UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

DIFFERENTIAL SENSITIVITY OF ANTIGEN- AND MITOGEN-STIMULATED HUMAN LEUCOCYTES TO PROLONGED INHIBITION OF POTASSIUM TRANSPORT

by

E. Pamela Wright

A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree of Ph.D. in Biology

UNIVERSITY OF OTTAWA
OTTAWA, CANADA, 1975

© E. Pamela Wright, Ottawa, Canada, 1975

Abstract

This work presents an investigation of the effects of ouabain on the activation of human peripheral blood lymphocytes induced in vitro by soluble antigens. The drug, which is a specific inhibitor of the membrane Na^+-K^+-ATPase, had previously been shown to inhibit reversibly the transformation induced by the nonspecific mitogen, PHA. Ouabain was found generally to inhibit antigen-stimulated cells in a manner similar to its inhibition of mitogen-induced activation, with an important exception. Exposure of the cultures to the drug for two days or longer rendered them irreversibly incapable of subsequent response to all of the specific antigens tried, yet such cells remained quite capable of response to the mitogen PHA. This treatment with the inhibitor was shown to affect neither viability nor the function of the macrophage population. Incorporation of uridine was as strongly inhibited as was thymidine incorporation. The mixed leukocyte reaction was found also to be irreversibly inhibited after a two-day incubation with ouabain, while response to the mitogens PWM or Con A was not. The treatment with inhibitor affected neither the presence of immunoglobulin on the cells nor the fraction of immunoglobulin-bearing cells. The concentration of the drug and the duration of the incubation with it were shown to be important for the persistent inhibition. The inhibition was neither prevented nor reversed by increasing the concentration of K^+ in the culture medium

(up to 26 mM) either during or after the incubation with ouabain. These results are discussed, some hypotheses are offered to explain them and further experimental approaches are suggested.

Résumé

Ce travail rapporte une étude des effets induits par l'ouabaïne lors de l'activation des lymphocytes circulant humains par des antigènes solubles in vitro.

On avait précedemment établi que la transformation induite par la PHA, mitogène non spécifique, était inhibée de façon réversible par ce produit qui est un inhibiteur spécifique de l'ATPase (Na^{+}-K^{+}) dépendante membranaire. On démontre que généralement l'ouabaïne inhibe de façon identique les activations induites par un antigène ou par un mitogène non spécifique avec cependant une importante restriction: une incubation des lymphocytes durant deux jours ou plus en présence d'ouabaïne, les rend incapables de répondre à tous les antigènes étudiés de façon irréversible, alors qu'ils peuvent encore être stimulés par le mitogène PHA. Ce traitement par l'ouabaïne n'affecte ni la viabilité cellulaire, ni la fonction des macrophages. L'incorporation d'uridine comme l'incorporation de thymidine est fortement atteinte. De même la MLC est irréversiblement inactivée si les cellules sont incubées deux jours en presence d'ouabaïne, alors qu'elles peuvent encore être stimulées par les mitogènes PWM ou Con A. Le traitement des cellules par l'inhibiteur n'affecte ni la présence d'immunoglobulines à leur surface, ni le nombre de cellules portant des immunoglobulines. On montre que comme la concentration en inhibiteur, la durée de l'incubation est important. L'inhibition ne peut être empêchée ni reversée en augmentant la concentration de K^{+}

(jusqu'a 26 mM) durant l'incubation en présence d'ouabaine ou après.

Ces resultats sont discutés et quelques hypothèses pour les expliquer sont presentées, ainsi que les approches expérimentales a réaliser pour leur vérification ultérieure.

Acknowledgements

Gratitude is first directed to Dr. Gordin Kaplan, who variously inspired, criticized, humored and entertained me throughout these years in his laboratory. His encouragement and generosity made it possible.

Thanks are also due to my committee members, Dr. C.M. Johnson-Lussenburg and Dr. Jules Harris, and to my colleague Dr. D.R. Stoltz, for helpful reviews of the research and of the thesis. The technical assistance, first from Marian Potjer, later from Lynn Hamilton, was greatly appreciated. Dr. Harris and Daphne Hyslop are to be thanked for their sharing of laboratory facilities and time. I am also indebted to Mr. L. Lefkovitch for advice and assistance with statistical analysis of the data.

Many other members of the Biology department contributed to my progress in different ways - they, too, were appreciated.

Table of Contents

		Page
Abstract		ii
Résumé		iv
Acknowledgements		vi
Table of Contents		vii
List of Figures		x
List of Tables		xi
List of Abbreviations		xiii
1. INTRODUCTION		
1.1	The Lymphocyte	1
1.1.2	Subpopulations and their interactions	1
1.1.2.1	T and B Cells *in vitro*	4
1.1.3.1	Macrophages	7
1.1.3.2	Red Cells and Platelets	8
1.2	Specific versus Nonspecific Response	9
1.2.1	Nonspecific Response	9
1.2.1.2	Kinetics of Mitogen Response	10
1.2.2	Specific Response	11
1.2.2.1	Kinetics of Response to Antigen	11
1.2.3	Mixed Lymphocyte Reaction	13
1.3	Events in Lymphocyte Activation	14
1.3.1.1	Early Metabolic Events	14
1.3.1.2	Proteins	15
1.3.1.3	Carbohydrates	16
1.3.1.4	RNA	16
1.3.1.5	DNA	17
1.3.2	Morphological Changes	18
1.3.3	Antigen-induced Events	19
1.3.4	The Lymphocyte Surface	20
1.4	Cation Transport	22
1.4.1	Transport of K^+ and Na^+	22
1.4.2	Cation Transport and Transport of Other Substances	23
1.4.3	Cation Transport and Cell Volume Regulation	24
1.4.4	Effect of Antibody on Cation Transport in LK Sheep Red Cells	25
1.5	Role of K^+ in Cell Functions	25
1.5.1	Monovalent Cations and Enzyme Activation	26
1.5.2	Potassium and Ribosome Integrity	27
1.5.3	Potassium and Chromosome Structure	28
1.6	Inhibition of K^+ Transport by Ouabain	29
1.6.1	Ouabain Interaction with the ATPase	30

		Page
1.6.2	Ouabain and Tissue Culture Cells	31
1.6.3	Ouabain and Lymphocytes	32
1.7	The Problem and the Approach	33
2.	MATERIALS AND METHODS	35
2.1	Leukocyte Culture Techniques	35
2.1.1	Source of Cells	35
2.1.2	Preparation of Cell Suspensions	35
2.1.2.1	Standard Procedures	35
2.1.2.2	Preparation of "Purified" Lymphocytes	36
2.1.2.3	Preparation of "Purified" Macrophages	36
2.1.3	Culture Conditions	37
2.1.4	Mitogens and Antigens	37
2.1.5	Inhibitors	38
2.1.6	Washing Procedures	38
2.2	Use of Radioactive Tracers	39
2.2.1	Addition to Cultures	39
2.2.2	Harvesting and Counting	42
2.3	Fluorescent Staining	43
2.4	Treatment of Data	43
3.	RESULTS	44
3.1	Culture Characterization	44
3.1.1	Cell Concentration	44
3.1.2	Antigen and Mitogen Concentration	47
3.1.3	Other Features of the Culture System	47
3.2	Time Course and Activation Levels	50
3.2.1	Time Course	50
3.2.2	Levels of Activation	50
3.3	Inhibition of Response by Ouabain	54
3.4	Prevention of Ouabain Inhibition by Increased K^+ in the Culture Medium	58
3.5	Reversal of Ouabain Inhibition by Washing and Medium Change	58
3.6	Washing Effects	62
3.7	Zoschke-Bach Effect?	65
3.8	Effect of Ouabain Alone day 0-day 2	65
3.9	Intermission	67

		Page
3.10	Viability	67
3.11	Effect of Ouabain 0-2 Uridine Incorporation in Antigen- and in PHA-treated Cells	69
3.12	Macrophages	72
3.13	Other Mitogens and Ouabain	73
3.13.1	Other Mitogens	77
3.13.2	MLR	77
3.14	Other ATPase Inhibitors--Do They Have The Same Effect	80
3.15	Surface Effects	87
3.16	Effect of Length of Exposure and Concentration of Ouabain on Responses to Antigen and Mitogen	89
3.16.1	Effect of One Day Exposure to Ouabain on Antigen Response	89
3.16.2	Effects of Two Day Exposure to Different Concentrations of Ouabain on Mitogen and Antigen Responses	81
3.16.3	PHA and 2 × 10^{-7} M Ouabain--Variation in Effect	94
3.17	Effects of K^+ on Ouabain Inhibition	96
3.17.1	Effect of K^+ on Ouabain Inhibition of PHA Response	98
3.18	The Effect of Pre-incubation in K^+-free Medium on Response to Antigen and to PHA	102
DISCUSSION		105
	Effect of K^+ on the Inhibition	107
	Is The Macrophage Function Affected by the Ouabain Treatment?	109
	Does the Ouabain Affect Only T Cells or Only B Cells?	110
	Is There Inhibition of Synthesis of a Secreted Molecule?	111
	Is This An Effect on a Surface Component?	112
DIRECTIONS		116

List of Figures

		Page
Fig. 1:	Effect of cell concentration on PHA response	45
2:	Effect of cell concentration on antigen response	46
3:	PHA dose response	48
4:	Antigen dose response	49
5:	Time course of PHA stimulation	51
6a:	Time course of SLO stimulation	52
6b:	Time course of VAR stimulation	53
7:	Effect of ouabain on control cultures	55
8:	Effect of ouabain on PHA stimulation	56
9:	Effect of ouabain on antigen-induced activation	57
10:	Effect of extracellular K^+ on ouabain inhibition of PHA response	59
11:	Effect of extracellular K^+ on ouabain inhibition of antigen response	60
12:	Effect of exposure to ouabain for 1,2 or 3 days on PHA stimulation	61
13:	Effect of exposure to ouabain for 1 or 2 days on response to antigen	63
14:	Effect of ouabain on PWM and Con A stimulation	75
15.	Effect of ouabain on the mixed leucocyte reaction	76
16a:	Effect of digoxin on PHA-stimulated cells	82
b:	Effect of acetylstrophanthidin on PHA-stimulated cells	83
17a:	Effect of digoxin on antigen-stimulated cells	84
b:	Effect of acetylstrophanthidin on antigen-stimulated cells	85

