracellular potassium content (fmoles/cell, see Table 3 for values) gives a value for influx (fmoles/cell.hr.).

Thus, mean net total influx for resting cells was calculated as 3.35 fmoles/cell.hr., which was activated by Con A to 8.96 fmoles/cell.hr. These values differ from those determined from the 30 minute points in Figure 3 because fractional uptake represents mean net uptake over a 2.5 period,

as opposed to the estimate of the initial rate obtained from the 30 minute uptake points.

This difference illustrates an inherent disadvantage of such a lengthy assay for measurement of influx. There is an increasingly large component of backflux of tracer from the cells to the medium with time. The earlier the time point chosen for influx measurement, the more accurate an estimate of initial influx is obtained. For this reason, influx was measured using a very short assay, during which backflux did not become significant.

Figure 5. Uptake of potassium by Balb/c splenocytes, both resting and stimulated with Con A for 14 hours. Total uptake time was 3 minutes. Counts contained in trapped medium have not been subtracted, this has no effect on the slopes (m values). Slopes were calculated by linear regression analysis. Points represent the means of 3 experiments, the bars are standard error of the mean.



fMoles K⁺ per cell
1.0
0.75
0.5
0.25
0
45
90
135
180
Time (seconds)
Con A
(m = 12.66 ± 0.8)
Resting
(m = 7.35 ± 0.6)

Figure 5 shows the results of such an assay. The total time for assay was 3 minutes. Tracer was added to the cells 1¼ hours after the initiation of culture. The uptake line generated was linear. This allowed an accurate determination of the rate of uptake; it also made correction for counts trapped in the pellet unnecessary, since this correction would affect only the intercept, and not the slope of the line. Slopes of these lines (whose correlation coefficients were all greater than 0.9) were calculated by linear regression. Influx into resting cells was 7.35 +/- 0.6 fmoles/cell.hr., that into Con A -activated cells was 12.66 +/- 0.8 fmoles/cell.hr. Con A therefore produced a 1.72-fold activation of potassium influx.

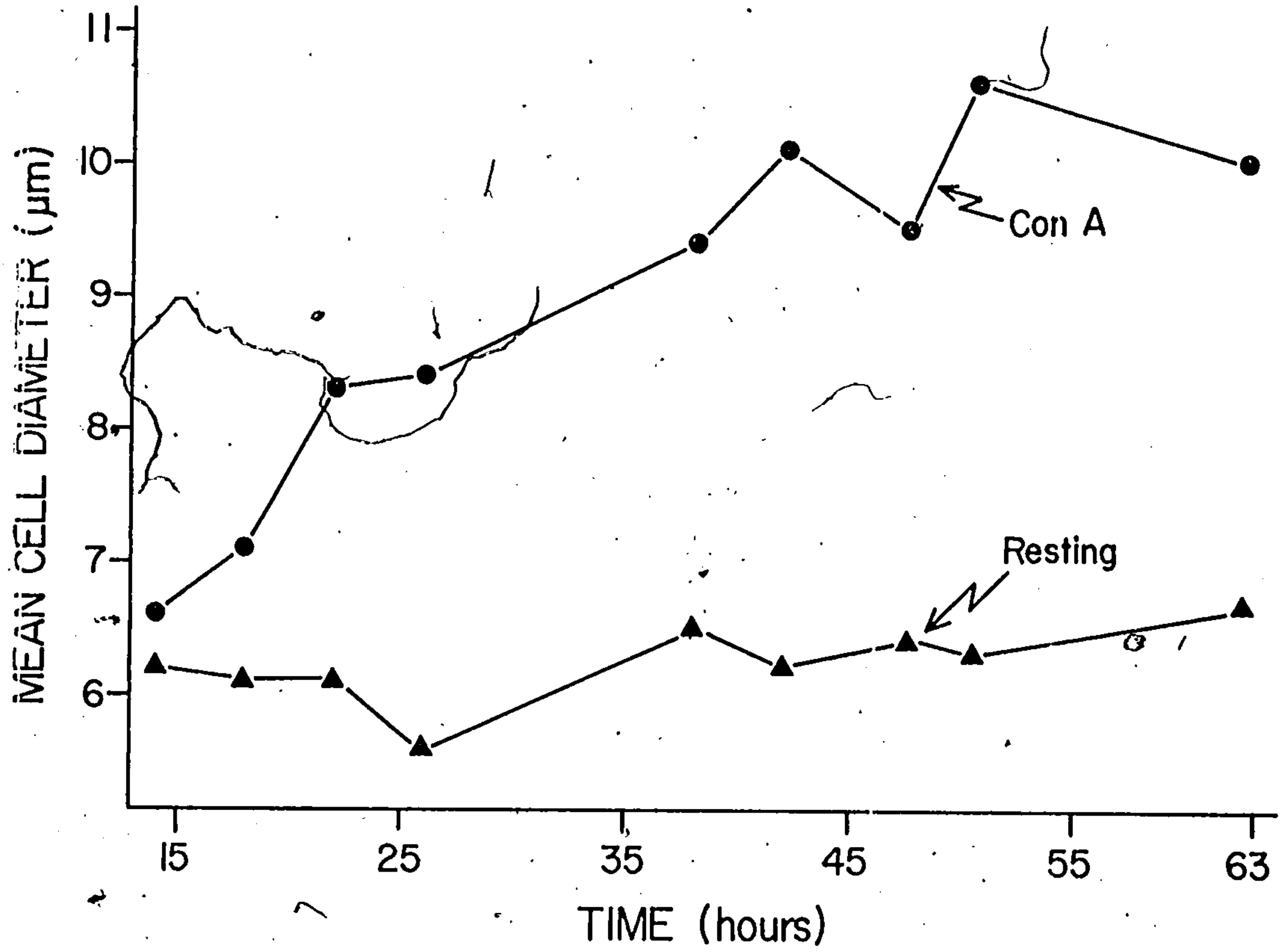
The degrees of activation measured by this method and by using the 30 minute time point on Figure 3 were similar, which justified the use of the 30 minute uptake points for the assessment of activation. That the rates were so different emphasizes the contribution of backflux, even as early as 30 minutes. In later experiments, the 2.5 hour assay was used, since activation can be assessed reasonably accurately by this method. Rates determined from these assays are intended for comparison of treatments; they are not absolute.

It was important to determine whether the activation of influx was due to an activation of transport activity, or to an increase in the number of transport units, associated with an increase in cell surface area.

3.1.3 Cell Size.

Cell size was measured in resting and Con A-stimulated cultures. Figure 6 shows mean cell diameter in these two treatments with time after initiation of culture. A mean diameter of 6.2 µm was measured for resting cells during the first 14 hours of culture; the mean cell diameter for Con A-stimulated cultures at 14 hours was 6.5 µm. Thereafter, the mean cell diameter for stimulated cells increased considerably, reaching a value of almost 11 µm after 48 hours. These mean values tend to obscure the fact that certain of the cells in the 48 hour cultures were as large as 20 µm in diameter, while there always remained a population of cells whose diameters were in the 6-8 µm range. However, since all other measurements were from populations which were similarly heterogeneous, these mean values are appropriate.

Figure 6. Mean diameter for both resting and Con A-stimulated cells with time after initiation of culture. Results from one experiment are shown. At least 50 cells were counted for each point. Cell diameters were measured using a calibrated eyepiece.



MEAN CELL DIAMETER (μm)
11
10
9
8
7
6
Con A
Resting
15
25
35
45
55
63
TIME (hours)

The mean cell diameter in stimulated cultures was only 1.04 times that of resting cells at 14 hours after initiation of culture, rising to only 1.16 times at 20 hours. Assuming that the number of transport sites/mm^2 of cell surface remains constant, the almost 2-fold activation of potassium influx which occurs at 6-8 hours cannot be ~~due to~~ an increase in the number of transport sites per cell which would result from the approximately 1.1-fold increase in cell surface area. Were potassium influx to have been measured at later times (such as 48 hours), much of the increase in influx per cell might well result from such a surface area increase.

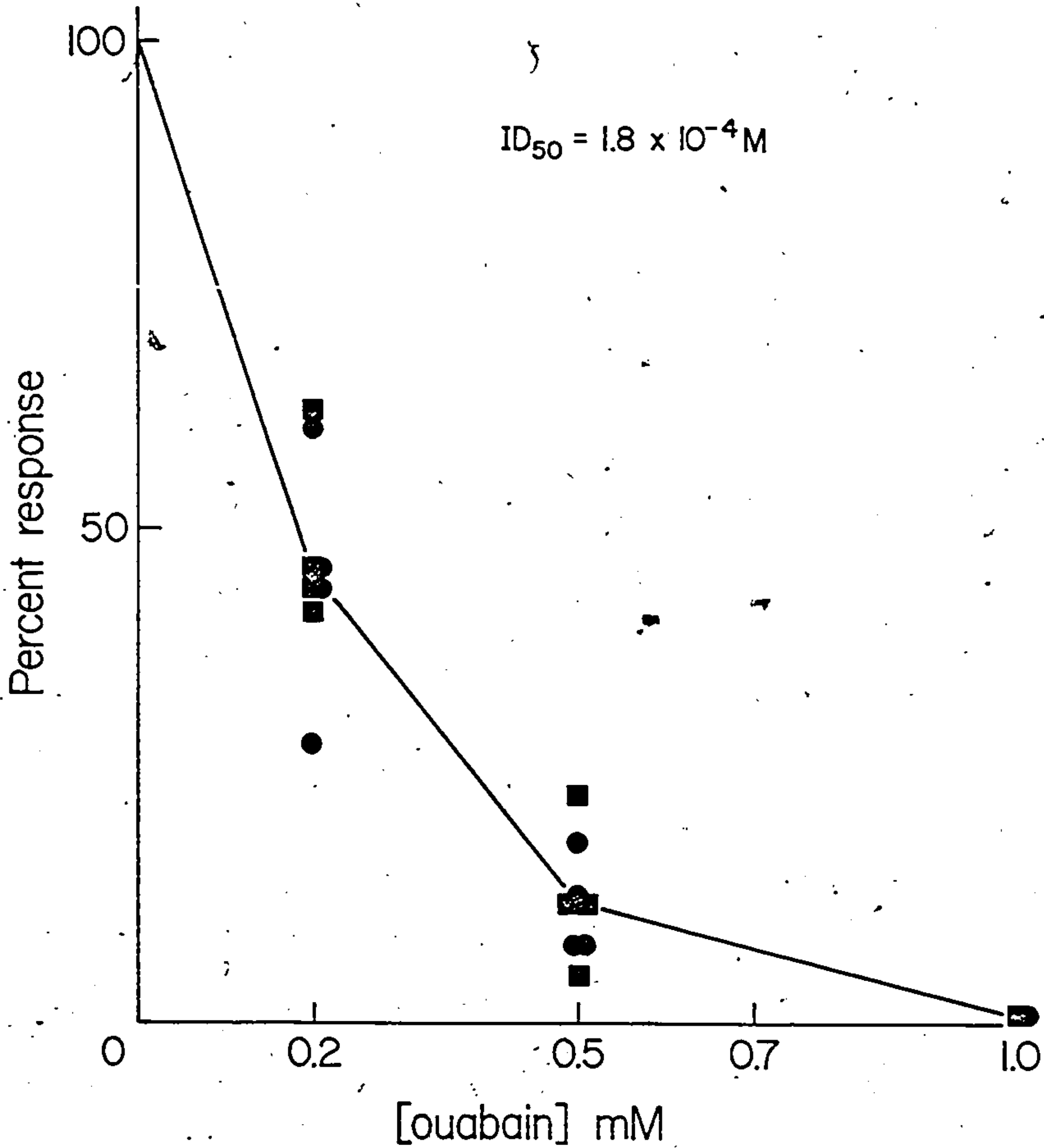
3.1.4 Ouabain Sensitivity

In order to determine the contribution of the (Na^+,K^+)-ATPase to this activation of influx, the inhibition of influx by ouabain was measured. Ouabain-insensitive influx was defined as that which could be detected in the presence of 1 mM ouabain. This represented about 20% of resting influx, and about 10% of Con A-stimulated influx; Con A did not affect ouabain-insensitive influx greatly. Subtraction of this from total influx gave ouabain-sensitive influx. Most of the Con A-activated influx was ouabain-sensitive, as can be seen by comparing Figures 3 and 9.

Figure 7 shows the dose-dependent inhibition by ouabain of (Na^+,K^+)-ATPase-mediated potassium influx into Con A-

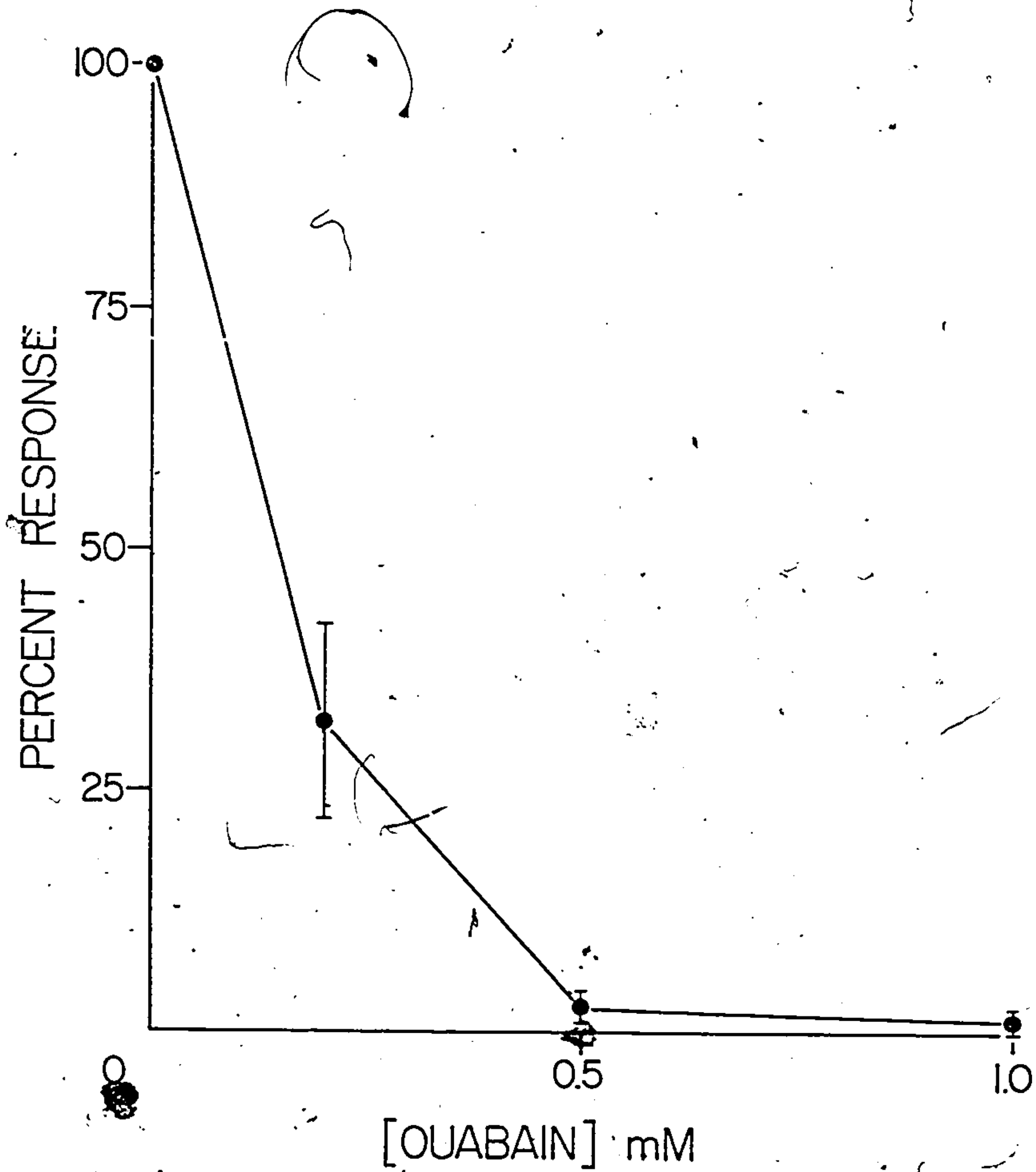
stimulated and resting splenocytes. Influx was measured at 10 and at 45 hours after initiation of culture, data from both times are included. The dose-dependence of the inhibition was the same at both times; the ID50 for inhibition was 1.8 X 10^{-4} M ouabain. Con A-activated potassium influx was reduced to resting levels by 3-4 X 10^{-4} M ouabain; this concentration abolished the subsequent activation of ^{3}H-thymidine incorporation.