List of Tables

		Page
Table 2.1 :	Sources of Materials	40
2.2 :	Standard doses of mitogens and antigens	41
2.3 :	Sources and doses of labelled precursors,	41
3.1 :	Effect of washing and medium change on lymphocyte activation	64
3.2 :	Response of ouabain + antigen-treated cells to PHA and to antigen	66
3.3 :	Effect of ouabain alone day 0-day 2, on lymphocyte activation	68
3.4 :	Viability of cells exposed to ouabain for two days.	70
3.5 :	Effect of two day exposure to ouabain on uridine incorporation in PHA- and antigen-treated cultures	71
3.6 :	Effect of macrophages on antigen response in ouabain-treated cultures	74
3.7 :	Effect of two-day exposure to ouabain on response to PHA, Con A and PWM	78
3.8 :	Effect of two-day exposure to ouabain on the MLR	79
3.9 :	Effect of two-day exposure to ATPase inhibitors on lymphocyte response	86
3.10:	Effect of two-day incubation with ouabain on binding of fluorescent anti-immunoglobulin by lymphocytes	88
3.11:	One-day versus two-day exposure to ouabain:effect on antigen response	90
3.12:	Effect of one-day exposure to 8×10^{-7}M ouabain on antigen response	92
3.13:	Effect of concentration of ouabain (0-2) on inhibition of PHA response	93
3.14:	Effect of concentration of ouabain (0-2) on inhibition of antigen response	95
3.15:	Effect of inclusion of K^+ (26mM) in the culture medium from day 0-day 2 on ouabain (0-2) inhibition of antigen response	97
3.16:	Effect of inclusion of K^+ (26mM) in the culture medium from day 2 to end of culture on ouabain (0-2) inhibition of antigen response	99
3.17:	Effect of inclusion of K^+ (26mM) in the culture medium from day 0-day 2 on ouabain (0-2) inhibition of PHA response	100
3.18:	Effect of inclusion of K^+ (26mM) in the culture medium from day 2-end of culture on ouabain (0-2) inhibition of PHA response	101

Page

3.19: Effect of incubation in K^+-free culture medium day 0-day 2 on response to PHA and antigen 103

3.20: Effect of incubation in K^+-free medium for last 48 hours of culture on PHA and antigen response 104

List of Abbreviations Used in the Text

AcStro: acetylstrophanthidin
ATP (ADP,AMP): adenosine tri (di, mono) phosphate
ATPase: membrane-associated Mg^{++}, Na^{+}-K^{+}-ATPase
ALS: anti-lymphocyte serum

"B" cells: bursal-equivalent or bone-marrow derived lymphocytes.

Con A: concanavalin A (mitogen from Canavalia ensiformis)
cpm: counts per minute of radioactive label

DH: delayed hypersensitivity
DNA: deoxyribonucleic acid
DTT: diphtheria-tetanus toxoids

HPL: human peripheral leukocytes or lymphocytes

LPS: lipopolysaccharide (mitogen from bacteria)

MLR OR MLC: mixed leucocyte reaction or culture.

PBS: phosphate-buffered saline
PHA: phytohemagglutinin (mitogen from Phaseolus vulgaris)
PPD: Purified protein derivative, antigen from tuberculosis bacteria.
PWM: pokeweed mitogen (from Phytolacca americana)

RBC: red blood cells
RNA: ribonucleic acid

SL-O: streptolysin O (streptococcal antigen)
SL-S: streptolysin S (mitogen from Streptococci)

T cells: thymus-derived or -dependent lymphocytes
TAB(T): typhoid-paratyphoid A,B. vaccine + tetanus toxoid

VAR: varidase, streptokinase-streptodornase, (antigen from Streptococci)

1.1 The Lymphocyte

The subject of this work is a population of small round cells, to be found circulating through the blood vessels of the human body. These cells appear to be quiescent, with little function or future, but their capabilities are many and surprising. Lymphocytes are an integral part of our defense against all the foreign material encountered by our bodies, all the time.

Peripheral blood lymphocytes are small (7 to 8 μ in diameter) but very motile cells. The chromatin of the nucleus is highly condensed; a nucleolus is visible only in thin sections. The narrow rim of cytoplasm contains few organelles; there are several mitochondria (less than 25), a Golgi apparatus, and free ribosomes sparsely distributed throughout the cytoplasm (Ham, 1969).

The name lymphocyte is conventionally applied to any cell with the same superficial morphology. However, it is increasingly obvious that this morphological group is functionally heterogeneous. Cells which appear morphologically similar may be functionally quite dissimilar, while functionally similar cells may have very different appearances, related to different states of activation. There is, moreover, much evidence that many aspects of immune functions are regulated by cellular cooperation within the lymphoid system. It is this mixed population with which I work, and which I shall discuss.

1.1.2. Subpopulations, and their Interactions

Lymphocyte subpopulations can now be distinguished

on the basis of many and diverse characteristics: longevity *in vivo*, homing propensities *in vivo*, derivation (thymic or bone marrow), presence of antigens or complement receptors on the surface, buoyant density, electrophoretic mobility, ability to produce antibody or a host of other lymphocyte products, ability to recognize antigen, competence in a variety of immunological assays, differential sensitivity to immunosuppressive agents - (for example see Gowans, 1967).

One of the most popular divisions, proposed by Claman and Chaperon (1969), gives us two major subpopulations, those derived from or dependent on the thymus tissue (T cells) and the rest, which in the chicken are of bursal origin and in mammals are called "bursal-equivalent" or sometimes "bone-marrow" (B) cells. Each of these may in turn be composed of subpopulations, but at this point, at least, functional distinctions between these two are fairly well established. Most of their features are described from studies on mice; much of this work was later seen to be relevant for man as well (Davies et al., 1971; Meuwissen et al., 1969; Roitt et al., 1969).

B cells are considered to be responsible for synthesis and elaboration of antibodies; T cells take care of everything else (i.e. cell-mediated reactions) as well as assisting B cells in their responses.

Immunological memory seems to reside in both populations; both show evidence of specificity in secondary responses. However, work with haptens and carriers has

demonstrated that the specificities of T and B cells to one antigen are directed at different determinants on the same molecule or particle (Schlossman, 1972). This observation led to a model of T cell-B cell interaction in antibody response in which the role of the T cell was to trap antigen with receptors specific for one determinant so as to form a local concentration at some critical site, thus facilitating the response of B lymphocytes to another determinant. However, current theories have been modified to account for the need for the macrophage or adherent cell, and the finding that contact between T and B cells was not necessary. We may suppose that T cells release a specific immunoglobulin which, combined with the antigen, binds to the surface of the adherent cell; this antigen-antibody complex would then be the activator of the B lymphocyte (Feldmann and Nossal, 1972). T cells have also been shown to elaborate some non-specific factors, which might in some instances help activate B cells (Andersson et al., 1972a).

T and B cells can be distinguished in mixed or pure populations by various means. One popular way is to label the cells, directly or indirectly, with fluorescent antibody, which is thought to attach specifically to the immunoglobulin receptors on the B cells. T's are thought to bear complement receptors, and in some species antibodies to a T-specific antigen are obtainable, although these may not label all T cells (Bianco and Nussenzweig, 1971; Haskill et al., 1972).

1.1.2.1 T and B Cells *In Vitro*

In experimental animals such as mice it is easy to obtain relatively pure preparations of one or the other subpopulation, by thymectomy, irradiation, bone marrow transplants, chemical treatments, or genetic selection (Miller et al., 1971). In less intensively studied systems and in man, it is usually easier to obtain T populations by eliminating B cells, by virtue of their binding to various anti-immunoglobulins, than it is to recover the B cells or to selectively eliminate T cells (Wigzell and Andersson, 1971; Wofsy, 1971). Patients with immunological deficiency disorders may be one source of human lymphocytes enriched in one or the other subpopulation (Douglas, 1972); these may not be reliable sources of pure populations, since the lesion may not result in the total absence of one cell type, but rather in its abnormal function, at <u>any</u> point in the immune reaction (Catovsky et al., 1972; Lischner et al., 1973; Wu et al., 1973).

The definitive work of Greaves and coworkers (Greaves and Janossy, 1972) on mouse lymphocytes showed that PHA, Con-A and lentil mitogen activate only T cells; some antilymphocyte sera (ALS) activate only T cells, some both T's and B's; lipopolysaccharide (LPS) activates only B cells; PWM activates both T and B cells. However, suitable manipulation, such as concentrating and insolubilizing Con A or PHA on, for example, sepharose beads, renders these T-specific mitogens stimulatory to purified B cells as well (Andersson et al., 1972b; Greaves and Janossy, 1972); soluble T cell products have also been

reported to render B cells receptive to activation by Con A (Andersson et al., 1972a). Since in most *in vitro*, and all *in vivo* situations there are matrices which can fulfill the role of the sepharose beads (for example macrophages, reticular cells, red cells), in this study in which I used a heterogeneous mixture of cells, none of the common mitogens can be considered specific for either subpopulation. Indeed, Phillips and Roitt (1973) reported that up to 30% of blasts induced by PHA in human lymphocyte cultures had surface markers characteristic of B cells. Phillips and Weisrose (1974) reported PHA response even in "purified" human B cells. Although Geha et al. (1974) and Greaves et al. (1974) were unable to confirm this result, they did agree that in cultures of mixed T and B cells, a fraction of the responding cells could be B cells. It thus seems most likely that mitogen activation of human peripheral lymphocytes (HPLs) *in vitro* involves both T and B cells.

Some *in vitro* assays of immune function are thought to reflect largely the activity of one or the other type of lymphocyte. Production of macrophage migration inhibition factor (MIF), for example, is attributed to T cells (Häyry et al., 1972), as is cytotoxicity of sensitized lymphocytes to allograft or tumor antigens on target cells (Golstein et al., 1972). B cells produce the antibody which is assayed by hemolytic plaque production in a lawn of heterologous red blood cells, and the antibody that is cytotoxic in complement-mediated cytotoxicity (Greaves and Hogg, 1971). Formation of rosettes with heterologous RBC is considered to be a T cell function

in a nonimmune system (in which the lymphocyte donor has not been sensitized) but in an immune system, with RBC coated with antibody and complement, the rosettes form around B cells (Jondal et al., 1972; Jondal and Klein, 1973).

In vitro stimulation by soluble antigen may involve contributions from both subpopulations. The usual approach to this question in man has been to attempt to correlate in vitro activation, as measured by, for example, DNA synthesis, with in vivo functions attributed to one or the other type of cell. Some authors have found correlations between in vitro blastogenesis and delayed hypersensitivity (DH) in vivo (Hirschhorn et al. 1963), while others have been able to correlate it with antibody production, but not DH (Fellner et al. 1967), while still others have been unable to show correlation of such activation with any other assay of immune function (such as MIF production, antibody titer)(Curtis and Hersh, 1973; Simons and Fitzgerald, 1969).

While some authors reported differences in appearance of the blasts between HPL cultures treated with different mitogens or antigens (Douglas, 1972), others found no major differences (Chalmers et al., 1967). Greaves and Roitt (1968) observed two types of cells in antigen stimulated cultures, the majority being like those in PHA cultures (putative T cells), the minority having well developed endoplasmic reticulum and staining with anti-immunoglobulin (putative B cells). Geha and Merler (1974), using purified human tonsil lymphocytes, found that although only T cells proliferated in response to antigen, B cells proliferated in response to supernatants from

responding T cells. Thus, it seems only safe to assume for the present, that the observed or measured response to antigen _in vitro_ probably includes components derived from both sub-populations.

1.1.3.1 Macrophages

There is general agreement in the literature that there is a requirement, in many immune functions, for a non-lymphoid cell type usually referred to as the macrophage. These are large mononuclear cells characterized by phagocytotic activity, high resistance to radiation and adherence to glass or plastic surfaces (Schwartz, 1970).

In vitro manifestations of secondary response show this requirement for macrophages, but their role is not entirely clear. Certainly several reports have shown that an adherent, radioresistant cell population is necessary for optimum, or perhaps even any, stimulation of HPLs by antigen (Cline and Swett, 1968; Hersh and Harris, 1968; Lake et al., 1971), as well as for their participation in mixed leukocyte reactions (MLRs) (Bach et al., 1971; Twomey et al., 1970) and for interferon (Epstein et al., 1971) or antibody production (Feldmann, 1972; Sjoberg et al., 1972). In most cases the macrophages had to be alive, healthy and in close contact with the lymphocytes. They could not be replaced by another adherent cell (for example, autologous fibroblasts) nor by various cell fractions or products (Epstein et al., 1971; Hersh and Harris, 1968).

Although earlier theories about macrophage function centred around a processing of antigen (modification and

possibly combination with a specific RNA before passage to lymphocytes) (Schwartz, 1970) some workers have suggested that the helper function might be a result of increased lymphocyte-lymphocyte contact as they cluster around the macrophage. Current dogma assigns a role of intermediate specificity to the macrophage in *in vitro* secondary response. It is thought to bind a complex of T-cell derived specific immunoglobulin and antigen; this complex, on the macrophage surface, might then stimulate B cells to synthesize antibody (Feldmann and Nossal, 1972).

1.1.3.2 Red Cells and Platelets

Several workers (Newlin, 1970; Tarnvik, 1970; Yachnin, 1972; Yachnin et al., 1972) have shown that autologous red cells and/or platelets could have a significant effect on transformation of HPLs. Yachnin's interpretation of these effects (Yachnin, 1972) was that in the case of mitogens, the potentiation phenomenon resulted from the ability of potentiating particles to construct a concentrated matrix of PHA molecules at their membrane surfaces, for presentation, and more irreversible binding, to sites on the surfaces of responsive lymphocytes. Inhibition of mitogen response by high concentrations of RBC or platelets was considered to be a result of competition for binding, so that most of the mitogen was involved in, for example, red cell-red cell binding and was not available to lymphocytes. Yachnin (1972) felt that the lack of effect of these formed elements on antigen response

simply reflected a lack of specific or nonspecific receptors for the antigen on those cells.

1.2 Specific versus Nonspecific Response

In the fifteen years since PHA was found to stimulate growth and division of blood lymphocytes (Hungerford et al., 1959), an impressive array of other agents has been shown to have broadly similar effects. These can be loosely categorized as either specific or nonspecific, according to their effects on lymphocytes in culture.

1.2.1 Nonspecific Response

The most visible effect of addition of a nonspecific activator (mitogen) to a lymphocyte culture is the appearance of a large proportion (up to 80%) of blast cells one or two days later, many of which will go on to divide. The classical mitogens are extracts from various plants, the most commonly used being PHA, Con A and PWM, although a number of others have been reported. Other mitogenic substances include ALS (Grasbeck et al., 1964; Woodruff et al., 1967), some heavy metals (Hg^{++} (Caron et al., 1970; Yachnin, 1972), Zn^{++} (Ruhl et al., 1971)), sodium periodate (Novogorodsky, 1972; Parker et al., 1972). The important distinguishing features of nonspecific response are its magnitude, which by any measure is many times larger than the specific response;

the time course; which characteristically shows an early peak of activity (for example of DNA synthesis on day 2 or 3 after addition of mitogen)(Douglas, 1972; Loeb and Agarwal, 1971; Rubin et al., 1969); independence of presence of other cell types (although this point has been debated in the literature) and lack of need for prior sensitization (even cord blood and that of neonates respond) (Lindahl-Kiessling and Book, 1964).

1.2.1.2 Kinetics of Mitogen Response

Nonspecific response to mitogens by HPLs is characterized by a rapid transformation of a large proportion of the small lymphocytes into large blast cells. The fraction of the cells which transforms is about 50 to 80% by the second day of culture, depending on such variables as the culture system and the donor of the blood. Since this large number of blasts can be seen before initiation of DNA synthesis in most cells, it can be assumed that it reflects direct or indirect transformation of much of the population and is not derived by replication of a smaller fraction. The large number of blasts persists for several days, and the cells seem to be able to keep proliferating after the first division. Marshall et al. (1969) never saw reversion of blasts to small lymphocytes in their cinematographic studies. The total cell generation time for dividing cells seems to be on the order of 20 hours (Sasaki and Norman, 1966; Sören, 1973).

Con A activation appears to have kinetics similar to that of PHA (Douglas, 1972).

Some investigators have shown the peak of PWM response to be delayed (Douglas, 1972), appearing about the 5th day rather than 2nd, 3rd, or 4th, as with the other mitogens. Others have not found this delay (Chalmers et al., 1967), and one report at least showed the early peak in unpurified cultures, but a delayed one in cultures of purified lymphocytes (Gajl-Peczalski et al., 1969). It seems therefore to depend on culture conditions, and in our hands PWM has the early response characteristic of mitogens.

1.2.2 Specific Response

In the context of this work, this refers to in vitro activation of blood lymphocytes by an antigen to which the donor has been previously sensitized (this is a requirement for response). This treatment also results in formation of blast cells (Pearmain et al., 1963; Schrek, 1963), but these are fewer in number early in the response (less than 5% on the second day) and even at the peak of response, 5 to 8 days after initiation of culture, are not as frequent as in mitogen treated cultures (Cowling and Quaglino 1965; Hirschhorn et al., 1963; Moorhead et al., 1967). There is in this case an absolute requirement for the presence of macrophages (as discussed in 1.1.3.1).

1.2.2.1 Kinetics of Response to Antigen

The central problem in kinetics of in vitro proliferative response to antigen by peripheral blood lymphocytes is whether the numbers of activated lymphocytes measured (by

any means) represent only those cells derived by clonal proliferation of specifically antigen sensitive cells, or includes a population, possibly also clonally proliferating, recruited by the antigen activated cells early in the response. Considering the plethora of products released into the culture medium by activated lymphocytes (Dumonde, 1970; Kasakura, 1970; Meuwissen et al., 1969; Pick and Turk, 1972), the latter seems not unlikely. Although Marshall et al., (1969) who were able to follow, cinematographically, individual human cells proliferating *in vitro*, felt that the number of blasts seen in their cultures could be accounted for solely by successive divisions of a few antigen sensitive cells, their work did not rule out recruitment during the first two days of response. Schellekens and Eijsvoogel (1971) have found good evidence of just such a phenomenon, in human peripheral lymphocytes, while Larralde (1970) had excellent cytogenetic evidence that recruitment accounted for up to 80% of proliferating antigen-treated rat cells. Lack of summation of response to different antigens (Cowling and Quaglino, 1965; Schellekens and Eijsvoogel, 1971) and the low number of cells seen to bind antigen in an *in vitro* culture (Bona et al., 1973; Jiminez et al., 1971; Raff, 1973) also suggest that recruitment plays a major role, if not in *in vivo* immune response, at least in our ability to detect and measure response *in vitro*.

The generation time of a dividing antigen-activated cell has been found to vary widely, from 7.5 to 38 hr. (Bach et al., 1969; Marshall et al., 1969). Marshall et al. (1969)

were able to follow individual cells through up to 6 successive divisions over 8 days in culture.

The late appearance of measurable numbers of blasts or levels of incorporation in specific activation is clearly not a result of a "slow" response, since the onset of mitotic response has been observed even at 48 hrs (Marshall et al., (1969)), but only a reflection of the smaller number of cells involved, so that instead of measuring activation after one or no divisions, we must wait until the activated cells have increased their numbers to the lower limits of sensitivity of our measuring techniques, which may take a few days.