Figure 7. Ouabain-sensitive uptake of $^{86}Rb^{+}$ into resting (■) and Con A-stimulated (●) Balb/c splenocytes, vs. concentration of ouabain in the medium. Uptake is expressed as a percentage of the uptake in the absence of ouabain. Uptake was measured at 10 and 45 hours after the initiation of culture, one point for each of two experiments is shown - there was no correspondence between the degree of inhibition and the time of its measurement.



Ouabain-sensitive 86Rb uptake
ID50 = 1.8 x 10-4 M
100
50
0
Percent response
0.2
0.5
0.7
1.0
[ouabain] mM

Figure 8. Incorporation of ^{3}H-thymidine into Balb/c splenocytes which had been incubated with Con A, and ouabain at various concentrations, for 45-48 hours. Incorporation is expressed as a percentage of that into cells cultured in the absence of ouabain, and plotted against the concentration of ouabain in the medium. Results from 3 experiments are combined, the bars represent standard deviations.



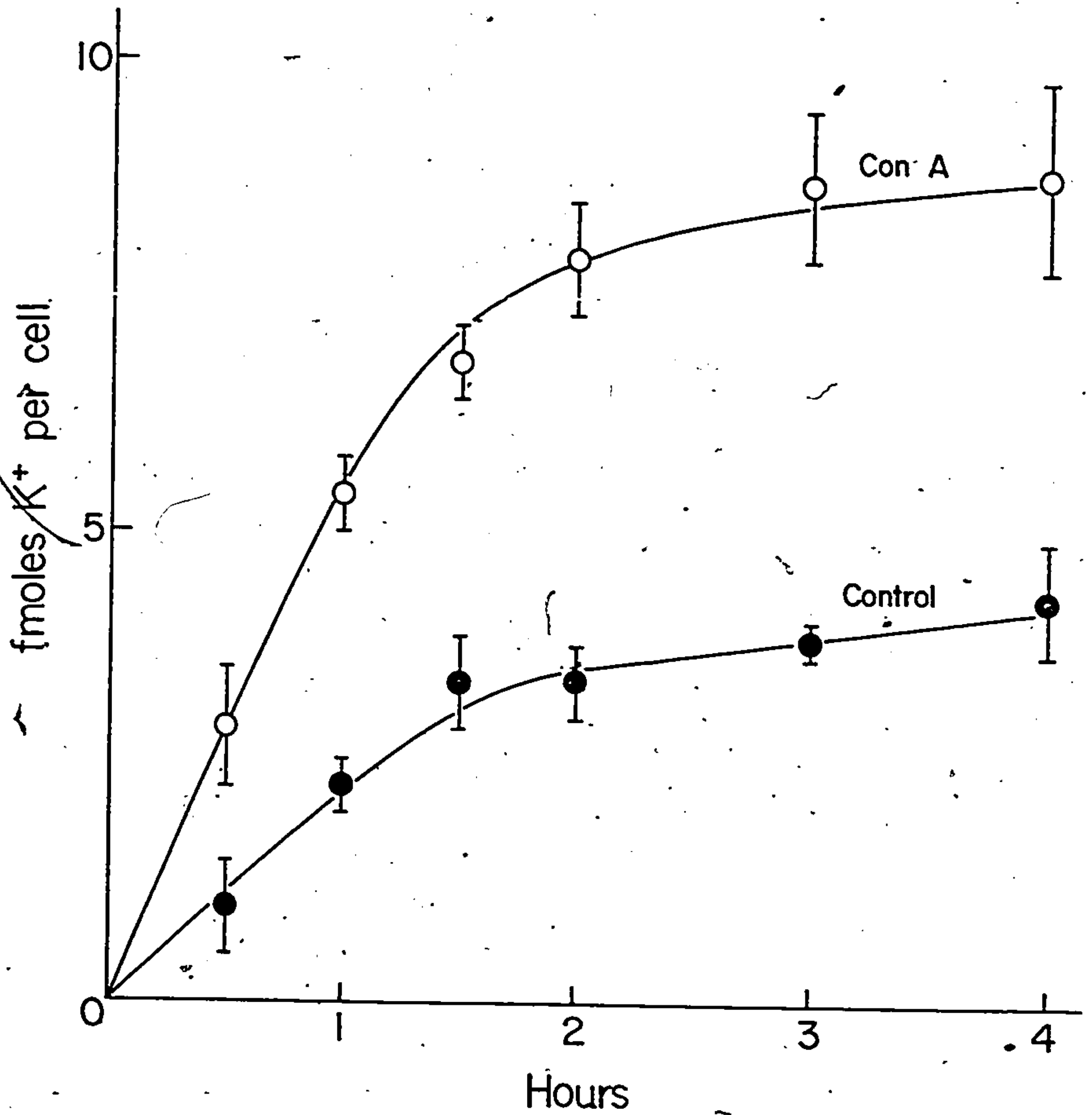
PERCENT RESPONSE
100
75
50
25
0
0.5
1.0
[OUABAIN] mM

In Figure 8, the dose-dependent inhibition by ouabain of ^3H-thymidine incorporation (measured at 48 hours) is shown. The ID50 for this inhibition was almost identical to that for the inhibition of potassium influx, at 1.4×10^{-4} M ouabain. These ID50 values are similar to those reported by Quastel & Vogelfanger (1971).

That the ID50 for both inhibitions was so similar suggests that the inhibition of ^3H-thymidine incorporation, assayed at 48 hours, was causally related to the inhibition of potassium influx at 10 hours. That is, ouabain, through its effect on potassium influx activation mediated by the sodium pump, inhibited the later activation of DNA synthesis.

In Figure 9, ouabain-sensitive potassium influx into resting and Con A-stimulated splenocytes is shown. Influx was measured between 10 and 14 hours after Con A addition. Using the 30 minute point as an estimator, it can be calculated that Con A increased ouabain-sensitive net potassium influx from 2.2 fmoles/cell.hr. in resting cells to 5.9 fmoles/cell.hr. in activated cells, a 2.7-fold increase.

<u>Figure 9</u>. Ouabain-sensitive potassium uptake into Balb/c splenocytes, both resting and stimulated with Con A for 10-14 hours. Results from 6 experiments are combined, bars represent standard error of the mean.



fmoles K⁺ per cell
10
5
0
Con A
Control
1
2
3
4
Hours

Table 1.

Reversibility of Ouabain Inhibition

[Ouabain]	Percent Response			
	^{86}Rb uptake		^{3}H-thymidine Incorporation	
(mM)	3 hours pre-wash	10-12 hours post-wash	3 hours pre-wash	17-21 hours post-wash
0	100	100	100	100
0.2	65 , 47	81 , 94	19 , 29	121 , 117
0.5	28 , 15	30 , 50	1.3, 2	43 , 44
1.0	3 , 12	17 , 20	0.5, 0.5	0.5, 2

Cells were incubated with Con A and ouabain for 48 hours, then washed 3 times with RPMI 1640, and resuspended in medium containing Con A.

Results from 2 experiments are shown, each was performed in triplicate.

3.1.5 Reversibility of Inhibition

Inhibition by ouabain of Con A stimulation is reversible, as shown in Table 1. Potassium uptake and thymidine incorporation were measured after 45 hours of culture. The responses to Con A of cells incubated with various concentrations of ouabain are expressed as a percent of the uninhibited response. At 48 hours, the cells were washed three times and resuspended with Con A. Potassium uptake was measured 10-12 hours after the wash, thymidine incorporation was measured 7-9 hours later. The degree of inhibition at all concentrations was much reduced by this wash; in the case of 0.2 mM ouabain, reversibility was complete. The inhibition due to ouabain therefore required the continuous presence of the drug.

There was one exception to this finding. The incorporation of ^{3}H-thymidine by cells which had been cultured with Con A and 1 mM ouabain for 48 hours did not recover following washing. They did however recover an appreciable proportion of their potassium uptake. This suggests that an irreversible change in some system essential for entry into S-phase, other than membrane cation transport, was induced by this treatment.

Table 2.

Activation of Potassium Efflux by Con A

Treatment	Time of Assay (hr)	Slope of Efflux Line (hr^{-1}) Mean ± SD	Efflux (fmoles/cell.hr.) Mean ± SEM	Number of Experiments
Resting	0	0.46 ± 0.12	6.42 ± 0.6	9
Con A	0	0.46 ± 0.14	6.42 ± 0.65	9
Con A	14	0.66 ± 0.07	10.6 ± 0.4	9
Con A; Mitogen removed	14	0.51 ± 0.1	8.1 ± 0.7	6

Slopes of efflux lines were calculated by libear regression: correlation coefficients were all greater than 0.9.

Efflux values were calculated from the slopes by their multiplication by intracellular potassium content, taken from Table 3 (see Segel & Lichtman, 1976).

3.2 ACTIVATION OF EFFLUX

Con A also enhanced efflux of potassium from splenocytes. This efflux was totally ouabain-insensitive, as befits a leak flux. Table 2 shows that the rate of tracer loss from cells that had all been preloaded for 14 hours with $^{86}Rb^{+}$ was increased 1.43-fold by Con A. Efflux was calculated from the slopes of fractional loss regression lines, (similar to those for influx, Figure 4), by the method of Segel & Lichtman, 1976. Resting cell efflux was 6.42 +/- 0.36 (SEM) fmoles/cell.hr., which was activated by Con A to 10.6 +/- 0.45 (SEM) fmoles/cell.hr. When slopes (hr^{-1}) were converted to efflux (fmoles/cell.hr.), the degree of activation of efflux became greater, due to the contribution of intracellular potassium content (which is greater in stimulated cells; Table 3) to the calculation. Efflux in resting cells was not significantly different ($p=0.3$) from resting influx (Figure 5). Influx and efflux are equal in resting cells (net flux = 0). That they were measured as equal therefore justifies the methods used for measurement (especially of influx; the efflux assay is not subject to backflux.

The activation of efflux by Con A was not seen if the assay was carried out directly after Con A addition, but was fully evident 14 hours later. Efflux was not measured at any intervening time.

Cells which had been preloaded with isotope in the presence of Con A had a higher isotope content after the 14 hour

loading period than did resting cells. If Con A was not included in the washing medium, an insignificant (p=0.3) increase in the slope of the efflux line was seen. Even when the slopes were converted to efflux, the resultant increase in the degree of activation, due to the higher potassium content of 14 hour Con A-treated cells (see above), was only significant at the p=0.1 level. The increase measured in the presence of Con A was significant at the p=0.001 level. Activated efflux in these cells was therefore due to the presence of Con A, and not to their higher isotope content.

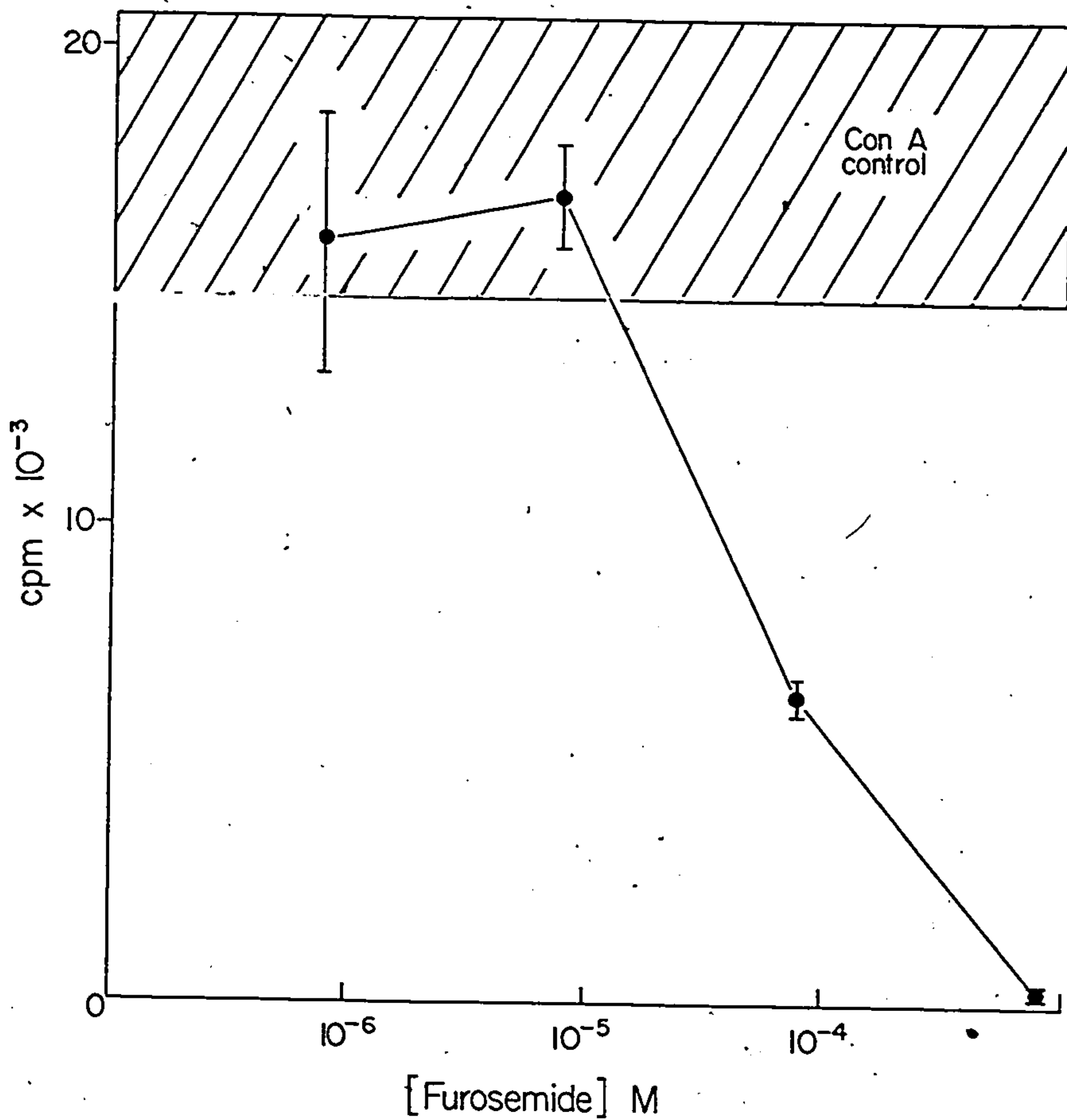
The degree of activation of efflux by Con A was similar to, but slightly less than that of, influx (1.66 fold vs. 1.7-1.85 fold). The activated rates (influx and efflux) were significantly different from each other (p=0.05).