1.2.3 Mixed Lymphocyte Reaction

This reaction, in which lymphocytes from genetically dissimilar donors proliferate when cultured together, exhibits characteristics of both of the above types of response. Like response to specific antigen, the reaction requires adherent cells and takes about 5 days to develop maximum response, which is generally of a lower level than the response to nonspecific mitogens. However, the frequency of responding units has been estimated to be much higher than for any antigen (Zoschke and Bach, 1971) and, as in mitogen response, prior sensitization is not required.

The MLR requires viable, metabolically active cells for stimulus as well as for response (Lindahl-Kiessling and Säfwenberg, 1972). The proliferation is apparently due to recognition of surface markers (other than histocompatibility antigens) on stimulating cells by

specific receptors on responding cells (Yunis and Amos, 1971)

1.3 Events in Lymphocyte Activation

Most of the detailed knowledge of the events following stimulation in lymphocytes has been acquired from cells treated with mitogens, usually PHA, simply because mitogens evoke changes in a large enough number of cells to enable sensitive biochemical determinations to be made. As far as is known, other means of activation (other mitogens, as well as antigens) induce a series of events which is in most respects qualitatively if not quantitatively the same. However, a few differences have been noted and these will be described at the end of the section.

1.3.1.1 Early Metabolic Events

The earliest measurable effects of mitogens on lymphocytes are changes in properties of the cell membrane. Directly after PHA administration the normally high electrical resistance of the membrane is reduced at sites of cell contact (Hülser and Peters, 1971). Within the first three minutes Fisher and Mueller (1968; 1971) found a fourfold increase in turnover of phosphatidyl inositol, almost completely restricted to the plasma membrane. Stimulation of de novo synthesis of lecithin and neutral fats is also very rapid (Resch et al., 1973; Resch and Ferber, 1972; Kay, 1968) and membrane glycoprotein turnover is doubled in 3 hours (Hayden et al., 1970). After five to thirty minutes, increases in ion

(K^+ (Quastel and Kaplan, 1970; Averdunk 1972) and Ca^{++} (Whitney and Sutherland, 1973; Allwood et al., 1971)), glucose (Peters and Hausen, 1971b; Averdunk, 1972), uridine (Peters and Hausen 1971a) and amino acid (Mendelsohn et al., 1971; van den Burg and Betel, 1973) transport are demonstrable, followed by increased uptake of phosphates (Cross and Ord, 1971) and later of folate (Das and Hoffbrand, 1970). Since the uptake of glucose, uridine, K^+ and α-aminoisobutyric acid did not depend on protein synthesis, it was concluded that in these cases pre-existing but inaccessible transport sites had been revealed.

Activity of membrane enzymes is also increased soon after mitogen contact, including adenyl cyclase (within 10 minutes)(Smith et al., 1971) and a Na^+-K^+-independent ATPase (after 30 minutes)(Novogrodsky, 1972). The activity of the former results in a cAMP level in the stimulated cell that is 2-3 times that of resting cells (Smith et al., 1971). However, cGMP levels are soon raised 20-50 fold; this may be a more important signal (Hadden et al., 1972).

1.3.1.2 Proteins

Mitogen-induced protein synthesis is first measurable after a few hours and peaks after 24-48 hours (Kay et al., 1971). Synthesis of new enzymes results in increased activity of carbohydrate metabolism (see 1.3.1.4), of lysozomal (Hirshhorn et al., 1967) and hydroxylating enzymes, and of DNA (see 1.3.1.5) and RNA (see 1.3.1.4) metabolism. After several hours a number of mediators of cellular immunity are formed, some of which are thought to be proteins (Dumonde,

1970; Meuwissen et al., 1969).

1.3.1.3 Carbohydrates

Stimulation of carbohydrate metabolism is detectable 15 minutes after PHA addition. Two to twenty-four hours later the rates of glucose consumption and lactate production as well as the levels of several intermediates of glycolysis were found to be raised. PHA causes a slight activation of the hexose monophosphate shunt in four hours as well as an increase in glycogen turnover. (Roos and Loos 1970, 1973; Loos and Roos 1973).

1.3.1.4 RNA

PHA increases the combined rate of synthesis of all forms of RNA about 4 times. It also increases the half-life of the labile heterogeneous nuclear RNA, changes the pattern of methylation of tRNA and increases the intracellular level of a ribonuclease inhibitor.

PHA affects ribosome activity by causing more efficient utilization of pre-existing ribosomes, by stimulating both the synthesis of the 45S precursor of rRNA and the processing of this precursor into 32S and 18S subunits, and by reducing the rate of 18S RNA wastage, which in resting cells amounts to loss of half the units before complete ribosome assembly.

This general stimulation of RNA metabolism may be partly a result of gene activation. Acceleration of acetylation

of histones and phosphorylation of nuclear proteins, and an increase in template activity of isolated nuclei have been reported, and the ratio of euchromation to heterochromatin changes from 0.56 to 10.8 in the stimulated cells. (Cooper, 1972).

1.3.1.5 DNA

Induction of DNA synthesis in HPLs is delayed for at least 24 hours after initiation of culture with PHA (Bender and Prescott, 1962; Mackinney et al., 1962; Tormey and Meuller, 1965; Soren, 1973). Bender and Prescott found mitoses only after 42 hours of culture; by 72 hours they had numerous mitotic cells, most undergoing their first division. However, Buckner and Pike (1964) and Sasaki and Norman (1966) reported a large proportion of cells in their second division in 72 hour cultures. Michalowski (1963) found very low incorporation of thymidine for the first 30 hours of culture, followed by an increase to a maximum at 60 hours. Sören (1973) reported that most cells started to synthesize DNA between 48 and 72 hours of culture, but that there was great variation in time of initiation: some were in first S at 24 hours, while others were in first S at 120 hours of culture.

Loeb and Agarwal (1971) reported that DNA polymerase activity in PHA-treated HPLs increased immediately prior to replication of DNA. From that time the activity was very closely correlated to the ability of the cells to incorporate ^{3}H-TdR into DNA. The polymerase activity increased 30- to 150-fold, and there was evidence that this resulted from

de novo synthesis of the enzyme (Loeb et al., 1968; Loeb et al., 1970). In a study of the thymidine salvage pathway, Rabinowitz et al., (1970) found that thymidine kinase activity increased in parallel with DNA synthesis.

Not all lymphoblasts go on to divide and it has been suggested that even all those which synthesize DNA do not divide, that some merely extrude their new DNA into the medium and revert to the resting state (Polgar and Kibrick, 1970; Rogers et al., 1972; Sarma and Zubroff, 1973).

1.3.2 Morphological Changes

Lymphocyte transformation consists of a gradual change from a small or medium sized lymphocyte to a large blast cell, so that subclassifications are inevitably arbitrary. Generally in a culture consisting originally of small and medium sized lymphocytes (7-10 μ), which have a dense nucleus, only a thin rim of cytoplasm and few organelles, addition of mitogen results in the appearance, about 24 hours later, of transitional cells. These larger cells (10-12 μ) still have a high nucleus/cytoplasm ratio, but the nuclear chromatin is less dense, and nucleoli may be present. In the following 24 hours, larger (15-30 μ) blast cells begin to appear, these having a lower nucleus/cytoplasm ratio, a leptochromatic nucleus, and a cytoplasm which is basophilic and rich in mitochondria, golgi, ribosomes, etc., and is often vacuolated (Biberfeld, 1971).

1.3.3 Antigen-induced Events

Although the lower level of the response to specific antigens precludes the performance of some of the studies just described for mitogen-activated lymphocytes, a few studies have shown differences between the two modes of activation.

Cooper and Rubin (1965) showed that the newly synthesized RNA molecules recoverable from PHA-stimulated human lymphocytes had a different size distribution from those found in SL-O-stimulated cells. In the antigen-treated cells, RNA's were largely the size of ribosomal RNA precursors, while in mitogen treated cells the new molecules were clearly non-ribosomal. Thus although both modes of stimulation resulted in increased RNA synthesis, there was a clear difference in the RNA metabolism involved.

Lucas (1970) looked at phospholipid changes in human tonsil lymphocytes stimulated with PHA, SK-SD and tetanus toxoid. He found that SK-SD, like PHA, stimulated incorporation of phosphate into phosphatidyl inositol, while tetanus toxoid stimulated incorporation not into phosphatidyl inositol but into phosphatidyl choline and phosphatidyl ethanolamine. He suggested that this was due to a diversity of membrane receptors for the different mitogens.

Souleil and Panijel (1972,1973) have reported a difference in the DNA replicative processes following mitogen or antigen treatment of guinea pig lymph node lymphocytes. Observations on BUdR-labelled DNA isolated during in vitro response to antigen suggested that in these cells the number

of separate DNA units undergoing replication simultaneously is higher than in non-specifically stimulated cells. However, Lanotte and Panijel (1973) found that DNA replication in cells activated in a MLR was similar to that in PHA stimulated cells, and unlike that induced by specific antigens.

There may indeed be more differences between specifically and non-specifically stimulated lymphocytes, but due to the paucity of cells available for study in the former, especially in humans, these remain to be elucidated.

1.3.4 The Lymphocyte Surface

Components of the outer membrane surface obviously play a crucial role in all reactions involving lymphocytes. However, the sequence of events at the surface and the relation of this to the more distal events in activation have yet to be elucidated. Lymphocytes have membranes which seem to fit the "fluid mosaic" model of Singer and Nicolson (1970). That is, a lipid bilayer studded with movable protein and glycoprotein complexes, such as enzymes, antigens and receptors of various types. Some of the differences between T and B lymphocyte capabilities undoubtedly reflect differences in the receptors or markers on their surfaces. For example, only B cells are thought to bear large amounts of surface immunoglobulins (Vitetta and Uhr, 1973), while only Ts are thought to carry the site for recognition of foreign antigens on other lymphocytes (Benacerraf and McDevitt, 1972). However, not all of the distinctions can be ascribed to surface variations; for example

murine Ts and Bs bind soluble PHA equally well, but only Ts respond by proliferating (Greaves et al., 1972; Stobo et al., 1972). Also, Loor (1974) was unable to detect surface binding of PWM to either T or B cells, although it activates both types of cells.

The native distribution of immunoglobulin receptors and of binding sites for lectins is apparently diffuse and random (de Petris and Raff, 1973; Loor, 1974). However, soon after administration of labelled lectin or anti-immunoglobulin, the distribution can be seen to change. First there may be formation of "patches" of label. As this can occur in the cold and in the presence of metabolic inhibitors, it is probably a process of passive diffusion. This may be followed by "cap" formation, when multivalent lectins or antigens are involved and if the cell is metabolically active. In this process, the patches are actively moved to one pole of the cell; how the patches are segregated from unlabelled membrane, or how they are moved, is not yet known. Pinocytosis of the labelled cap usually follows, although in some cases the material accumulated in the cap is shed. Pinocytosis can also occur in the absence of capping, (de Petris and Raff, 1973; Loor, 1974). That endocytosis of bound mitogen or antigen is not a prerequisite for activation is evident from studies with mitogen or antigen linked to sepharose beads: these can still stimulate lymphocytes (Geha and Merler, 1974; Greaves and Bauminger, 1972).

Loor (1974) showed that lectin binding sites on murine lymphocyte membranes could move independently of immunoglobulin receptors, although caps of both formed at the same pole of the cell.

Thus, although it is clear that one result of treating lymphocytes with mitogens is interaction with membrane receptors and movement of these receptors, it is not clear which specific event(s) could trigger transformation.

1.4 Cation Transport

The distribution of the monovalent cations K^+ and Na^+ is invariably different inside and outside living cells. The extracellular environment has a relatively high Na^+ concentration and a low K^+, while inside the cell the situation is reversed. Since the cell is permeable to both cations, K^+ must be pumped into the cell and Na^+ out in order to maintain a state inside the cell which is largely independent of the composition of the extracellular electrolytes. This process is important in regulation of cell volume, transport of other substances and certain other cellular activities.

1.4.1 Transport of K^+ and Na^+

Since the sodium and potassium ions are being moved across the membrane against a concentration gradient, energy must be expended to accomplish the exchange. This energy is obtained by hydrolysis of ATP, which binds on the inside of the membrane, where the reaction products, ADP and P_i, are

liberated. This hydrolysis and the coupled transport of K^+ into and Na^+ out of the cell constitutes an enzyme complex known as the "membrane-associated magnesium-dependent sodium and potassium activated adenosine triphosphatase", generally referred to as the Na^+-K^+-ATPase or the sodium-potassium pump. This system requires the combined presence of sodium and potassium for activation, and in the intact cell the effect of sodium is from the inside and that of potassium from the outside. There is competition between the two ions for both sites but the respective affinity ratios are quite different (Skou, 1973; Schwartz, 1972).

Such detailed knowledge as exists about the activities of the pump <u>in situ</u> has been obtained largely from studies with isolated squid giant axons and red blood cells from various species. However, Lichtman et al., (1972) have accumulated evidence which points to the existence of a system of pumps and leaks in the human lymphocyte qualitatively similar to that of the red cell for maintaining intracellular ion concentrations.

1.4.2 Cation Transport and Transport of Other Substances

Transport of K^+ and/or Na^+ has been shown to be intimately involved in the transport of other substances into the cell against a gradient. Riggs et al., (1958) found that in the case of amino acid uptake into Ehrlich ascites cells, the presence of internal K^+ and external Na^+ was critical. They also found that K^+ ions migrated outward in exchange for Na^+ ions during the uptake of the amino acids. Crane et al., (1961) found that accumulation of actively transported

sugars into hamster intestinal tissue required active transport of Na^+ ions. Both the Na^+ and K^+ ion concentrations were critical for uphill transport of galactose in rat kidney slices (Almendares and Kleinzeller, 1971; Kleinzeller and Kotyk, 1961) while both Csaky et al. (1961) and Crane et al. (1961) observed that inhibitors of alkali metal cation transport were also inhibitors of intestinal monosaccharide transport. It is evident, therefore, that in tampering with K^+ and Na^+ transport one must be aware that there is also an effect, even if indirect, on transport of various other substrates.

1.4.3 Cation Transport and Cell Volume Regulation

For a cell to maintain a fixed volume, it must limit the free diffusion of salts and water between the cell and its environment. The mechanism of regulation must be sensitive to very slight changes in the intracellular concentrations of some of these. Animal cells in general regulate their volume by ion pumps that oppose the leakage of ions across the membrane. Thus the continued function of the sodium and potassium transport system regulates cell volume (Robinson, 1968).

Jung and Rothstein (1967), who studied cation metabolism in relation to cell size in an exponentially growing mouse lymphoma cell line, pointed out that in growing cells, as opposed to the steady state red cell in which most of the groundwork has been done, the continuous increase in size presumably reflects a continuous increase in the cellular osmotic content. Since sodium and potassium make up a large proportion of the cellular osmotic content, growth in volume may reflect,

in large part, an accumulation of these cations (and accompanying anions). Growing cells must therefore be geared to continuous accumulation of electrolytes, rather than to maintenance of a steady state.

1.4.4 Effect of Antibody on Cation Transport in LK Sheep Red Cells

In populations of sheep the RBC of some individuals contain a K^+ concentration typical of animal cells (about 80 mM) while the majority of individuals have red cells with a low K^+ concentration (about 13 mM) (Evans and King, 1955). These two phenotypes are determined by a single genetic locus with two alleles; the allele for low K^+ (LK) is dominant. The major difference is apparently in the number of membrane pump sites, the HK cells having about six times the number on the LK cells (Dunham and Hoffman, 1971). Lauf et al. (1970) found that treatment of LK cells with an antiserum to a membrane antigen present only on cells of homozygous LK sheep stimulated active transport in the LK cells both by changing the kinetics of the pump and by increasing the number of pump sites. This finding is interesting because it demonstrated that interaction of a membrane antigen and its antibody can affect membrane transport.

1.5 Role of K^+ in Cell Functions

As the dominant intracellular cation, K^+ would be expected to have a significant role in cellular functions.

It has been shown to be involved in cell respiration, glycolysis, activity of many enzymes, macromolecular synthesis and fat metabolism (Kernan, 1965). However, it is sometimes difficult to differentiate among the effects of changes in the activity of the cation pump, the effects of the actual level of the cation in the cell, and the effect of the cation on the transport of other substances.

For example, in Lubin's (1967) report of the inhibition of macromolecular synthesis caused by low intracellular K^+, this low K^+ was achieved not by inhibition of the pump but by increasing leakage through the membrane, and Byrnes et al.'s (1973) finding of stimulation of DNA polymerase activity by K^+ was in a cell-free system. However, other workers have suggested that the ouabain sensitivity of their systems might be due to the inactivation of the pump in turn affecting the levels of ADP in their cells (Whittam and Blond, 1964; Pena et al., 1969).

1.5.1 Monovalent Cations and Enzyme Activation

Enzymes activated by K^+ are involved in a great variety of reactions and in all organisms (Lubin, 1964). Some such as pyruvate phosphokinase taken from sources in several phyla were found to be uniformly K^+ dependent, while others, such as S-adenosylmethionine synthetase are K^+ dependent in eukaryotes (where studied) but not in bacteria (Lubin, 1964).

Cations may participate in the reaction as part of a complex with the substrate (for example, with ATP in phosphoryl transfer reactions) or they may be instrumental

in maintenance of a specific protein conformation necessary for optimum catalytic activity (for example, pyruvate kinase) (Suelter, 1970). Suelter (1970) compiled a list of monovalent cation-activated enzymes, which he classified into a few types of reactions. He felt that the sum of data suggested an interaction of the cation with the substrate and enzyme to form a functional ternary complex.

It is noteworthy, in the light of the widespread importance of cAMP levels in cells, that at least in some tissues, (including rat diaphragm (Lundholm et al., 1967), fat cells (Mosinger and Kujalova, 1966) brain (Suelter, 1970) and thyroid (Burke, 1970)) the activity of adenyl cyclase can be augmented by high levels of K^+.

It is evident that K^+ is intimately involved in a number of reactions critical to cell function.

1.5.2 Potassium and Ribosome Integrity

Potassium is apparently critical in the maintenance of ribosomal structural integrity. *In vitro* studies of mammalian ribosomes have shown that as a result of K^+ deficiency, monoribosomes lose their ability to use poly-U as an artificial message for synthesis of polyphenylalanine and dissociate into subunits or are transformed into smaller particles. These effects are not reversed upon incubation in medium containing K^+ (Näslund and Hultin, 1970).

Although endogenous amino acid incorporation by polyribosomes is more resistant to the effects of K^+ depletion, this treatment does result in a characteristic gradual change

in their sedimentation patterns, suggesting that mixed aggregates are formed consisting of normal and structurally modified particles. This destabilization of ribosomal internal structure may be due in part to the absence of the stabilizing effect of monovalent cations on the secondary structure of RNA (Näslund and Hultin, 1971). In any case, if this disruption of ribosome structure is also a result of depletion of cellular K^+, such treatment could be expected to have diverse effects, some perhaps quite subtle.

1.5.3 Potassium and Chromosome Structure

Alterations of chromosome morphology, visualized as condensation or uncoiling of chromatin, are directly related to changes in gene activity. In the polytene chromosomes of dipteran salivary glands, gene transcription can be visualized as puffs of expanded chromatin at particular sites along the chromosome, functionally related to the transcription of particular genes. During embryogenesis these puffs appear and disappear at consistent and specific stages, but various treatments can induce some puffs to appear off-schedule. Berendes et al. (1965) and Kroeger (Kroeger, 1963; Kroeger and Miller, 1973) have done this with high potassium solutions. Kreuger (1963) hypothesized that the treatment with salts mimicked the effect of the hormone ecdysone. He suggested that the hormone altered the membrane potential, causing a stimulation of the sodium pump, thereby increasing cellular K^+ (at the expense of Na^+), which increase resulted in a changing of the puffing pattern and release of a specific

selection of messenger RNAs to accomplish specific changes in cell metabolism.