3.3 FUROSEMIDE

Figure 10 shows the dose dependent inhibition by furosemide of ^{3}H-thymidine incorporation into splenocytes incubated with Con A and furosemide for 68 hours. Con A stimulation was completely inhibited by 1 mM furosemide - this same concentration reduced Con A-activated potassium influx to the resting cell level (data not shown). This indicates that there is a component of the activated influx, required for entry into S-phase, which is furosemide-sensitive. The ouabain-sensitivity of this component was not determined; neither was the effect of furosemide on efflux measured. Aver-

dunk & Gunther (1980B) have confirmed the inhibition by furosemide of Con A activation of human lymphocytes.

Figure 10. Incorporation of ^{3}H-thymidine into Balb/c splenocytes which had been incubated with Con A and furosemide for 64 hours. Incorporation is expressed as a percentage of that into cells incubated in the absence of furosemide, and plotted against the concentration of furosemide in the medium. Results from 1 experiment are shown, bars are standard deviations.



20
10
0
cpm x 10^{-3}
Con A
control
10^{-6}
10^{-5}
10^{-4}
[Furosemide] M

Table 3.

Potassium Contents of Resting and Con A-stimulated Balb/c splenocytes

Time in Culture (hr)	Potassium Contents (fmoles/cell) Resting Cells (duplicates)	Con A-stimulated Cells (mean ± SEM of 4 replicates)
1.5	14.2 14.2	14.7 ± 0.4
5	14.2 14.6	14.05± 0.25
10	14.6 12.9	15.6 ± 0.3
14	13,6 12.5	16.3 ± 0.65

Mean Resting cell Potassium Content = 13.96 ± 0.2 (SEM) fmoles/cell.

Mean Con A-stimulated Potassium Content at 10 and 14 hours = 16 ± 0.35 fmoles/cell.

The 10 and 14 hour values are not significantly different ($p=0.3$).

Con A-stimulated and Resting Contents were significantly different ($p=0.01$).

Amiloride also inhibited ^{3}H-thymidine incorporation in a dose-dependent manner, but was quite toxic to the cells (Owens & Kaplan, 1979).

It was important to determine whether these changes in potassium fluxes produced any change in the intracellular ionic environment, especially potassium concentration. For this reason, intracellular potassium content was measured.

3.4 INTRACELLULAR POTASSIUM CONTENT

Table 3 shows the potassium content (fmoles/cell) of resting and Con A-stimulated lymphocytes. Contents were measured by flame photometry, as described in Materials & Methods. During the first 5 hours of culture, there was no significant difference in this parameter between resting and Con A-activated cells. A mean potassium content of 14.0 +/- 0.2 (SEM) fmoles/cell was measured. When measured 10 and 14 hours after the addition of Con A, potassium contents were significantly ($p=0.01$) higher than in resting cells, with a mean value of 16 +/- 0.35 (SEM) fmoles/ cell.

However, because of the small increase in cell diameter (and therefore in cell volume) which has occurred by this time, there is no change in $(K^+)c$. Potassium content was converted to concentration by assuming a cell to be a smooth sphere, 78% of whose volume is water (Segel & Lichtman, 1976). $(K^+)c$ for resting cells was calculated as 140.7 +/- 7 mM; $(K^+)c$ for Con A-stimulated cells was 142.7 +/- 3mM.

There was no appreciable change in the intracellular concentration of potassium resulting from the flux activations.

3.5 SPECIFICITY OF FLUX ACTIVATIONS

All of the above determinations involved unfractionated and therefore heterogeneous populations of splenocytes. All parameters measured so far are expressed per cell present, even though all of these cells may not participate in the activation events. It was important therefore to determine the specificity of the activation by Con A of potassium fluxes. In particular, it was necessary to know whether only the cells activated into mitosis showed the flux activations, or whether fluxes were activated in all cells which bind Con A.

3.5.1 Proliferative Response

To answer this question, cells from the spleens of athymic (nude) mice were used. Con A binds to all these cells (Greaves & Janossy, 1972), but does not activate them. Table 4 shows the proliferative response of nude splenocytes to Con A and to LPS. While these cells responded excellently to LPS, their proliferative response was in fact depressed by Con A. Con A had no effect on their viability.

3.5.2 Potassium Influx

Figure 11 shows the effects of Con A and LPS on potassium influx into cells from RNC nu/nu and Balb/c spleen. This is total influx, as was shown in Figure 3. While Con A caused a doubling of potassium influx into Balb/c cells (curve B, measured at 14-18 hours), it had no effect on influx into nude spleen cells (curve A, measured at 8-16 hours). LPS, however, activated potassium influx into nu/nu cells by about 1.4-fold (curve C) ; this response was significantly different (p=0.02) from the resting response (curve A). LPS had no effect on potassium influx into Balb/c cells (Curve A, measured at 8-16 hours); neither had it any activating effect on DNA synthesis; the mean stimulation of ^{3}H- thymidine incorporation was threefold in 6 experiments.

Table 4.

Effects of Con A and LPS on ^{3}H-thymidine Incorporation by nu/nu splenocytes

Experiment Number	Assay Time (hr)	Resting cpm ± SD	Con A cpm ± SD	Con A SI	LPS cpm ± SD	LPS SI
1.	46.5	5,950 ± 700	1,640 ± 80	0.3	78,000 ± 6,600	13
2.	53.5	6,700 ± 940	1,700 ± 240	0.25	55,100 ± 4,100	8
3.	71	4,280 ± 300	1,700 ± 300	0.35	266,000 ± 49,000	60

SI (Stimulation Index) = (cpm in mitogen-treated cells)/(cpm in resting cells).

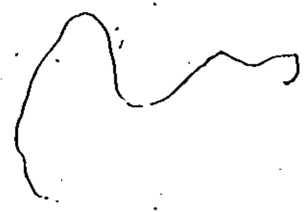
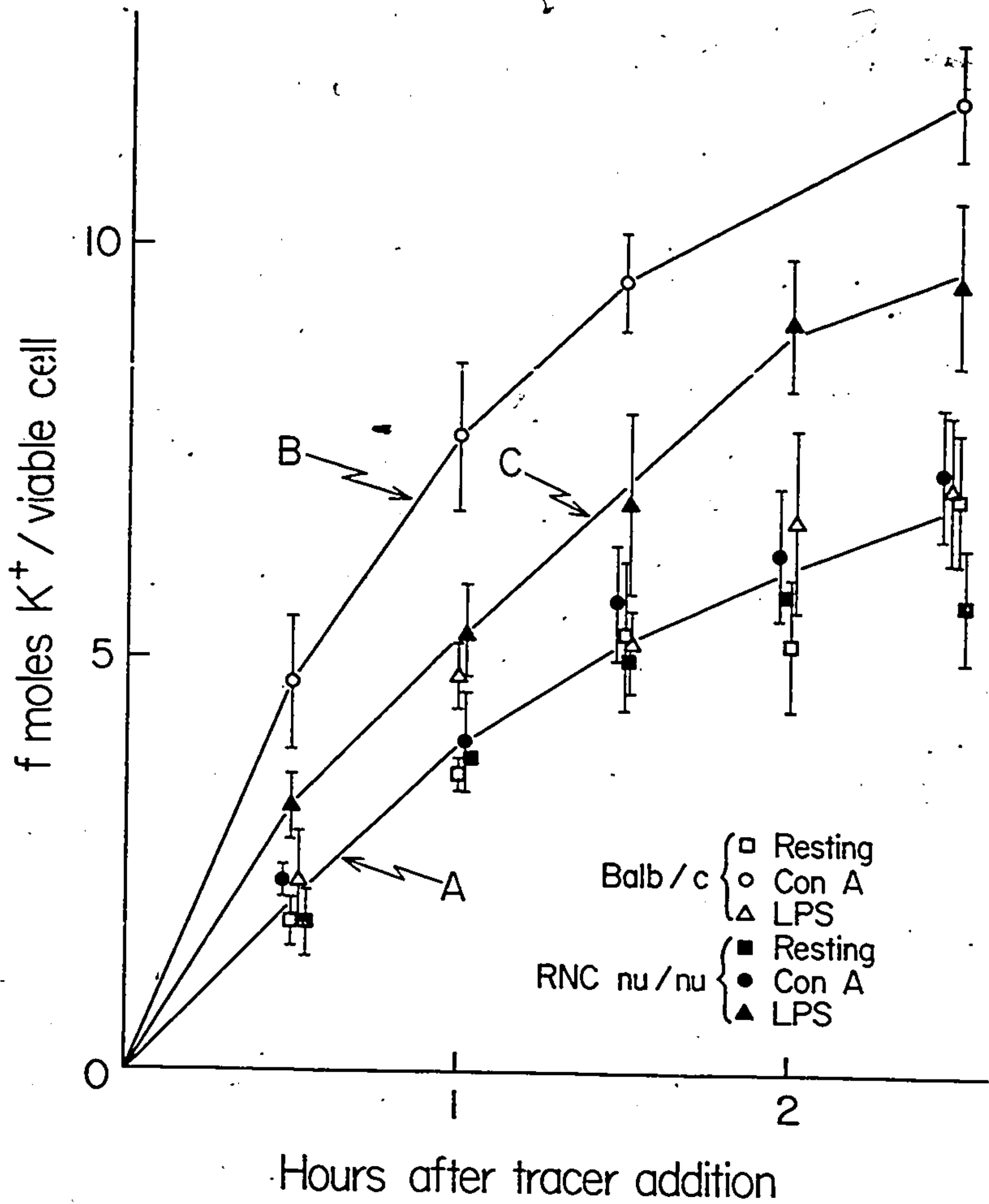


Figure 11. Potassium uptake by cells from RNC nu/nu and Balb/c spleen. (A): Potassium uptake by Balb/c cells, either resting (□), or incubated with LPS for 8-16 hours (Δ), and by nude spleen cells, either resting (■), or incubated with Con A for 8-16 hours (●). (B): Uptake by Balb/c cells which were incubated with Con A for 8-16 hours (○). (C): Uptake by nude spleen cells which were incubated with LPS for 8-16 hours (▲). Combined results from 3 experiments are shown, bars represent standard error of the mean.


f moles K+ / viable cell
10
5
0
1
2
Hours after tracer addition
B
C
A
Balb / c
Resting
Con A
LPS
RNC nu / nu
Resting
Con A
LPS

The activation of potassium influx by a mitogen is therefore restricted to those cells which are stimulated to divide, and does not result simply from binding of the mitogen.

This experiment was repeated using Balb/c cells which had been fractionated into B- and T-enriched populations. This fractionation was accomplished using WGA, by the method of Bourguignon et al (1978). The purity of these populations was assessed by anti- Ig immunofluorescence; the T-cells were routinely 90-95% Ig-negative, while the B-cells were at least 70% Ig-positive. Table 5 shows the effect of Con A on ^{3}H-thymidine incorporation by these cells. There was usually an increase in both counts and stimulation index in the T-cells, and a decrease in both in the B-cells. It is important to note that there was not found routinely an enhancement for the T-cells or a diminution for the B-cells of the response which was proportional to their purification as assessed by immunofluorescence. Neither was there ever complete removal of response to Con A in the B-cell population.

Table 5.

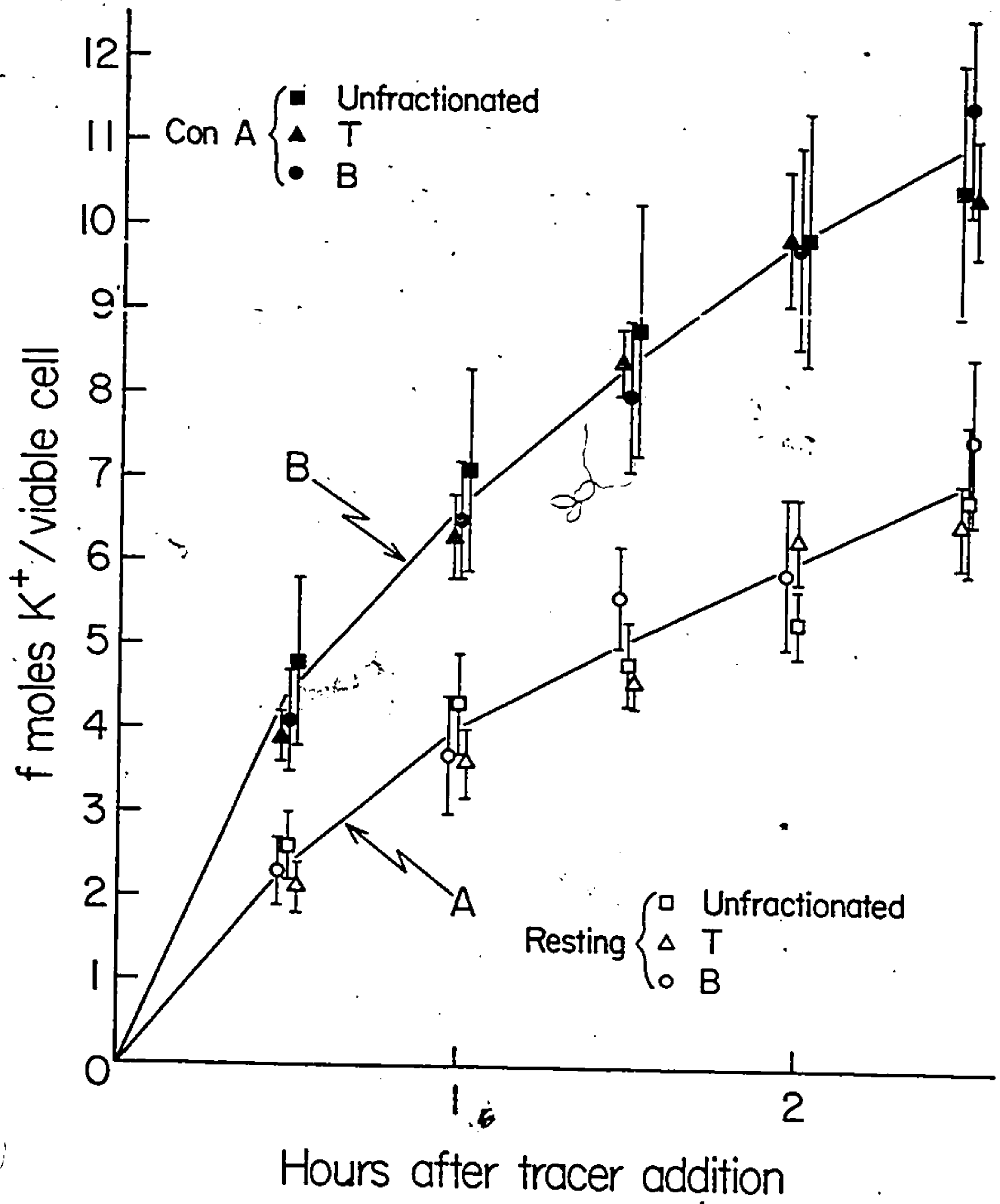
Effect of Con A on ^{3}H-thymidine incorporation by Balb/c splenocytes, both unfractionated, and separated by WGA.

Experiment Number	Time of assay	Stimulation Index		
		Unfractionated	T-Enriched	B-Enriched
1.	51.5	11	154	34
2.	67	45	148	7
3.	71.5	33	14	14
4.	70	NM	43	6
5.	48	NM	10	4

Stimulation Index = (cpm in mitogen-treated cells)/(cpm in resting cells).