1.6 Inhibition of K^+ Transport by Ouabain

Cardiac glycosides such as ouabain (strophanthin), from *Strophanthus*, and a variety of digitalis compounds, have long been used in treating congestive heart failure, but their cellular effects have just begun to be clarified. One glycoside, ouabain, is widely used as a specific inhibitor of the Na^+-K^+-ATPase. It was first observed by Schatzman (1953) that the active movements of Na^+ and K^+ in RBC were prevented by low concentrations of strophanthin-K (ouabain), one of the glycosides. The compound did not seem to affect energy-yielding reactions, and since it had no effect on cation movements when cells were kept cold, was not likely to be affecting only membrane permeability. It was noticed that half maximal inhibition of cation transport occurred at concentrations similar to those required for inhibition of the Na^+-K^+-activated ATPase (Bonting et al., 1962; Post and Albright, 1961). Caldwell and Keynes (1959) showed that the site of action of ouabain was not inside the cell but at the outer surface. Several workers (Bonting et al., 1964; Dunham and Glynn, 1961; Glynn, 1957; Kinsolving et al., 1963) showed that only the active transport of K^+ was affected and that increasing extracellular K^+ decreased the ouabain inhibition. Studies on ouabain binding to RBCs revealed a competition between ouabain and K^+ for binding sites, with binding of ouabain strikingly paralleled

by inhibition of the Na^+ pump (Baker and Willis, 1970). Since ouabain doesn't seem to enter the cell, and since its effects are largely preventable or reversible by high concentrations of K^+, any results of its application are considered to be directly or indirectly a result of its inhibition of active transport of Na^+ and K^+.

1.6.1 Quabain Interaction with the ATPase

It is a characteristic of the enzyme system described in 1.4.1 that it is inhibited by cardiac glycosides (Skou, 1973). Matsui and Schwartz (1968) showed that the binding of tritium labelled digoxin to a heart ATPase required ATP and magnesium, was stimulated by Na^+ and depressed by K^+. Some workers have suggested that combination of quabain and the enzyme is irreversible under physiological conditions (Albers et al., 1968); however this is not in agreement with other work (Baker and Willis, 1970). The consensus now is that ouabain binding is reversible (Hokin and Dahl 1972). There are some reports of a stimulatory effect of ouabain on the enzyme, when the drug was at low concentrations (10^{-10}-10^{-7} M -- which is within the therapeutic range) under conditions where higher concentrations were inhibitory (Bonting et al., 1964; Palmer et al., 1966). Others have failed to obtain this result (Kinsolving et al., 1963).

At least at higher concentrations, the effect of glycosides, including ouabain, on this enzyme system is clearly one of inhibition due to binding of the drug such that potassium cannot bind.

1.6.2 Ouabain and Tissue Culture Cells

This drug has not been widely used on cells growing in tissue culture; most studies of its effects are done on slices of heart or skeletal muscle. Some of the studies that have been done involved common cell lines derived from mice, which are quite refractory to ouabain effects, *in vivo* and *in vitro*. Such studies are not necessarily relevant to work on human cells, and will not be discussed here.

Vaughn (1972) treated HeLa cells with ouabain for a few hours at a time, and found that within 3 hours enough new ouabain-binding sites were generated that K^+ transport was fully restored. Of course, this recovery was observed only as long as transport was not totally inhibited. He felt that the cell was responding to the localized damage to normal function resulting from ouabain binding by synthesizing new enzyme systems.

Lamb and McCall (1972) grew HeLa and Girardi (human heart) cells for up to 24 hours in various concentrations of ouabain. They reported a rise in intracellular sodium and an equal fall in potassium, proportional to the ouabain concentration used (above 5×10^{-8} M). Inhibitor-treated cells attained new steady-state concentrations of Na^+ and K^+; the rate of attainment also depended on the concentration of ouabain, but the new equilibrium was reached after 8 to 24 hours. Once the changed level was reached it was maintained for at least five days (Boardman et al., 1972). They found that ouabain in the culture medium affected the growth rate of the cells, but not their volume. The effects were exacerbated by lowered

extracellular K^+ and prevented by increased extracellular K^+. The drug had no effects on Cl^- content and fluxes, nor on Ca^{++} content and fluxes, nor on membrane potential.

1.6.3 Ouabain and Lymphocytes

The work of Quastel and Kaplan showed that ouabain, at relatively low concentrations (down to 10^{-8} M) had a profound inhibitory effect on RNA, DNA and protein synthesis, respiration, and blast transformation of HPLs stimulated *in vitro* by PHA (Quastel and Kaplan, 1968; Quastel and Kaplan 1970a,b,; Quastel et al., 1970). This inhibition was antagonized by the inclusion of excess K^+ (up to 26 mM) in the culture medium, and could be reversed simply by washing the cells. Studies on K^+ transport showed that PHA had no effect on K^+ efflux but caused an increased influx, which could be inhibited by ouabain. They suggested that this increased K^+ transport was necessary for lymphocyte transformation, which would account for the great sensitivity of activated lymphocytes to ouabain.

Averdunk (1972) also studied K^+ transport in human lymphocytes, treated with PHA or ALS. He found an increase in K^+ influx which, in combination with a slight reduction in the rate of K^+ efflux, resulted in increased concentrations of K^+ within the cells.

Kay (1972) looked at the immediate increase in RNA synthesis after addition of PHA in HPL cultures. Ouabain did not affect this early increment in uptake of labelled precursors although it did inhibit the later, larger increase. This lack

of inhibition was observed even in cells preincubated with the drug for many hours. He suggested that the sodium/potassium balance was the critical factor, and that inhibition of RNA synthesis might be secondary to inhibition of protein synthesis.

Van den Burg and Betel (1974) reported that the Con A-induced increase in α-aminoisobutyric acid uptake in rat lymphocytes, although dependent on energy generation and on protein synthesis, was not affected by 1 mM ouabain (which resulted in 80% inhibition of the Na^+-K^+ ATPase).

1.7 The Problem and the Approach

Previous work of Quastel and Kaplan showed conclusively that inactivation of the membrane ATPase by specific inhibitors caused arrest of all the parameters of PHA-stimulation of HPLs that were investigated: synthesis of protein, RNA and DNA, transport of K^+, respiration, blast transformation and mitosis. Since PHA caused an increase in K^+ influx but no change in its rate of efflux, and since this did not depend on protein synthesis, and was not associated with an increase in the affinity of existing enzymes, they hypothesized that one effect of PHA might be to change the configuration of the surface glycoproteins such that previously cryptic transport sites were revealed and activated. The increase in binding of tritiated ouabain after PHA stimulation seemed to support this hypothesis, which would account for the great sensitivity of transforming lymphocytes to the inhibitor.

The work of Quastel and Kaplan demonstrated that K^+ transport was important in PHA-induced lymphocyte transformation. Considering the diversity of nature and function in the lymphocytes in human peripheral blood, it was of interest to determine whether lymphocytes stimulated in other reactions would be similarly sensitive to inhibition of K^+ transport. Human peripheral leukocyte cultures and soluble antigens to which the cell donors were sensitized were used to investigate the ouabain sensitivity of antigen-induced activation. It was found that although in many respects responses to antigen and to mitogen seemed equally sensitive, under some conditions response to antigen was irreversibly lost, while mitogen response was relatively unaffected. This work is thus concerned with elucidation of the details of this differential sensitivity and demonstrates that it shows promise as a tool for the dissection of lymphocyte actions and interactions in *in vitro* immune responses.

2. Materials and Methods

2.1 Leukocyte culture techniques

2.1.1 Source of cells

Donors were volunteers, apparently healthy, most male, most aged 20 to 30 years. Peripheral venous blood was withdrawn into heparinized syringes, then distributed into glass vials to which had been added dextran, to give a ratio of blood:dextran 7.5:1. These were left at 37°C for 1/2 to 1 hour, until the red cells and plasma were clearly separated and each about half the total volume. All further preparations were done at room temperature.

2.1.2 Preparation of cell suspensions

2.1.2.1 Standard procedures

In experiments done in tubes, the leukocyte-rich plasma was added so as to be 15% of final culture volume. The cells, therefore, were added in varying numbers, depending on the cell count of the donor. The WBCs in the final culture suspension were counted and a plasma smear was made and later stained with Wright's stain for a differential count. Thus, although the lymphocyte concentration was not controlled, it was known. It was usually 0.5 to 0.8 × 10^6 per ml.

In experiments done in plates, the cells were prepared so that in the culture suspension there were 10^6 lymphocytes per ml., unless a variation was part of the experimental design. The leukocyte-rich plasma was removed from the settling vials and centrifuged, the plasma removed

and reserved and the cell button resuspended in medium. An aliquot of this cell suspension was diluted in Turk's solution (2% acetic acid, 0.01% gentian violet in water), which stained the nuclei so that the mononuclear cells could be distinguished. Aliquots of cell suspension were added to culture medium (medium 199 + 15% autologous plasma) to give the appropriate cell concentration. Cell viability was checked using exclusion of trypan blue as the criterion for life.

2.1.2.2 Preparation of "purified" lymphocytes

These were prepared by passage of leukocyte-rich plasma through a glass syringe partly packed with glass wool, in a modification of the Rabinowitz technique (Rabinowitz, 1964), developed by Quastel (1970). Cells recovered from this column after a 1/2 hour incubation appeared to be at least 99% pure lymphocytes. Recovery of lymphocytes was never close to 100% but was generally between 50 and 80%. Usually, cells prepared in this manner showed normal PHA response but no response to antigens.

2.1.2.3 Preparation of "purified" macrophages

These were prepared according to Alter and Bach (1970). Unpurified leukocytes were plated with medium 199 + 30% autologous plasma in plastic petri plates and incubated in a humidified, 5% CO_2 in air atmosphere for 4 to 6 hours. Nonadherent cells were then removed by washing twice with PBS + 10% plasma. After a further two days of

incubation, plates were washed with PBS and trypsinized to remove adherent cells. These were washed and resuspended in medium + heparin (to prevent clumping) and counted. Appropriate numbers were then added to cultures of purified lymphocytes, to give a ratio of macrophages:lymphocytes of between 1:100 and 1:10.

2.1.3 Culture conditions

For tube experiments, culture medium consisted of 15% leukocyte-rich plasma + 85% TC 199 (without antibiotics, to reduce unplanned antigen responses). Aliquots of 3 or 2.5 ml. were distributed to 75 mm. plastic screw-capped tubes. These were tightly closed and incubated in a vertical position until harvested, usually 3 to 7 days later.

For microplate experiments, culture medium was made up with 15% fresh autologous plasma, the appropriate volume of cell suspension, and enough TC 199 to make up the rest. Aliquots of 200 λ were distributed by Oxford pipettor to the wells of a microplate (capacity 0.330 ml.). The plates were covered and kept in a small plastic box with a humidified 5% CO_2-in-air gas phase in an incubator until harvested.

K^+-free medium, used in some experiments, was custom-prepared medium 199, with NaCl but no KCl. (Gibco)

2.1.4 Mitogens and Antigens

These were added to the culture medium before distribution to the tubes or wells when possible. Otherwise

they were added as 0.05 ml. to 2.5 ml. cultures, or as 25 or 50 λ of a solution diluted in medium to microplate cultures. Standard doses were selected on the basis of dose response experiments, excep[illegible]r Con A and PWM, where doses used were the same as thos[illegible] by my colleagues. Table 2.2 is a list of the standard doses, given as ml. of stock (made up according to manufacturer's instructions) per ml. of culture. Antigens which came as liquid preparations were dialyzed overnight against at least 100 volumes of PBS, to remove toxic preservatives.

2.1.5 Inhibitors

In most experiments ouabain was used as a specific inhibitor of the membrane Na^+-K^+-ATPase. In some, digoxin and/or acetylstrophanthidin (Acstro) were used for comparison.

If the purpose of the experiment was to totally inhibit the cells, the inhibitor was added either at the time of initiation of culture or one day before harvesting. In either case, stock of each concentration was made up so as to add 0.02 ml. stock per ml. culture. Inhibitors to be added to microplate cultures were diluted in medium so as to add 25 or 50 λ per culture.

In experiments in which the ouabain was included in the culture medium from day 0 until the medium was changed, it was added as 0.02 ml. stock per ml. culture before addition of culture to the cells of the microplate.

2.1.6 Washing procedures

Critical to the results is the procedure used to

wash the cells after their two day exposure to ouabain. Cultures in the tubes were centrifuged gently, all but 0.5 ml. of the medium was removed by suction through a pasteur pipette, fresh medium was added, cells were resuspended by mixing on a vortex mixer then spun again. This was repeated and after the second wash, fresh culture medium, with or without mitogen or antigen was added. The caps were tightened, the cultures mixed and reincubated.

Cultures in microplates had about 150 λ of their medium gently removed and 150 to 200 λ of wash medium gently added. The plates were then left to sit at least 15 minutes to allow mixing of the wash and culture media and settling of any disturbed cells. The wash medium was removed and replacement medium added. Both wash and replacement media contained 15% autologous plasma, and antigens or mitogens were added to the replacement medium before distribution.

The removed culture and wash medium contained so few cells that it was not possible to count them even when concentrated 100 to 200 times.

2.2 Use of radioactive tracers

2.2.1. Addition to cultures

In most experiments, incorporation of ^{3}H-thymidine, presumably into DNA, was used as the measure of activation. In a few experiments, ^{3}H-uridine, or ^{14}C-thymidine was used. Table 2.3 gives the specific activities and amounts used in culture for all of these. They were added to the tubes as 0.2 ml., and to the microplates as 25 or 50 λ, diluted

Sources of Materials 2.1

Name	Number	Source	
heparin	50770	General Biochemicals	Chagrin Falls, Ohio.
dextran (Macrodex, 6% in saline)		Pharmacia	Montreal
TC 199	1135H	Gibco	Grand Island, N.Y.
culture tubes		Falcon Plastics	
microplates	3040	Falcon Plastics	Oxnard, California
lids	3041	Falcon Plastics	Oxnard California
M.A.S.H.		Otto Hiller	Madison, Wisconsin
LSC vials		New England Nuclear	Dorval, P.Q.
minivials & carriers		Picker Nuclear	Ottawa
Aquasol	NEF 934	New England Nuclear	Dorval, P.Q.
Econofluor	NEF 941	New England Nuclear	Dorval, P.Q.
radioactive precursors : see table 2.2			
PHA phytohemagglutinin-M	05-2857	Difco	Detroit Mich.
PWM	536	Gibco	Grand Island, N.Y.
Con A	234567	Calbiochem	LaJolla, Calif
DTT		Wyeth	Marietta, Pa.
TABT		Connaught Med. Res. Lab.	Willowdale, Ontario
VAR(SKSD)		Lederle	Pearl River, N
SLO streptolysin-O reagent		Gibco	Grand Island, N.Y.
ouabain (strophanthin-G)	O3125	Sigma	St. Louis, Mo.
digoxin	D6003	"	" " "
acetylstrophanthidin	A3259	"	" " "
mitomycin C	M0503	"	" " "

Table 2.2

Standard doses of mitogens and antigens

mitogen	ml. stock/ml. culture volume used
PHA	0.02
Con A	0.01
PWM	0.015
DTT	0.01
TABT	0.01
SLO	0.02
VAR (SK-SD)	0.02

Table 2.3

Standard doses of radioactive precursors

precursor	catalogue number[1]	specific activity	concentration in culture
Thymidine-methyl-^{3}H	NET 027	6.7 Ci/mmole	1 µCi/ml.
Thymidine-methyl-^{3}H	NET 027X	20 Ci/mmole	1 µCi/ml.
Thymidine-methyl-^{14}C	NEC 568	54.7 mCi/mmole	0.02 µCi/ml.
Uridine-^{3}H (G)	NET 028	5-15 Ci/mmole	1 µCi/ml.
Uridine-5-^{3}H	NET 174	>25 Ci/mmole	1 µCi/ml.

[1]New England Nuclear, Dorval, Canada

in 199 in each case.

Originally a 2 hour period for incorporation was used, but although this was adequate for good separation of mitogen and control results, a longer incorporation time proved better for distinguishing the lower levels of antigen activation. This was generally 4 hours. Isotopes were added using sterile technique and further incubation was at 37°C.

2.2.2 Harvesting and counting

Cultures in tubes were harvested by centrifugation and washing, once with PBS, twice with cold 5% TCA. The resulting precipitate was solubilized overnight in hyamine hydroxide then mixed with 15 ml. Aquasol (a dioxane-based premixed cocktail) in standard glass or plastic scintillation vials.

Microplate cultures were harvested on the multiple automatic sample harvester (MASH) designed for that purpose (Hartzmann et al., 1972). Brief washes with saline or water and methanol sufficed to give a labelled precipitate on glass fiber filter strips. The filters were left to dry, then counted in 3 ml. of Econofluor (a toluene-based premixed cocktail) in minivials.

Most of the counting was done on a Beckman LS 233 machine, at ambient temperature. Samples were automatically counted for 5 minutes or to 1% efficiency, whichever came first.

2.3 Fluorescent staining

Detection of immunoglobulin-bearing cells was done using a fluorescent anti-human immunoglobulin (Burroughs-Wellcome) according to the method of Sheldon et al. (1973). Briefly, the glass-wool purified lymphocytes were washed and incubated for 30 minutes at 4°C with the anti-immunoglobulin. The preparations, made in duplicate, were observed under normal and under fluorescent light and 200 cells per slide (i.e. per preparation) were counted and scored for fluorescence.

2.4 Treatment of data

There were always more than two replicate samples: in the tubes, 3 or 5, in the microplates generally 4. These numbers were selected for convenience of handling, in the racks or on the plates.

Data analysis consisted of a comparison of treated and control means, using the least significant difference (lsd) to determine whether there was activation in a given set of cultures. The comparisons were made on square root transformed data, since untransformed data do not satisfy the assumptions for this kind of analysis, i.e. they are not normally distributed and the variances are not independent of the means. The lsd is basically a student's t test using a pooled error variance, obtained in this case from an analysis of variance (anova) on the transformed data from 26 experiments, as follows.