NM = not measured.

Figure 12. (A) (open symbols): potassium uptake by resting Balb/c splenocytes which are unfractionated (□), and separated (using WGA) into T-enriched (△) and B-enriched (○) populations. (B) (closed symbols): Potassium uptake by replicate samples of the cell populations in (A) which had been incubated with Con A for 14-18 hours. Results from 6 experiments are combined, bars represent standard error of the mean.

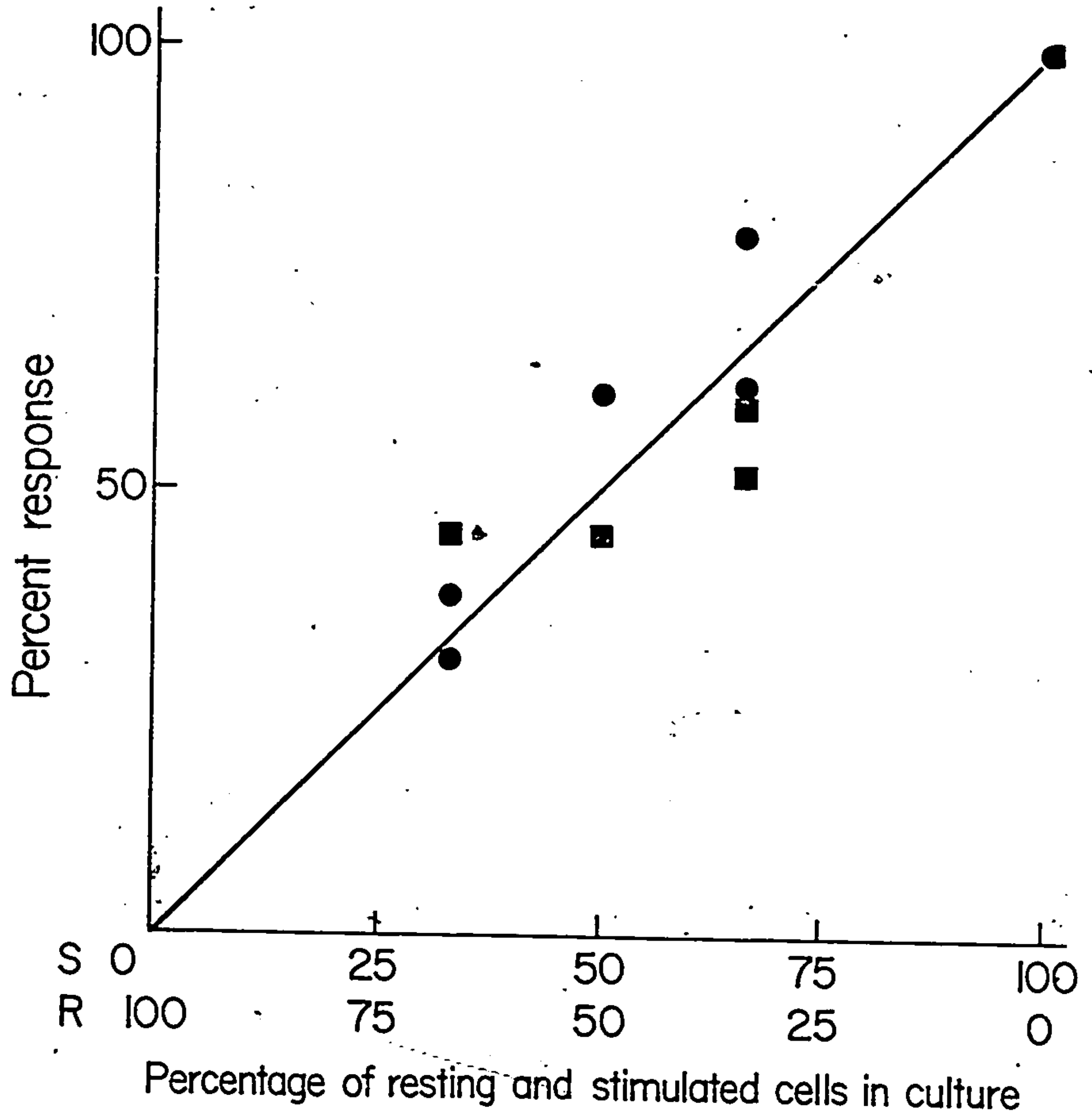


f moles K+/viable cell
Hours after tracer addition
Con A
Unfractionated
T
B
Resting
Unfractionated
T
B
A
B
0
1
2
3
4
5
6
7
8
9
10
11
12
1
2

Figure 12 shows that Con A activates potassium influx into these populations to an extent indistinguishable from that into unfractionated Balb/c cells. In all 3 populations, a 1.85-fold increase in total influx was observed. Pretreatment of nu/nu cells with WGA and N-acetyl D-glucosamine did not cause any activation by Con A of potassium flux in these cells. The results shown in Figure 12 are not therefore an artifact of the fractionation procedure.

It was important to ensure that the influx and thymidine incorporation assays were capable of detecting differences in the number of responding cells, such as were expected as a result of the WGA fractionation. Results of experiments to test this are shown in Figure 13. WGA-enriched T-cells and nu/nu cells were cultured with Con A, separately; there was no co-culture. Immediately prior to assay (potassium influx at 14-18 hours, ^{3}H-thymidine incorporation at 48 hours), they were mixed in various proportions, so as to dilute the T-cells with non-responding nude cells. There was observed a reduction in the percent response almost exactly proportional to the percent T-cells present. The line in Figure 13 is the response predicted for a dilution of the observed response proportional to the number of activated cells.

<u>Figure 13</u>. Dilution of response in absence of co-culture. Uptake or incorporation into undiluted nude cells was arbitrarily set to zero percent, and that into Con A-stimulated T-cells to 100 percent response. Results from two experiments are shown for each isotope. (■): potassium uptake, 14 hours in one and 18 hours in the other experiment; (●): ^{3}H-thymidine incorporation, at 48 hours. S and R on the abscissa refer to the percentages of resting (nude) and stimulated (T) cells, respectively.



100
50
Percent response
S 0
R 100
25
75
50
50
75
25
100
0
Percentage of resting and stimulated cells in culture

Figure 12 then indicates that, despite the WGA fractionation, the number of responding cells in all three populations was the same. WGA fractionation never completely removed Con A response (DNA synthesis) from the B-population. A likely explanation for the results in Figure 12 is that the contaminating T-cells were activated by Con A, and then secondarily activated the B-cells. To test this possibility, a model system was devised. Cells from RNC nu/nu spleen, by themselves devoid of Con A response, were mixed in varying proportions, at constant cell density, with cells from RNC nu/+ spleen, that had been enriched for T-cells. Con A was added to these mixtures and potassium uptake (at 14-18 hours) and DNA synthesis (at 48 hours) measured.

3.6 SECONDARY ACTIVATIONS

3.6.1 Potassium Influx

Table 6(A) contains the results of a representative experiment measuring potassium uptake into these mixed cultures. The uptake period used was 1 hour. In figure 14, the uptake data from a number (shown in parentheses) of experiments have been combined, and presented as the ratio of the uptake observed (cpm/pellet, after a one-hour incubation) to that expected were there to be no interaction between the two populations (see Table 6A). This latter would be the sum of 1); the proportional response of a population whose potassium uptake has been activated 1.75-fold, and 2); the propor-

tional response of a population whose potassium uptake was unaffected by Con A i.e. a resting response. A ratio of unity, shown by the dashed line, indicates absence of interaction. By definition, this ratio applies to the unmixed populations. While the maximal activation is 1.75-fold, the maximal observed/expected ratio is less than that, since a culture containing no Con A-responsive cells cannot be activated.

Line a) in figure 14 shows the response to Con A of cultures of T-cells, mixed with nu/nu cells that had been irradiated with ultraviolet light (84 ergs/mm2) before mixing. This irradiation completely abolishes the capacity of a cell to respond to any mitogen, without affecting its viability (Castellanos, 1980). Such cultures showed only the activation of potassium influx that would be expected from the T-cells present.

When the T-cells were mixed with nu/nu cells that had not been irradiated (i.e. were responsive to LPS), response ratios greater than unity were observed (Figure 14, line b); Table 6A). These ratios increased until the proportion of T-cells in the mixtures fell to below 10%. There was thus considerable interaction between the cells in these mixed cultures. An activation of potassium uptake greatly in excess of that expected on the basis of the Con A- responsiveness of the added cells was produced. The combined data in figure 14 indicate that as long as there were more than 10%

T-cells in the mixtures, an approximately maximal response to Con A ensued. The observed/expected ratios predicted for the maintenance of a maximal response as the T-cells are reduced would fit on a line that joins, roughly linearly, unity and 1.75; the data points also fit such a line.

3.6.2 Thymidine Incorporation

The results of a typical experiment measuring ^{3}H-thymidine incorporation in response to Con A in these T/nude cell mixtures are shown in Table 6B. The combined results from a number of such experiments are shown in Figure 15. Responses are expressed as a percentage of the T-cell response. In line a), the squares show the response of mixtures of nu/nu cells which had been UV-irradiated before mixing, and T-cells which were untreated. There was a co-linear dilution of the response with dilution of the T-cells. The line drawn corresponds to that in Figure 13, and represents the predicted response were there to be no interaction. In line b), the triangles show the response of T-cells that were diluted without the addition of nu/nu cells; the cell density in these cultures decreased with the percent T-cells. The reduction in response, although similar to that in line a), was more severe. This reflects an effect of cell density on Con A activation.

In line c), the circles show the response of T-cells mixed with untreated nude spleen cells. As in line b), Figure

14, there was no appreciable reduction of the response below that of a pure T-cell culture until the proportion of T-cells was reduced to less than 10%.

There was therefore an interaction between the two populations which resulted in both excess potassium uptake and DNA synthesis in response to Con A (without Con A, no response at all was observed since these were syngeneic cells). This interaction did not occur if the nude cells were inactivated before mixing.

Table 6.

Cell Interaction in Potassium Influx and DNA synthesis

Percent T-cells in mixture	(A) Potassium Influx (fmole/cell.hr.)			(B) ^{3}H-thymidine Incorporation (cpm)		
	Observed	Expected on basis of dilution	O/E	Observed	Expected on basis of dilution	O/E
100	8.2 ± 0.6		1.00	16,000 ± 3,600		1.0
50	8.1 ± 0.4	5.90	1.36	17,600 ± 3,500	8,300	2.1
25	6.9 ± 0.1	4.75	1.45	21,000 ± 900	4,450	4.7
17	5.5 ± 0.2	4.40	1.25	18,000 ± 1,600	3,218	5.6
12.5	5.1 ± 0.3	4.20	1.21	12,700 ± 3,100	2,525	5.0
10	5.0 ± 0.4	4.06	1.23	12,000 ± 950	2,140	5.6
0	3.6 ± 0.2		1.00	600 ± 100		1.0

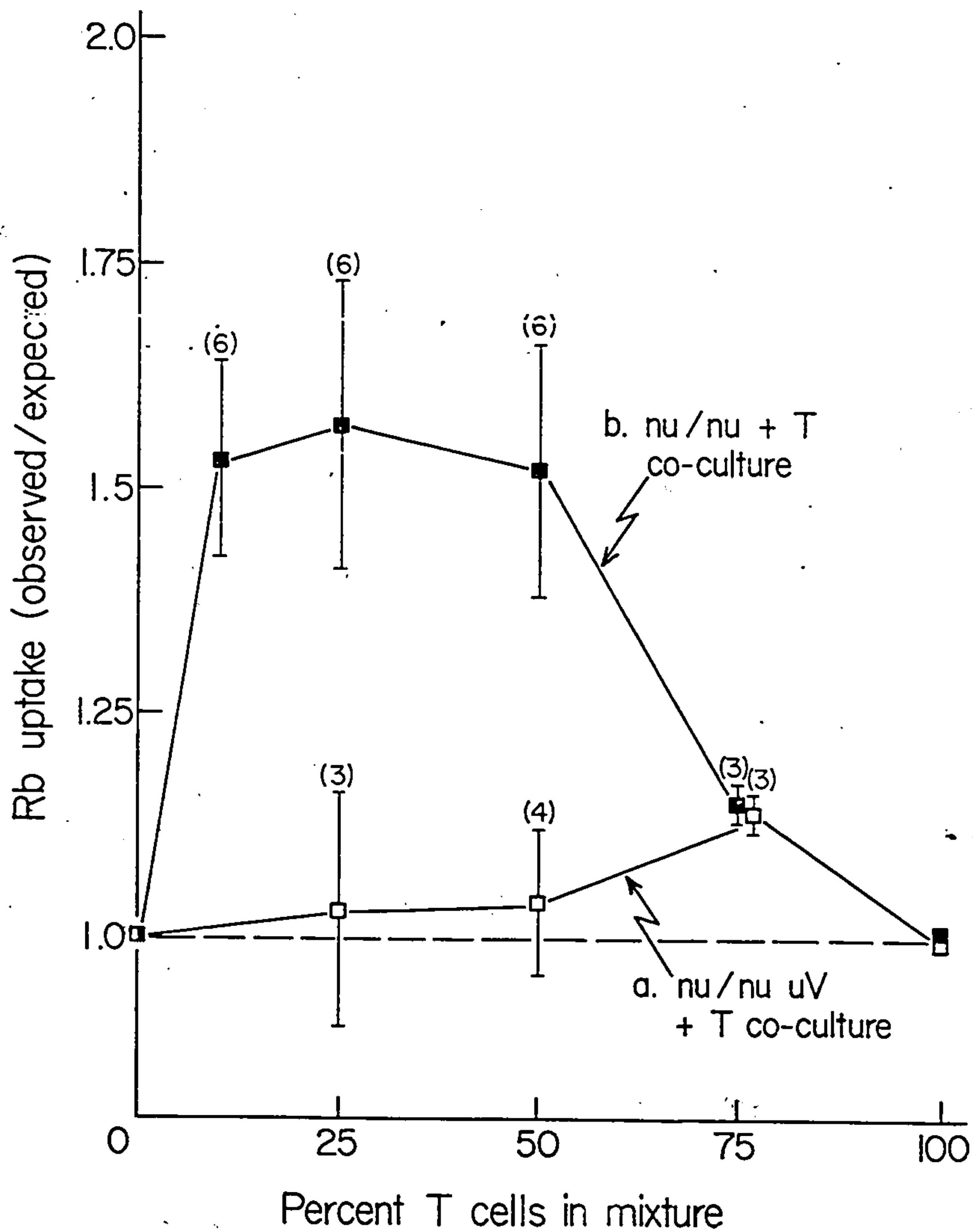
Values are means of triplicates ± SD.

Potassium Influx was measured 15 hours after addition of Con A.

^{3}H-thymidine Incorporation was measured 48 hours after addition of Con A.

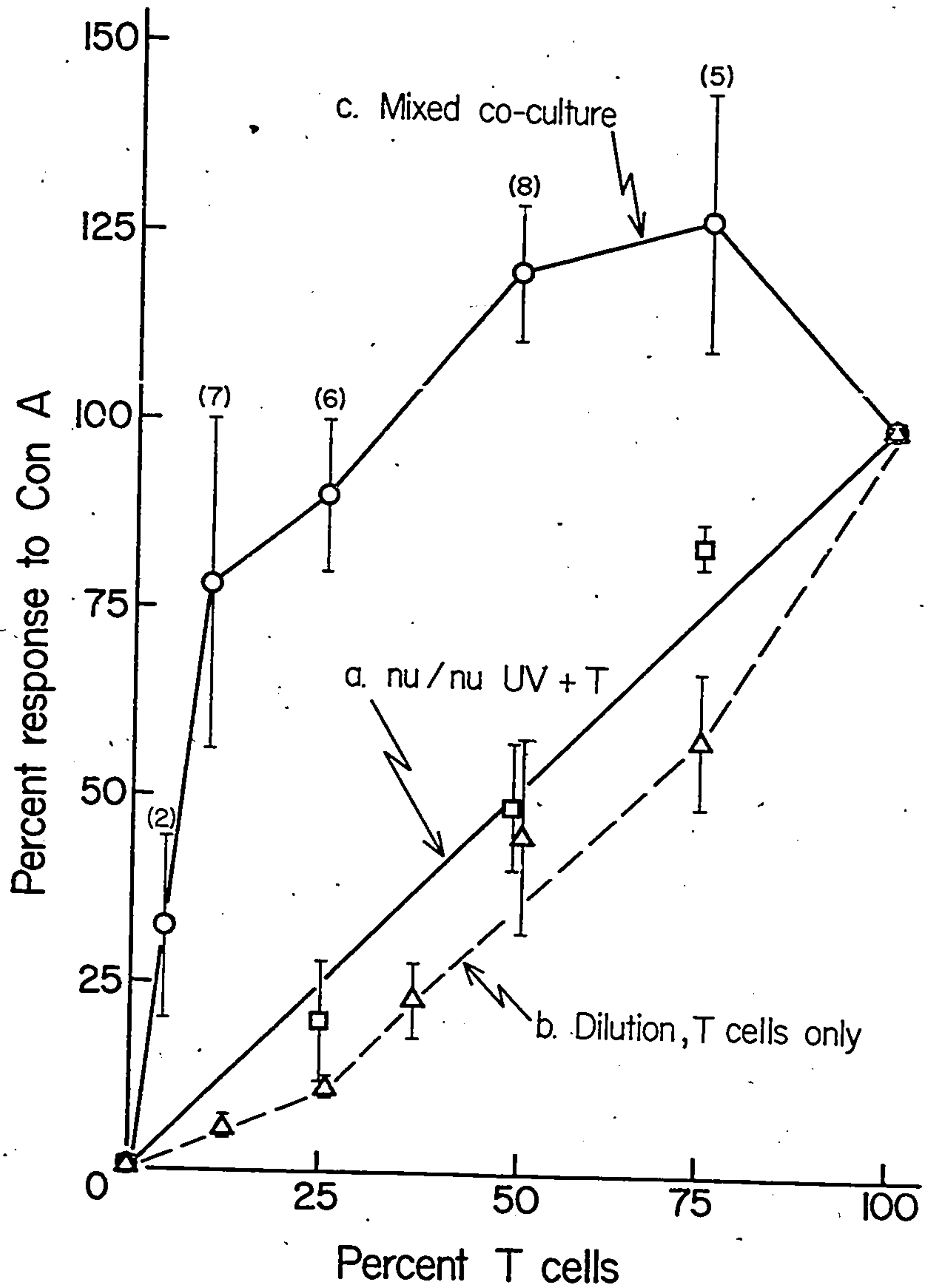
O/E : Observed response/response expected on the basis of dilution of T-cells with unresponsive B-cells. A ratio greater than unity indicates interaction between the cells.

Figure 14. Excess potassium uptake by mixtures of T-cells and nude cells, cultured with Con A for 15 hours. (a) (□): uptake ratios for mixtures of T-cells and nu/nu cells; the nude cells had been UV-inactivated before mixing. (b) (■): Response of mixtures of T-cells and nude cells, neither of which had been treated before mixing. The points represent the means of the numbers of experiments shown in parentheses. The bars show the standard error of the mean.



2.0
1.75
1.5
1.25
1.0
Rb uptake (observed/expeced)
(6)
(6)
(6)
(3)
(4)
(3)
(3)
b. nu / nu + T
co-culture
a. nu / nu uV
+ T co-culture
0
25
50
75
100
Percent T cells in mixture

Figure 15. ^{3}H-thymidine incorporation into mixtures of T-cells and nu/nu cells, measured 48 hours after addition of Con A, and expressed as a percentage of the T-cell response. (a) (□): The line drawn is that predicted were the response to Con A to diminish proportionally to the number of T-cells added to the mixture. The squares show the response of mixtures of nu/nu cells which had been UV-inactivated before mixing, and T-cells, at constant cell density. (b) (Δ): Response of cultures containing only T-cells, whose density was progressively reduced; these points represent experiments in which the cell density was not maintained constant, unlike those represented by lines a) and c). (c) (O): Response of mixtures of T-cells and nu/nu cells, neither of which were treated before mixing. Each point shows the mean of the number of experiments shown in the parentheses. The bars show the standard error of the mean.



Percent response to Con A
150
125
100
75
50
25
0
25
50
75
100
Percent T cells
c. Mixed co-culture
a. nu / nu UV + T
b. Dilution, T cells only
(2)
(7)
(6)
(8)
(5)

Table 7.

Effect of UV-irradiation of T-cells before mixing on the response to Con A of mixtures containing 20% T-cells, 80% nu/nu cells.

Experiment Number	^{3}H-thymidine Incorporation (cpm) Untreated T-cells	UV-irradiated T-cells	nu/nu cells	Mixed cultures	O/E
1.	10,150 ± 1,530	140 ± 30	380 ± 150	770 ± 240	2.4
2.	13,000 ± 990	170 ± 100	190 ± 50	710 ± 190	3.8
3.	16,800 ± 780	800 ± 100	570 ± 70	1,410 ± 520	2.2

O/E = Observed counts / Counts expected were there to be no interaction between the two populations.

Con A was added to all cultures; ^{3}H-thymidine incorporation was measured after 48 hours.

Values are means of triplicates ± SD.

3.6.3 Requirement for Active T-cells

That this interaction was mediated through T-cell activation, was shown by UV- irradiating the T-cells prior to mixing. In Table 7, the results of 3 experiments are shown. In T-cells, thymidine incorporation at 48 hours was reduced to resting levels by their prior irradiation. Nude cells of course did not respond to Con A. In a mixture containing 25% T-cells, there was almost no response above that expected on the basis of additivity. The interaction which did occur, indicated by the observed/expected ratios, was too small to be considered significant.

3.6.4 T-Cell Supernatants

In three experiments, supernatants from Con A-activated whole spleen cultures (collected 8-16 hours after addition of Con A) had no effect on the response of nu/nu cells, with or without added Con A. Similarly, neither supplementing the medium with IL-2 (a gift from Dr. K.A. Smith) nor with T-cell growth factor (prepared in our lab by Dr. P.K.Lai), had any effect on cultures of nu/nu cells, whether or not Con A was included in the medium.

3.6.5 Demonstration that nu/nu Cells respond

In some experiments, more blast cells were counted in mixtures of 10%T/90% nude cells, at 50-60 hours, than T-cells were originally added (as much as twice as many), suggesting

that much of the excess response came from nude spleen (Table 8). To show that the cells which contribute the excess response came from nude spleen, it was necessary to be able to discriminate in culture between cells from different spleens. The fact that the Y or male chromosome can be distinguished from the other 39 chromosomes in the mouse was exploited. Its centromere is not differentially stained with Hoechst dye 33258, whereas the other 39 are (Nesbitt & Francke, 1973; Latt & Wohlleb,1975). Plate 1,a), shows metaphase chromosomes from a male (RNC nu/nu) mouse. The Y chromosome, indicated by an arrow, is distinguishable from the others on the basis of its size (it is the third smallest), its lack of obvious banding, and the fact that there is no differential staining of the centromere. The other 39 chromosomes, and all 40 in Plate 1,b), showing chromosomes from a female, RNC nu/+ mouse, show a centromere which stains brighter than the rest of the chromosome. Many are also banded. Thus, metaphases from male and female mice can be distinguished.

Table 8.

Number of Blast Cells in mixtures containing 10% T-cells/90% nu/nu cells, which had been co-cultured with Con A for 2 days.

Experiment Number	Number of Cells Counted	Number of Blast Cells per mL of culture	Number of T-cells initially added
1.	50	4.3×10^5	2×10^5
2.	70	5.4×10^5	2×10^5
3.	55	2.5×10^5	2×10^5

Blast Cells were defined as cells greater than 8 um in diameter.

Blasts were counted between 48-70 hours after the addition of Con A.

Plate 1. a) Metaphase chromosomes from a male BNC nu/nu spleen. Cells had been cultured for 50 hours with LPS before preparation of this slide. The Y chromosome, whose centromere is not stained differently from the chromosome as a whole, is indicated by the arrow. b) Metaphase chromosomes from a female, BNC nu/+ spleen. Cells were cultured for 70 hours with Con A before preparation of this slide. All 40 chromosomes show a differentially-stained centromere. Final magnification is 1700 X.

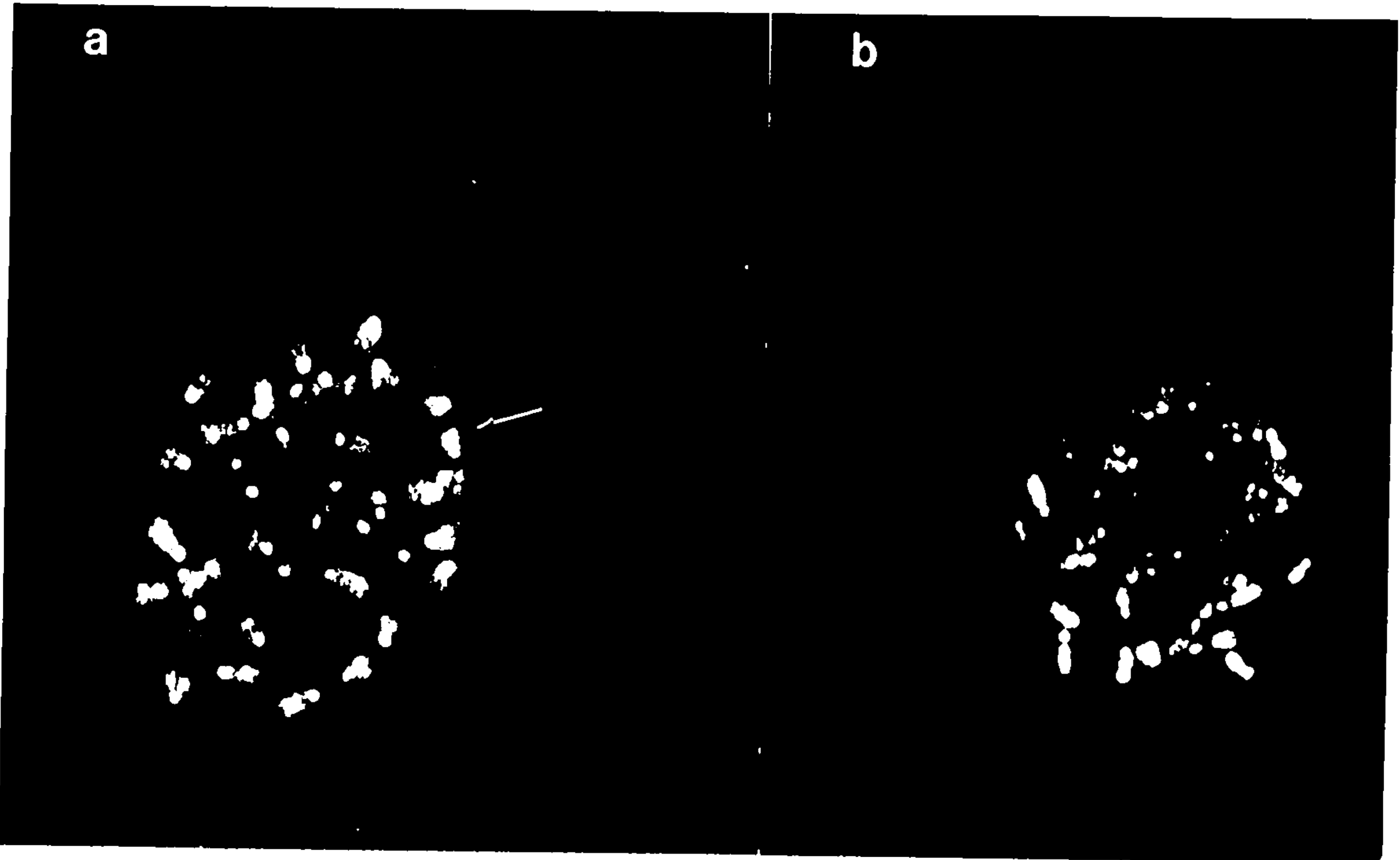

a
b

Table 9.

Identification of the Spleen of Origin of Metaphase Cells in Mixed Cultures of T-cells (from a female mouse) and nu/nu cells(from a male mouse), that were cultured with Con A for 2 days.

Percent T-cells in culture	Mitogen	Number of Metaphases counted	Percent Activated Cells from nu/nu spleen
100	Con A	50	4
50	Con A	63	32
20	Con A	75	40
10	Con A	75	59
0	LPS	50	100

Mitogens were added immediately after mixing, and metaphases were counted after 2 days.

T-cell/nude cell mixtures such as those decribed in Figures 14 and 15 were set up, with one difference; the T-cells came from a female mouse, and the nude cells came from a male mouse. After 2 days co-culture with Con A, metaphases were prepared and stained with Hoechst dye 33258. They were then scored as male or female, thus indicating their spleen of origin. Results of this scoring are shown in Table 9. It is clear that many of the responding cells were from nu/nu spleen. As the proportion of cells from nude spleen was increased, the proportion of metaphases from nude spleen increased. When the T-cells were reduced to 10%, as many as 59% of the responding cells were from nude spleen.

3.6.6 Nature of the Responding Cell

Table 10 shows that treatment of cells from nu/nu spleen with monoclonal anti-Thy 1.2 and complement (2 cycles) before they were mixed with T-cells had no effect on the subsequent response to Con A. Therefore, Thy 1.2-bearing cells in nude spleen are not responsible for the interactive response. The response to Con A of unfractionated cultures which had been treated with anti-Thy 1.2 and complement before addition of the mitogen was 18 - 30% that of complement-treated cultures (Table 11). The possibility that cells which express Thy 1.2 too weakly for lysis are activated cannot be tested, due to the impossibility of their discrimination from the T-cells added in the constitution of the mixtures.

Table 10.

Effect of Treatment of nu/nu cells with anti-Thy 1.2 and complement before mixing on the response of mixtures of these cells and T-cells to Con A.

Treatment	Percent T-cells in mixture	Percent Response Experiment 1.	Experiment 2	Experiment 3.
Complement	100	100	100	100
	50	137	63	109
	20	NM	40	23
	10	33	28	16
Anti-Thy 1.2 plus complement	50	147	30	76
	20	110	30	30
	10	62	14	13

^{3}H-thymidine incorporation was measured 48 hours after the addition of Con A.

Responses of mixtures are expressed as percentages of the pure T-cell response.

All assays were performed in triplicate.

NM = not measured

Table 11.

Effect of treatment of unfractionated spleen cells with anti-Thy 1.2 and complement on their proliferative response to Con A

Experiment Number	Spleen	^{3}H-thymidine Incorporation (cpm) Complement	Anti-Thy 1.2 + complement	Percent
1.	RNC	10,190 ± 420	3,220 ± 260	32
2.	Balb/c	17,580 ± 1,080	4,110 ± 1,010	23
3.	Balb/c	8,035 ± 2,390	1,480 ± 300	18
4.	Balb/c	7,770 ± 1,880	2,350 ± 480	30

Anti-Thy 1.2 treatment was carried out before Con A was added.

^{3}H-thymidine incorporation was measured after 48 hours.

Values are means of triplicates, ± SD.

That the responding cells from nu/nu spleen were B-cells was shown by anti-Ig immunofluorescence. Cells from mixtures co-cultured with Con A for 48 hours were stained with FITC-goat anti-mouse Ig, then fixed and scored. The results of 3 experiments are shown in Table 12.

Table 12.

Identification of the nature of the responding cells in T-cell/ nu/nu cell mixtures.

Treatment		Percent Blasts	Percent Ig +ve cells	Percent Ig +ve blasts	Number of cells counted
T-cells, Con A	a.	12	5	4	204
	b.	31	4	5.5	116
	c.	42	0	0	100
20% T-cells, 80% nu/nu cells, Con A	a.	15	52	45	207
	b.	22	58	63	108
	c.	30	41	50	482
nu/nu cells, LPS	a.	18	75	89	102
	b.	22	85	100	62
	c.	24	61	100	234

Results from 3 experiments are included in this Table; they are shown by rows a, b, and c, for each treatment.

Cells were stained, fixed and scored 48 hours after the initiation of culture.

Blast cells were defined as those cells whose diameter was greater than 8 um.

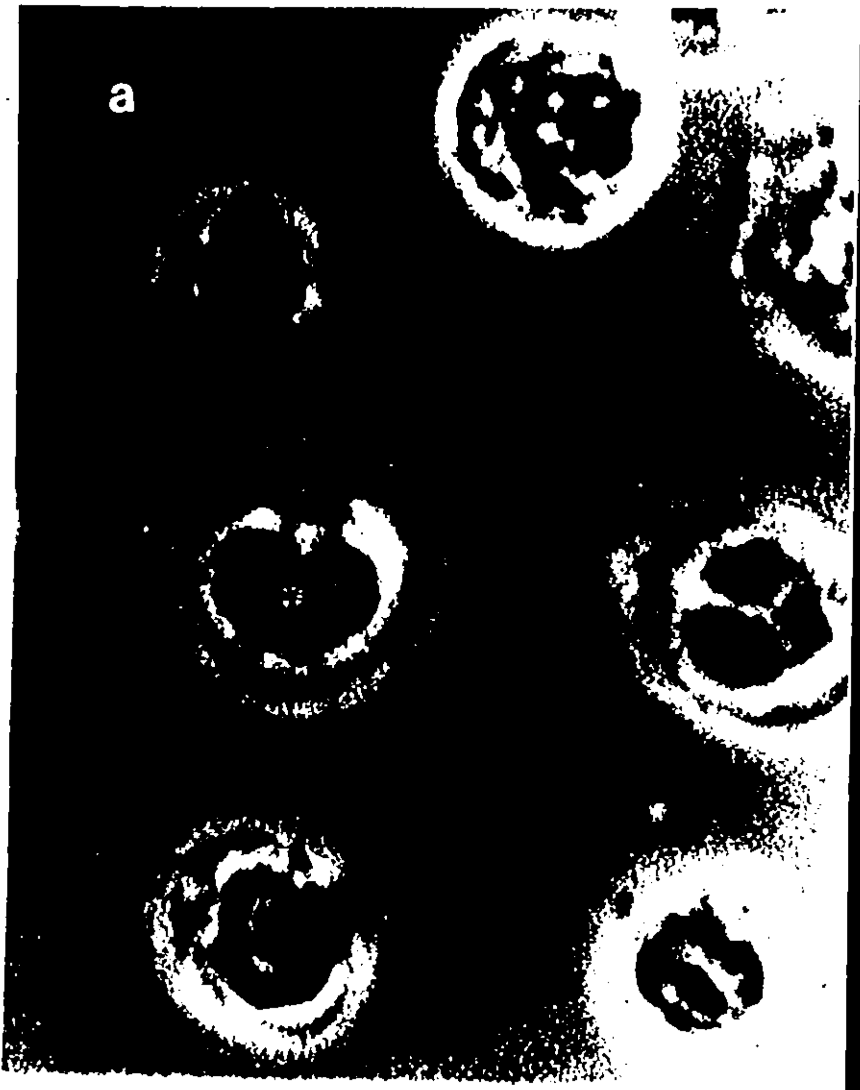
Table 13.

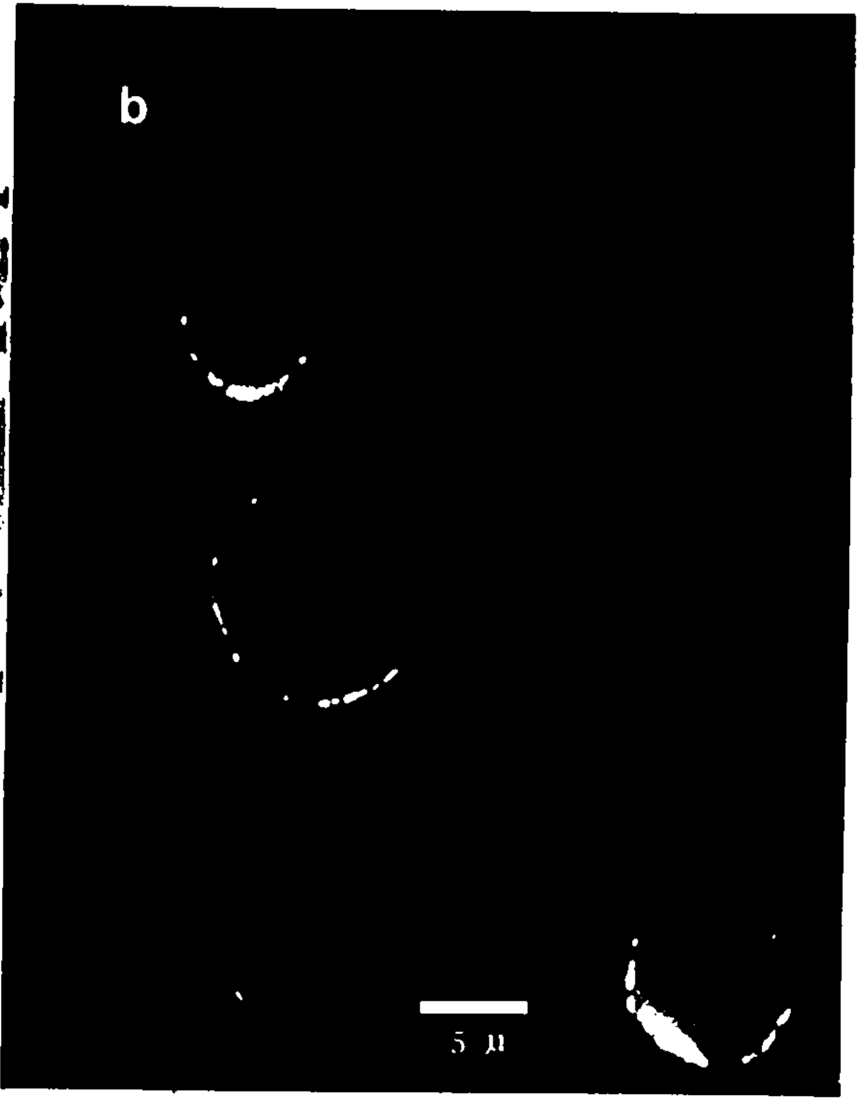
Proportion of Ig-bearing cells in a 1:4 (T-cell:nu/nu cell) mixture that had been cultured with Con A for 65 hours, then treated with anti-Thy 1.2 and complement before staining with FITC-anti-Ig.

Treatment	Percent Blasts	Percent Ig +ve cells	Percent Ig +ve blasts	Number of cells counted
Complement	31	63	38	102
Anti-Thy 1.2 + Complement	22	92	100	160

Cells used for this experiment were from the same culture used for Experiment c), Table 12.

Plate 2. a) Phase contrast micrograph of cells from a mixture containing 20%T/80% nude cells, which had been incubated with Con A for 48 hours prior to fixation and FITC-anti Ig staining. Two blast cells are visible. b) The same field as in a), photographed under UV light. Final magnification is 2600 X; the bar represents 5 um.


a


b
5 μ

The proportion of blasts in all 3 activated cultures was about equal. The very low percentage of Ig-positive cells in the T-cell cultures showed the high level of purity of this population, and also indicated that there was almost no non-specific binding of antibody to blast cells; the percentage of blasts which were Ig-positive was very low in these populations. In the nude cell population, there was a high percentage of Ig-positive cells, and in two out of three experiments, all of the blasts were Ig-positive. In a mixture to which was added 20% T-cells, between 45 and 65 percent of the blasts were Ig-positive (the mean was 50%).

Plate 2 a) shows a phase contrast micrograph of a field of cells from such a mixture. Two of the cells are enlarged blast cells. In Plate 2 b), this same field is shown photographed under UV. One of the blasts is strongly Ig-positive, the other is quite unstained. Only cells with a staining comparable to the former were scored as Ig-positive for Table 12.

In one experiment, treatment of a 20%T/80% nude cell mixture that had been incubated with Con A for 48 hours, with 2 cycles anti-Thy 1.2 and complement, raised the percent Ig-positive cells and blasts to 92% and 100% respectively, when these cultures were stained for surface Ig. Thus, all of the non-fluorescent cells were Thy 1.2- bearing (Table 13).

Table 9 showed that in a 1:4 T-cell:nu/nu cell mixture, 40% of the metaphases were from nude spleen. Table 12 shows

that 50% of the blasts in this mixture were Ig-positive, while almost none of the blasts or the cells from the T-cell population were Ig-positive. It seems therefore that all of the excess response can be attributed to B-cells from nude spleen.

3.6.7 LPS Does Not Produce Secondary Activation

When LPS was added to mixtures of T- and nu/nu cells, there was no interaction (Table 14). The LPS response diminished in proportion to the nude spleen cells. There was no activation of cells from the T-cell population. The interaction described above was therefore one-way. An activated T-cell can activate a B-cell, but the reverse is not true.

These results show then that an apparently maximal response to Con A, as measured by the activations of potassium influx and ^{3}H-thymidine incorporation, can be produced through the activation of B-cells by activated T-cells.

Table 14.

Response of Mixtures of T-cells and nu/nu cells to LPS

Experiment	Percent nu/nu cells in mixture	Percent Response
1.	100	100
	40	54
	20	29
	0	0
2.	100	100
	50	36
	25	23
	10	9
	0	0

^{3}H-thymidine Incorporation was measured 66 hours after the initiation of culture. Incorporations are expressed as a percentage of that into nu/nu cells; incorporation into T-cells (=resting counts) has been set to zero percent.

SECTION IV

DISCUSSION

4.1 FLUX ACTIVATIONS

Con A activates both influx and efflux of potassium in mouse splenocytes. Mouse lymphocytes are therefore similar to human lymphocytes, and to many other cell types, in that elevation of monovalent cation fluxes accompanies their activation to proliferate.

4.1.1 Rates

All rates (influx and efflux), and accompanying parameters (cell size, potassium contents) were measured in unfractionated, heterogeneous populations; values are therefore mean population values. Since all are expressed on the same basis, comparisons between them are valid.

The 30 minute uptake points (as from Figure 3), give a useful estimate of the degree of activation of influx (1.85-fold). However, they do not allow precise determination of the rate of uptake, due to the effect of backflux. This is avoided by using the short time assay, shown in Figure 5. Gargus *et al*, (1978) found that influx was maximal and linear over a ten minute period in mouse L cells, so the three minute assay used in this study must also exclude backflux. The fact that resting cell influx and efflux were measured as equal further justifies the use of this method.

Previous measurements of resting efflux and influx as unequal was probably a result of backflux, (Owens & Kaplan, 1980A). Resting fluxes were measured as equal in studies with human cells, using longer assay times for influx, (Hamilton & Kaplan, 1977; Segel et al, 1976). In these the 15 minute point was used for influx estimation, as opposed to the 30 minute estimator used in this study; thus,more accurate influx estimates were obtained. The earlier influx is measured, the less the result will be compromised by backflux.

The measured rates are consequently higher than those reported previously for mouse (Owens & Kaplan, 1980A), and human lymphocytes (Segel & Lichtman, 1976; Quastel & Kaplan, 1970B). The degrees of activation determined are similar (about 2-fold), this is probably because the relative effect of backflux on resting and stimulated uptakes is similar within the first 30 minutes. The increased degree of activation of influx measured over a longer period (Figure 4) indicates that this similarity does not hold over 2.5 hours. Activation is therefore more accurately measured from the 3 minute uptake.

A 1.72-fold activation was measured by the short uptake method (Figure 5); that from the 30 minute point was measured as 1.85-fold. A degree of activation of 1.75-fold was assigned to the elevation of total potassium influx by Con A.

4.1.2 Efflux Activation

Con A activates potassium efflux, with what can be assumed to be a similar time course to the influx activation (Table. 2). The degree of activation as determined from the slopes of efflux plots is considerably less than that of influx (1.43 vs. 1.75 -fold). Even when rates (fmoles/cell.hr.) are compared, (thus increasing the degree of activation of efflux due to the increased potassium content of a stimulated cell, Table 3), the degree of activation of efflux (1.66-fold) is less than that for influx.

While the degree of activation of efflux, as measured from the slopes of fractional loss plots, is absolute, being calculated from tracer counts alone, the actual efflux values (fmoles/cell.hr.) depend on the intracellular potassium content, which can change with time. Since this is so, it is very important for the interpretation of flux data that all assumptions which are used in calculation be verified. For instance, assumption that splenocytes are cells of diameter 7 um with a $(K^+)c$ of 130 mM, constant from 0-14 hours, gave erroneous values both for efflux itself and for the degree of its activation (Owens & Kaplan, 1980A). The only assumption that was made in the calculation of the rates presented in Table 2 was that cells are smooth spheres, 78% of whose volume is water; both Segel & Lichtman (1976) and Gargus et al (1980) have verified the latter assumption, for human and mouse cells respectively.

The difference in the degree of activation of influx and efflux is apparently peculiar to the rodent lymphocyte; such differences, although predicted, have not been seen in human cells (Kaplan, 1978; Kaplan & Owens, 1980). Segal et al (1976) showed that PHA produced a 1.27-fold activation of potassium efflux in rat thymocytes, in experiments where they measured a 2-fold activation of efflux in human lymphocytes; they did not measure influx in rat cells.

Segel et al (1976) found that potassium efflux in unstimulated rat thymocytes was almost twice that in human lymphocytes, measured in the same study. They calculated a Ke value (see Segel & Lichtman, 1976, for method) for this efflux of 7.8 nm/min. This is very similar to the Ke value which can be calculated from the slopes in Table 2 (8.1 nm/min). Efflux and influx in resting human lymphocytes are equal (Segel et al, 1976; Hamilton & Kaplan, 1977); these rates are all about 4 fmoles/cell.hr. Resting fluxes in rat and mouse lymphocytes are almost twice this value, at about 7 fmoles/cell.hr. Rodent lymphocytes therefore differ in three respects from human lymphocytes: they are 4 orders of magnitude less sensitive to ouabain; their resting potassium fluxes are almost twice those in human cells; and, the degree of activation of efflux by a mitogen is substantially less than in human cells.

4.1.3 Potassium Contents & Concentration

There is a measurable imbalance between influx and efflux, or a net influx. This predicts an increased intracellular potassium content (assuming that tracer fluxes reflect chemical fluxes). There is, in fact, a significant (2 fmoles/cell) increase in potassium content, detectable at 10, but not at 5 hours after Con A addition (Table 3). Intracellular potassium concentration does not change, because of a compensatory increase in cell volume. Constancy of $(K^+)c$ was also shown by Segel & Lichtman (1978) for PHA-stimulated human lymphocytes. Negendank & Collier (1976) measured $(K^+)c$ in stimulated human lymphocytes, and found that it decreased; decreases were also reported by Averdunk (1976), and by Averdunk & Gunther, (1980A), for mouse and human lymphocytes, respectively. Decreases in $(K^+)c$ may be due to the increase in membrane leakiness reported by Segel et al (1975), which precludes washing of cells in such studies. In the only study other than this of K^+ contents in mouse lymphocytes (that by Averdunk, 1976), a Con A concentration was used which in my hands is not mitogenic. There is general agreement that there is no increase in $(K^+)c$ following stimulation.

4.1.3.1 Imbalance

The minimum net potassium influx that must occur to produce, between 5 and 10 hours, an increase in potassium content of

2 fmoles/cell is 0.4 fmoles/cell.hr. The observed net influx of almost 2 fmole/cell.hr., measured at 14 hours, is more than adequate. That potassium content has not increased further may simply indicate that net influx was not maintained over the entire 5-hour period.

This imbalance between influx and efflux is sufficient to maintain $(K^+)c$ constant during blast transformation. Since potassium concentration is critical for protein synthesis (Cahn & Lubin, 1978), it may be assumed that, as was shown for the 0-14 hour period, there is no significant change in $(K^+)c$ during blastogenesis (10 - 48 hours). In this time, the mean cell diameter of these unfractionated populations increases from 6.24 to 10.6 um (see Figure 6) - this produces an approximately 5-fold increase in mean population volume. To maintain $(K^+)c$ constant in the face of this increase, potassium content must increase 5-fold. This requires the net entry of 4 times the original potassium content, or 56 fmoles/cell. The required mean net influx, over a 38 hour period, is then 1.5 fmoles/cell.hr. The observed net influx, almost 2 fmoles/cell.hr., is sufficient to maintain $(K^+)c$ constant in the face of a 6.5-fold mean population volume increase, if maintained for 38 hours.

In this system, therefore, the predicted imbalance between influx and efflux, required for the maintenance of $(K^+)c$ in the face of a large mean population volume increase, can and has been detected.

4.1.4 Components of Activated Influx

About 80% of total potassium influx is ouabain-sensitive. Con A activates ouabain-sensitive influx by about 2.9-fold (Figure 9). Most of the activated influx is therefore ouabain-sensitive, and so is mediated by the (Na^+,K^+)-ATPase. There is also a furosemide-sensitive component (Figure 10); this may represent Na^+-K^+ co-transport, as has been shown for L cells by Gargus & Slayman (1980). Chloride has been suggested as a counter-ion for these movements (Bakker-Grunwald et al, 1980). Averdunk & Gunther (1980B) equate furosemide- sensitive $^{42}K^+$ influx in human lymphocytes with K^+:K^+ exchange, which is Na^+-insensitive, and unaffected by Con A (this however does not mean that its inhibition cannot affect Con A stimulation). Segel & Lichtman (1976) found no evidence for K^+:K^+ exchange in human lymphocytes. Since the furosemide sensitivity of efflux was not measured in this study, and the ouabain-sensitivity of the residual flux was not determined, interpretation of the inhibition by furosemide of both potassium influx and Con A-induced proliferation is necessarily speculative. However, since it is accepted that furosemide inhibits a ouabain-insensitive component of influx, this result shows that, although the (Na^+,K^+)-ATPase may be the principal effector of activated influx, other components are also activated. It is possible that all components of cation fluxes are activated and that these activations are interdependent, so that inhibition of any one

component will lead to inhibition of the others, and therefore of the entire activation sequence.

4.1.5 Inhibition by Ouabain

The ID50 for the inhibition by ouabain of both potassium influx (mediated by the sodium pump) and ^{3}H-thymidine incorporation is identical (Figures 7 & 8). Therefore, the effect of ouabain on the entry of cells into S phase can be attributed entirely to its earlier inhibition of the (Na^{+},K^{+})-ATPase. Segel & Lichtman (1980) have shown that although the ID50 for the inhibition by ouabain of potassium influx (280 nM) is greater than that for the inhibition of DNA synthesis (40 nM), it decreases with time of incubation with ouabain, due to the slow binding of the drug at low concentrations, so that the inhibitory doses for both parameters of activation become equal (40 nM) when they are measured after 24 hours. Szamel et al, (1980) claim that even at 48 hours, the ID50's are different, and they maintain that ouabain inhibits ^{3}H-thymidine incorporation in a cell cycle-dependent manner by some mechanism other than its action on the sodium pump. However, they used a 6-hour assay for $^{86}Rb^{+}$ uptake, which may have led to erroneous estimates of the degree of activation (and therefore of inhibition) of potassium influx, as was suggested above. Since ouabain was only present for 6 hour periods, the slow binding at low doses noted by Segel & Lichtman (1980) could also account

for the discrepancy. Mills & Tupper (1976) measured the ouabain and furosemide sensitivities of K+ influx during the cell cycle in Ehrlich ascites cells. Pump-mediated influx increased during S-phase, then declined in G2 (both relative to G1). Szamel et al also found that influx was maximally sensitive to ouabain during S-phase.

In this study, not only was the ID50 equal at 45-48 hours, as in human cells, but that for the inhibition of potassium influx at 10 hours was no greater i.e. there was no difference over the period of incubation. This is a consequence of the 4 orders of magnitude difference in the sensitivity of mouse and human cells to ouabain; at concentrations approaching 1 mM, maximal ouabain binding is attained quickly.

4.1.5.1 Reversibility of Ouabain Inhibition

The reversibility of the inhibition by ouabain of both potassium influx and DNA synthesis by washing shows that ouabain does not produce a permanent effect on the cell; constant presence of the drug is required for its effect. This was not true, however, for those cells which were incubated for 48 hours with 1 mM ouabain. Although there was some recovery of potassium influx in these cells following washing, there was no recovery of the proliferative response (Table 1). These cells have been irreversibly inhibited. Wright et al (1973) showed that the mitogen responsiveness of human

cells, lost following incubation for 48 hours with concentrations of ouabain as high as 8 x 10^{-3}M, could be restored by washing with high (K^+) medium (20 mM). Such washes were not used in this study. Quastel & Kaplan (1970A) showed that at doses sufficient to inhibit mitogenesis (10^{-8} - 10^{-7}M), the inhibition by ouabain of human lymphocytes was reversible. The same thing was shown by the reversibility of the inhibition by 0.2 mM ouabain in this study (Table 1). Presumably, prolonged shut-down of the sodium pump changes the intracellular ionic environment drastically, and so causes an irreversible loss of ability to respond, whereas lower concentrations, which inhibit activation only while the drug is present, do not produce such changes.

4.1.5.2 Requirement for Pump Activation for Proliferation

Results presented in this thesis show that the inhibition by ouabain of proliferation results entirely from its inhibition of the sodium pump. Ouabain exerts all of its effects through its binding to the (Na^+,K^+)-ATPase (Robinson & Flashner, 1979). Inhibition of the (Na^+,K^+)-ATPase, whether it was previously activated or not, must have profound consequences for all cellular activity.

Negendank has suggested that membrane ion pumps are not as important for the regulation of intracellular ionic activities as is the adsorption of ions onto fixed anionic sites within the cell (Negendank & Shaller, 1980). While

some results may be difficult to reconcile with the membrane pump hypothesis, the evidence that the (Na^+,K^+)-ATPase translocates Na^+ and K^+, against their electrochemical gradients, and is also electrogenic, is by now overwhelming. Any effect of ouabain (a drug specific for this enzyme) is therefore conclusive evidence for a role of the sodium pump in ionic regulation in that system.

That ouabain inhibits Con A-induced proliferation therefore indicates a requirement for a functioning sodium pump during blast transformation, as was suggested for human lymphocytes by Quastel & Kaplan, (1970A). Lever et al (1980) have shown that neither activated ^3H-uridine nor $^{86}Rb^+$ uptakes are sufficient to induce proliferation in 3T3 cells. However, in the same paper are presented data showing that there was never proliferation without prior activation of potassium uptake. So, while the activation of influx may not be sufficient to induce proliferation, it is indeed necessary.

There was never detected in this study activation of proliferation which was not preceded by activation of ouabain-sensitive potassium influx. Furthermore, the same concentration of ouabain which reduced the activated potassium influx to resting levels (3- 4 mM) also reduced ^3H-thymidine incorporation to resting levels. Maintenance of resting cell pump flux is therefore not sufficient for mitogenesis; potassium fluxes must be activated. Similar conclusions have been

reached by Hammarstrom & Smith (1979), and by Hart (1981), who used K^+ congeners to demonstrate (Na^+,K^+)-ATPase activity in stimulated lymphocytes from mouse and hamster.

4.1.6 Mechanism of Flux Activation

The mechanism of the influx activation was not determined. An early cell rounding, such as that shown for rat thymocytes by Hume et al, (1979), could lead to exposure of new, previously cryptic transport sites, so causing an elevation of potassium influx. However, cell rounding was measured within 30 minutes of Con A addition, whereas influx is not activated in this system until 6 hours later. There is no significant increase in cell size until after 12 hours (Figure 6; LaBadie et al, 1979), so the 1.75-fold influx activation at 6 hours cannot result from a cell surface area increase.

It was not possible to accurately measure intracellular sodium, so the hypothesis that the sodium pump is activated in response to an increased $(Na^+)c$ was not tested. However, the inhibition by furosemide may suggest sodium entry, if indeed this drug inhibits Na^+-K^+ cotransport. Inhibition by amiloride (Owens & Kaplan, 1979) would be compatible with the same hypothesis, but its toxicity clouds the interpretation. One result which is supportive of sodium entry is the demonstration of activated potassium efflux; this is ouabain-insensitive, and represents a leak. Sodium enters the cell by the analogue of this leak flux.

Activated sodium entry and potassium efflux have both been shown in a number of cell types that are activated to proliferate (Leffert, 1980; see Introduction). There is in all these systems a general membrane permeability increase, as was shown for mouse splenocytes by the Con A- induced depolarization of the membrane potential (Kiefer et al, 1980), and by the enhanced uptake of Hoechst 33342 by splenocytes that have been activated either in MLR or by mitogens (Lalande & Miller, 1979, 1980). These observations complement those previously made of increased potassium efflux (Kaplan, 1978). Since any permeability increase will produce an elevated $(Na^{+})c$, the sodium pump must respond. Therefore, at least part of the pump activation probably results from an elevation of $(Na^{+})c$.

Demonstration of the activation of $^{86}Rb^{+}$ uptake by treatment of human lymphocytes with EGTA, a Ca^{++} chelator, implicates Ca^{++} in the control of this influx activation (Quastel et al, 1980). Intracellular Ca^{++} has been shown to regulate potassium fluxes in red cells by the Gardos effect (Gardos et al, 1975). The early increase in potassium influx in human lymphocytes is independent of both translation and transcription, (Quastel et al, 1970), so is probably mediated by a combination of physiochemical events such as those described above; it is likely that similar mechanism(s) operate in the mouse system.

4.1.7 Timing

The activation of potassium influx in mouse splenocytes was not routinely detectable until 6-8 hours after the addition of Con A. This suggests that it may be related to a "second signal" of activation, rather than to Con A binding *per se*, which is essentially complete within 30 minutes (Repacholi, 1981). The fact that activation of protein synthesis could, in some experiments, be detected before the activation of potassium influx, and in others was simultaneous with it, might be explained by association of the flux activation with the secretion of some soluble factor. The timing is appropriate for its being associated with the production of IL-2 (Gronvik & Andersson, 1980). Since Puri *et al* (1980) have shown that IL-1 (LAF) increases the lipid viscosity of T-cell membranes from mouse splenocytes, IL-1 is less likely to be a flux-activating factor. Influx is activated a little later than the depolarization reported by Kiefer *et al*, (1980).

Why the activation of influx should be later in mouse splenocytes than in human PBL is not known. It may reflect either a species or a tissue difference.

4.1.8 Specificity of Flux Activations

That activation of potassium influx is not a result of Con A binding *per se* was demonstrated by the results presented in Table 4 and Figure 11. Con A binds to RNC nu/nu splenocytes

(Greaves & Janossy, 1972), but activated neither potassium influx nor DNA synthesis in them. LPS activated both. The association of cation flux elevation with LPS activation has not previously been shown. In Balb/c cells, the reverse was true; Con A activated both parameters, LPS neither. The activation of potassium influx is therefore absolutely linked to the activation of a cell to proliferate. The Con A-induced depolarization in mouse splenocytes was also specific to those cells stimulated to proliferate (Kiefer _et al_, 1980). If the influx activation is indeed associated with a soluble factor, it is probably then not produced simply by interaction of the factor with a cell, as the specificity of factor interaction is considered to be conferred by the mitogen (Smith _et al_, 1979).

4.1.9 Why is Influx Activated?

The activation of influx can be seen to have three goals:

1) to balance efflux;
2) to regulate cell volume;
3) to maintain a constant $(K^+)c$ in the face of the volume increase of blastogenesis.

1) assumes that efflux activation is specific to those cells which proliferate, and that it initiates the influx activation. Both these assumptions seem reasonable. It has been shown in the preceding discussion that the activation of influx is sufficient in magnitude and appropriate in

character for these three goals. The furosemide-sensitive component may function in volume regulation; much of the remainder of influx is ouabain- sensitive, and can both balance efflux and maintain a constant $(K^+)c$.

The depolarization shown by Kiefer et al (1980) in mouse lymphocytes probably reflects the activation of efflux. Moolenaar et al (1981) find that inhibition of Na^+ entry and prevention of sodium pump activation by amiloride in serum-stimulated neuroblastoma cells does not have any effect on the serum-induced depolarization of these cells. The electrical events may be distinct from radiotracer fluxes. The depolarization seen in mouse lymphocytes occurs 2-4 hours after mitogen addition, while that in neuroblastoma cells is immediate, and repolarization is complete within minutes. These depolarizations probably reflect different activation events. In lymphocytes, the repolarization may reflect the activation of influx, which repolarizes and subsequently hyperpolarizes the cell, as a result of activation of the pump for 24-48 hours. It seems likely that such hyperpolarizations have a general role in activation. Wondergem & Harder (1980) showed that regenerating rat liver cells are hyperpolarized; Sachs et al (1974) correlated an increase in membrane potential with entry of Chinese hamster lung cells into S-phase.

4.2 ACTIVATION OF INFLUX AND PROLIFERATION IN B-CELLS

The results discussed so far have shown that activation of potassium influx is an essential parameter of the mitogenic activation of mouse splenocytes. The fact that the activation is not a consequence of Con A binding, but of some event(s) occurring some hours afterwards, indicates that it represents a 'second-signal' activation parameter. It has been shown to be a specific event, restricted to those cells which subsequently proliferate.

4.2.1 Cell Interactions in WGA B-cell Populations

4.2.1.1 A Paradox

At first glance, the results shown in Table 5 and Figure 12 seem to contradict those in Table 4 and Figure 11. Fractionation of Balb/c splenocytes with WGA removes almost all of the Ig-bearing cells from one population (the 'T' cells, see Table 11), and substantially enriches the 'B' population for these cells. One would predict, on the basis of results in Figure 11 and Table 4, that populations so altered in terms of their proportional content of T- and B-cells would respond differently to a T-cell mitogen. While differences in the proliferative response were observed (Table 5), there was no difference in the activation by Con A of potassium uptake in these populations (Figure 12). One inference from this finding is that activation of potassium uptake is not necessarily proportional to mitogenesis. It was not initial-

ly clear why a response which had been shown to be restricted to those cells which proliferate in response to Con A should not be reduced when the number of those cells was reduced.

Unlike cells from nude spleen, WGA B-cells showed a measurable proliferative response to Con A. Bourguignon et al (1979) identified 10% of their B-population as theta-bearing; this can be taken as a minimum estimate of the percent T-cells in such cultures. B-populations used in this study contained 70-80% Ig-bearing cells, and therefore 20-30% T-cells. It was through these T-cells that Con A activation was mediated.

4.2.2 T-cell/Nude cell Mixtures

As a model system for the interaction of Con A-responsive and non-responsive populations, T-cells from BNC nu/+ mice were cultured with nu/nu cells and Con A. Nude spleen contains some T-cells (Kindred, 1979), but, since nude splenocytes do not respond to Con A (Table 4), they are functionally equivalent to B-cells. Figures 14 and 15, and Table 6, show that these mixed co-cultures responded to Con A as though there had been no reduction in the number of T-cells, as long as there were more than 10% T-cells present.

4.2.2.1 A Paradox Lost

Figure 14 and Table 6(A) show that potassium uptake is enhanced by these mixtures to a level sufficient to account for the apparently maximal response of a B-enriched population (Figure 12). As the proportion of T-cells was reduced, the excess, or unexpected potassium uptake (represented by an observed/expected ratio greater than unity) increased. The maximum activation of potassium influx by Con A is 1.75-fold. In a typical WGA B-cell population, there are 25% T-cells, and 75% B-cells. Addition of Con A will raise the T-cell contribution to the population K^+ influx from 0.25 to (0.25).(1.75) = 0.44. The B-cell contribution will remain unchanged, at 0.75. In the absence of any interaction, therefore, population K^+ influx will rise from (0.25 + 0.75) = 1.0, to (0.44 + 0.75) = 1.19. Through cell interaction, influx is enhanced, as shown by the observed/expected ratios in Table 6(A). Potassium influx becomes (1.19).(1.45) = 1.72. This is almost equal to the 1.75-fold maximal activation, and indistinguishable from it. Thus, due to interaction between B-cells and a small number of Con A-activated T-cells, an apparently maximal activation of potassium influx can be produced in that population, despite its lowered content of Con A-responsive (T-) cells.

The excess, or interactive uptake, that is required to maintain a maximal response as the number of T-cells is reduced will generate observed/expected ratios which fall on a

line which joins, almost linearly, unity (for 100% T-cells) and 1.75 (for 0% T-cells - note that since this response is impossible, this is a theoretical ratio). The data points (Figure 14, line b) fit such a line, until the T-cells are reduced to less than 10%. Since there were always at least 10% T-cells in the WGA B-populations, the maximal response seen was therefore a consequence of co-culture of T- and B-cells. This also explains why the response of purified T-cells was no greater than that of unfractionated cells (which contain approximately equal numbers of T- and B-cells). Figure 13 shows that this response is only obtained if the cells are co-cultured. Mixing T-cells and nu/nu cells immediately prior to assay gave the reduction of both K^+ influx and ^3H-thymidine incorporation expected for the reduction in the number of responding (T-) cells.

4.2.2.2 Requirements for Active Cells

Lines a) in both Figures 14 and 15 show that responsive nu/nu cells were required for these responses; when they were inactivated prior to mixing, the response was reduced exactly in proportion to the number of T-cells. Line b) in Figure 15 shows that reduction of the both the number of T-cells and the absolute number of cells in culture (by decreasing the cell density), caused a more severe reduction than did dilution with inactivated nu/nu cells. There was therefore an effect of T-cell density on their response to Con A, such

that the response was not directly proportional to cell number (see Stewart & Hamill, 1981). It was only when T-cells were cultured with responsive nu/nu cells that a response in excess of that expected from the T-cells alone was seen.

The production of this excess response in T-cell/nude cell mixtures was mediated through the T-cells. Table 7 shows that if the T-cells were rendered unresponsive to Con A by UV-irradiation before mixing, there was essentially no response of the mixtures to Con A. The response seen was higher than that expected on the basis of additivity of counts, but the counts themselves were too low to be considered meaningful.

4.2.2.3 Response of Cells from Nude spleen

Both blast counts and Hoechst 33258 identification of the spleen of origin of mitotic cells showed that cells from nu/nu spleen were activated in these mixtures (Tables 8 & 9). The excess cation influx activation and thymidine incorporation are therefore a result of there being more cells engaged in activation and not to a 'superactivation' of the T-cells.

The excess, or unexpected $^{86}Rb^{+}$ uptake seen in Figures 12 and 14 is most likely due to these cells from nu/nu spleen; activated potassium uptake has already been shown to be an essential component of the proliferative response, and nude cells were shown to proliferate (Table 9, Plate 1).

4.2.2.4 B-cells Respond

While cells from nude spleen were used as B-cell equivalents on the basis of their non-response to Con A, rather than their identity, the cells from nu/nu spleen which responded were in fact B-cells (Table 12, Plate 2). Comparing Tables 9 and 12, it can be seen that the proportion of Ig-bearing blasts in a 20% T-cell mixture is as great as the proportion of metaphases from nude spleen in similar cultures. It is quite probable then that all of the nude cells which respond are B-cells.

If T-cells from nude spleen were activated, they did not express Thy 1.2 very strongly. Table 10 shows that treatment of nu/nu spleen cells with anti-Thy 1.2 and complement before mixing had no effect on the reponse to Con A of the mixtures. Table 11 verifies that this treatment lyses Con A-responding cells. It has been reported that T-cells from nude spleen express Thy 1.2 more strongly when they have been activated (Letarte <u>et al</u>, 1980; Dennert & Hyman, 1980). However, it was not possible to exploit this property here, since, once mixed, Thy 1.2-bearing cells from nu/nu and nu/+ spleen could not be distinguished. T-cells from nude spleen could not therefore be formally excluded from the response.

There have been two other reports of the generation of excess proliferative response to T-cell mitogens by co-culture of T- and B-cells; both used human PBL. Kasahara <u>et al</u>, (1979) showed no diminution of response to Con A or to PHA

of T-cells diluted with B-cells, until the T-cells constituted less than 20% of the mixtures; they found optimal response at ratios between 80% and 40% T-cells. When they inactivated the B-cells with mitomycin c, the response was unchanged; they concluded that T-cell activation was enhanced. Han & Dadey (1978) mixed X-irradiated T-cells with B-cells, in a 1:1 ratio, and found a significant proliferative response to PHA. My results differ from both in that inactivation of either population totally prevents the interaction. This may be at least partly due to differences in the effects of UV- and X-irradiation on T-cells.

4.2.2.5 Lack of Interaction in LPS Response

Figure 14 shows that the activation by LPS of K^+ influx in nu/nu cells was not as great as that by Con A in Balb/c or in BNC nu/+ cells. This is probably due to the fact that LPS does not produce any secondary interactions, as shown in Table 14. The response of nu/nu cells to LPS is reduced as the proportion of these cells is reduced by co-culture with nu/+ T-cells. The activation of B-cells by T-cells is therefore one-way. The 1.4-fold activation of K^+ influx by LPS represents activation in B-cells only. The finding by Goodman & Weigle (1979) that T-cells can enhance LPS response was perhaps due to their inclusion of 2-mercaptoethanol in the medium.

4.2.3 Immunology of Secondary Activations

The activation of B-cells by activated T-cells is possibly a non-specific T-helper activity. Similar activations of B-cells have been described in a number of systems. Con A has been shown to induce polyclonal antibody synthesis in mouse splenocytes, cultured *in vitro*. There was no such effect on cells from nude spleen, showing that T-cells mediated this B-cell activation (Coutinho *et al*, (1973). A similar activation of antibody synthesis by Con A was shown by Markham *et al*, (1977); here, Con A was administered *in vivo*, with antigen, and the PFC response to that antigen assayed *in vitro*. In neither of these studies was it conclusively shown that either T- or B-cells proliferated, although one assumes that Con A mediated its effects via T-cell activation.

The results in this thesis are the first demonstration of the mitogenic activation of nude mouse B-cells by activated T-cells. Such polyclonal 'non-specific' activations may in fact be a collection of individual specific activations. This could be verified by assessing the antibody response of these mixed cultures. This work is presently in progress.

4.2.4 Mechanism

The mechanism of the secondary activation is unknown. Supernatants from Con A-activated cultures had no effect on nu/nu cells. Attempts to produce activations in a Marbrook-type system were unsuccessful. It seems likely that soluble fac-

tors are involved, as has been shown for other B-cell activations (Chen & Leon, 1976; Primi *et al*, 1979; Bergenstock *et al*, 1981; Potash, 1981), but direct cell-cell contact cannot be excluded. Addition of neither IL-2, a TCGF preparation, nor Con A supernatants had any effect on nude spleen cells. Both the supernatants and the TCGF may have contained suppressive components (see Primi *et al*, 1979). If nu/nu cells were cultured with IL-2 and with UV-inactivated T-cells, the interaction might have been restored; IL-2 has been shown to restore the activity of UV-treated cells in MLR (Uotila *et al*, 1978).

4.2.5 Limits to the Response

The T-cell/nude cell mixtures closely model the potassium uptake response of WGA B-cells. However, the proliferative responses of the two systems are quite different. This may be explained by considering the regulation of the level of these responses.

4.2.5.1 Proliferation

The proliferative response of T/nude mixtures to Con A was essentially unchanged above a threshold number of T-cells. There were therefore two limits to the response; the T-cells were limiting when they were reduced to less than 10%, and some other cell type was limiting above this level. This cell was probably the macrophage. Miller *et al* (1979) have

shown that T-cell response (assayed by CTL production) could be elevated by the addition of syngeneic nu/nu spleen cells; they showed that it was the macrophage addition which was responsible. WGA T-cells are not depleted of macrophages, as is evidenced by their responsiveness to Con A. The plateau of response from 10-100% T-cells in nude/T-cell mixtures can be explained by there being an equivalent proportion of macrophages in both populations, so that their content in the mixtures is constant. This constant proportion then sets a limit on the level of the proliferative response (see Figure 15). The shape of the response curve (Figure 15) makes it unlikely that T-suppressors are limiting; they would be reduced by dilution at low T-cell proportions.

4.2.5.2 Flux Activation

The same unity of response was not seen for proliferation of the different WGA-fractionated populations. Although K^+ influx was activated to the same (maximal) extent in all three populations, whose T- and B-cell compositions differed, their proliferative responses to Con A were not equal (Table 5). This apparent discrepancy may be explained by assuming that the B-cells were macrophage-depleted, relative to unfractionated or T-cell populations. This would not necessarily affect T-B interactions, but would set a lower limit on the attainable response. The fact that K^+ influx was acti-

vated equally in all 3 populations would suggest either that macrophages are not required for flux activation, or that the minimum requirement is less than were actually present, and less than that for entry into S-phase.

4.2.6 Early vs. Late Activation

This would predict that the early events of activation (such as flux activation) are less dependent on macrophages than the later events. Support for this hypothesis can be found in the literature. Resch & Gemsa (1979) showed that reduction of the macrophage content of lymph node cultures to less than 0.2% (the normal level is about 1%) completely inhibited their proliferative response to Con A or PHA. However, if macrophages were restored to depleted cultures after 24 hours in presence of the mitogen, a normal response ensued: the time of peak response was not changed. Similar results were obtained by Dixon et al (1981), using oxidative mitogenesis of human lymphocytes, and so culturing in the absence of a mitogen. Once again, the peak response was changed neither quantitatively nor temporally by the depletion of macrophages in the first 24-30 hours of culture. Iwata & Osawa (1980) showed that adherent cells were required for entry of mouse splenocytes into S-phase, and that these cells were Ia-bearing. They identified the requirement period as post-12 hours, and showed that cell-cell contact was necessary for this accessory cell function. This

would explain the higher requirement for macrophages for S-phase entry. If early macrophage functions were mediated by soluble factors, and the later functions via cell-cell contact, then a lower macrophage/lymphocyte ratio would be effective for the early activations (such as flux activation) than for entry into S-phase. Activation by Con A therefore comprises two stages, which can be differentiated on the basis of their requirement for macrophages. Cells which have accomplished the activation events of the first, low-macrophage requiring stage, cannot enter the second, high-requirement stage unless sufficient macrophages are present.

While Shiozawa et al (1980) have shown that macrophages can be completely replaced by an adherent-cell supernatant in B-lymphocyte activation, thus making cell-cell contact seem unnecessary, supernatants from activated T-cells were also present. It could be argued that the requirement for cell-cell contact had previously been fulfilled in that system.

Larsson et al (1980) have discussed the problem of differentiating between no and very few macrophages in a culture. It was suggested above that K+ flux activation requires either no macrophages, or fewer than for S-phase entry. It is unlikely that WGA B-cells were completely depleted of macrophages, since some proliferative response to Con A remained. It is also unlikely that macrophages are not required for the activation of K+ influx. If this were

so, then, by the current model for T-cell activation (Gronvik & Andersson, 1980; Larsson et al 1980), the flux activation, being independent of soluble factors, would be produced by Con A alone. This is not the case.

Given that K+ influx activation in T-cells may be soluble factor- mediated, and that K+ influx activation in B-cells is mediated by T-cells, at a similar time, it follows that B-cell activation is probably mediated by a T-cell soluble factor.

It has been reported that WGA can induce macrophages to produce a supernatant which 'helps' nude B-cells in T-dependent responses, in the absence of T-cells (Bernabe et al, 1979). The responses described above, however, all require Con A-activated T-cells. WGA pretreatment of nu/nu cells did not induce in them any responsiveness to Con A.

4.3 CONCLUSIONS

Results presented in this thesis are compatible with the hypothesis that potassium influx is activated in response to the interaction of T-cells with a soluble factor, whose production is mediated by macrophages. This factor is possibly IL-2. The macrophage requirement for this activation is not very stringent, although they are essential. Activated T-cells can then induce B-cells in the same culture to proliferate; it seems likely that a T-cell soluble factor mediates this activation. Subsequent entry of these cells into S-

phase requires more macrophages than for the flux activation, possibly due to a requirement for cell-cell contact.

Con A binding is essential for the flux activation in T-cells, it is not known if this is the case for B-cells. Only those cells which are activated towards proliferation show the activation of K+ influx.

All activations of potassium influx need not lead to proliferation ; the same B-cell populations which showed a maximal activation of potassium influx (Figure 12) showed a markedly reduced proliferative response (Table 5). This reflects differences in adherent cell density.

It seems likely that the other parameters of activation (efflux, changes in polarization etc.) will also be detected in secondarily activated cells. The mechanism of activation is probably universal (Monard, 1979).

The activation of B-cells by Con A-activated T-cells shown in the T/nude model system, and inferred from results with partially- purified cultures, undoubtedly takes place in all but totally purified cultures which are activated by mitogens. That this can produce activations of potassium influx and DNA synthesis that are indistinguishable from those in pure T-cell cultures emphasizes that, although Con A is a T-cell mitogen, all responses of these cultures cannot be assumed to be T-cell responses.

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