Analysis of Variance - Results

Source of variation	D.F.	S.S.	MS
sources	25	226691.2082	9067.6483
treatments	2	244149.5021	122074.7511
duplicates	3	243.2463	81.0821
S × T	50	282085.8364	5641.7167
S × D	75	6442.0595	85.8941
T × D	6	1335.7230	222.6205
S × T × D	150	14319.8260	95.4655
error	234	775267.4015	95.4737

$$S = \sqrt{\frac{2s^2}{r}} = 6.9092$$

$$lst\ .05 = t\ .05\ S\bar{d} = 14.2606$$

3. RESULTS

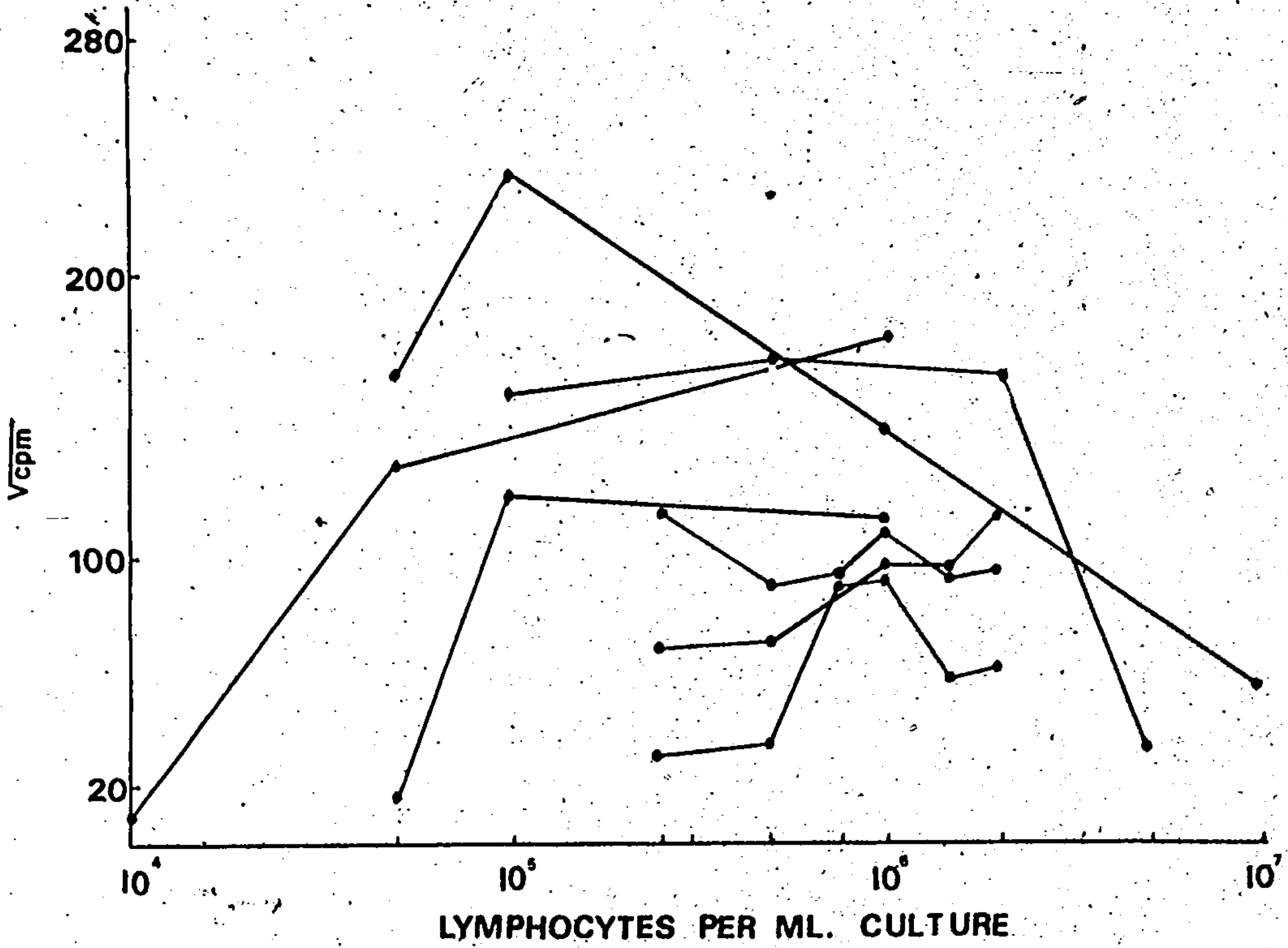
3.1 Culture characterization

3.1.1 Cell concentration

One of the most important variables in the culture system is the cell concentration. Since this was not standardized in the first experiments, done in tubes, it could not be shown that effects were not due to suboptimal culture conditions. One tube experiment on cell concentration versus response showed that PHA response varied little between 0.2 and 1.1×10^6 lymphocytes per ml., whereas SLO response was very low at 0.2×10^6 and high between 0.6 and 1.1×10^6 lymphocytes per ml. The control value did increase a little with higher concentrations.

When work was begun on the microplates, several experiments were done to determine optimum cell concentration. Control counts did not vary much with changing cell concentration. The optimum for PHA response on day 5 showed a great deal of interindividual variability (Fig. 3.1) but since antigen (SLO or VAR) response was usually greatest at or about 1×10^6 lymphocytes per ml. (Fig. 3.2) and PHA also gave high counts at this concentration, this was used as the standard. It might be noted at this point that response to antigen is still observed when the cell numbers are halved, although very much more dilution leads to no detectable response. This will become relevant in discussion of the effect of ouabain on the cells' response to antigen.

Figure 1: Effect of cell concentration on PHA response. ^{3}H-thymidine incorporation in PHA-treated microplate cultures. PHA was added on day 0, cultures were harvested on day 5. Each line represents one experiment.



280
200
100
20
√cpm
10^4
10^5
10^6
10^7
LYMPHOCYTES PER ML. CULTURE

Figure 2: Effect of cell concentration on antigen response. ^{3}H-thymidine incorporation in antigen-treated microplate cultures. Antigen was added on day 0 and cultures were harvested on day 5 or 6. Each line represents one experiment.
SLO ●———●, VAR O----O.

√cpm

140
130
120
110
100
90
80
70
60
50
40
30
20
10

10^4
10^5
10^6
10^7

LYMPHOCYTES PER ML. CULTURE

In summary, the cell concentration used in most of the experiments (1×10^6 lymphocytes per ml. culture) was that found to give maximum antigen response in all donors tested, on the days tested.

3.1.2 Antigen and Mitogen Concentration

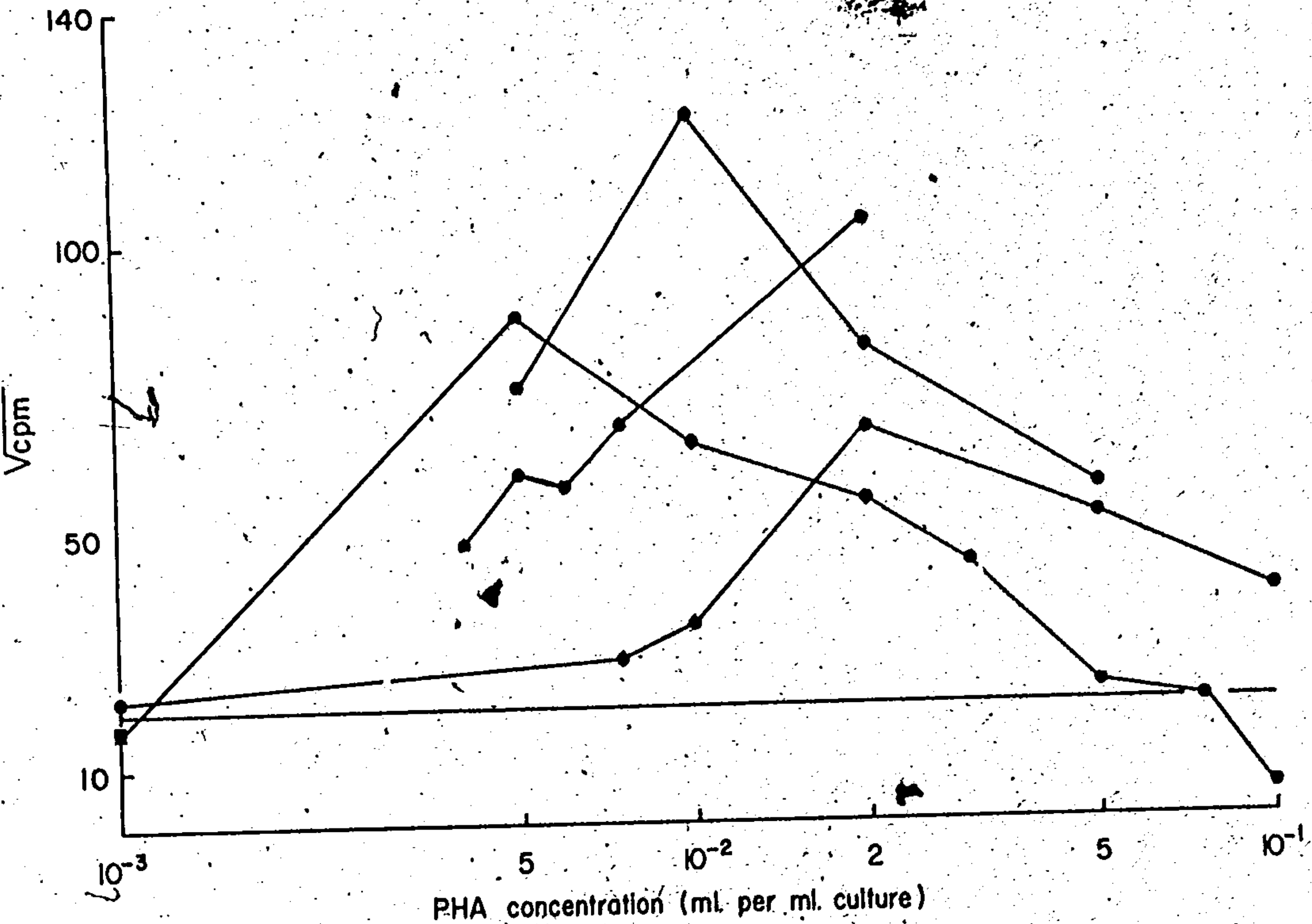
Perhaps the other most important factor in the culture technique is the concentration of antigen. It must be high enough to give maximum stimulation without being toxic to the cells. In the earlier experiments in tubes, a narrow range of antigen and mitogen concentrations was tested, comparable to those used in published work, and in other laboratories. A few experiments in the microplate system showed these to be optimum (Figs. 3.3 and 3.4): 0.01 to 0.02 ml. stock per ml. culture for SLO, VAR, and PHA.

3.1.3 Other features of the culture system

These include choice of medium, choice and concentration of autologous plasma, choice of gas phase (air or 5% CO_2 in air), culture vessel and harvesting technique. These were selected on the basis of the previous experience of my colleague M.R. Quastel (1970) and modified on the advice of F.H. Bach (personal communication). In all cases techniques used were comparable to those used in many other laboratories doing similar work.

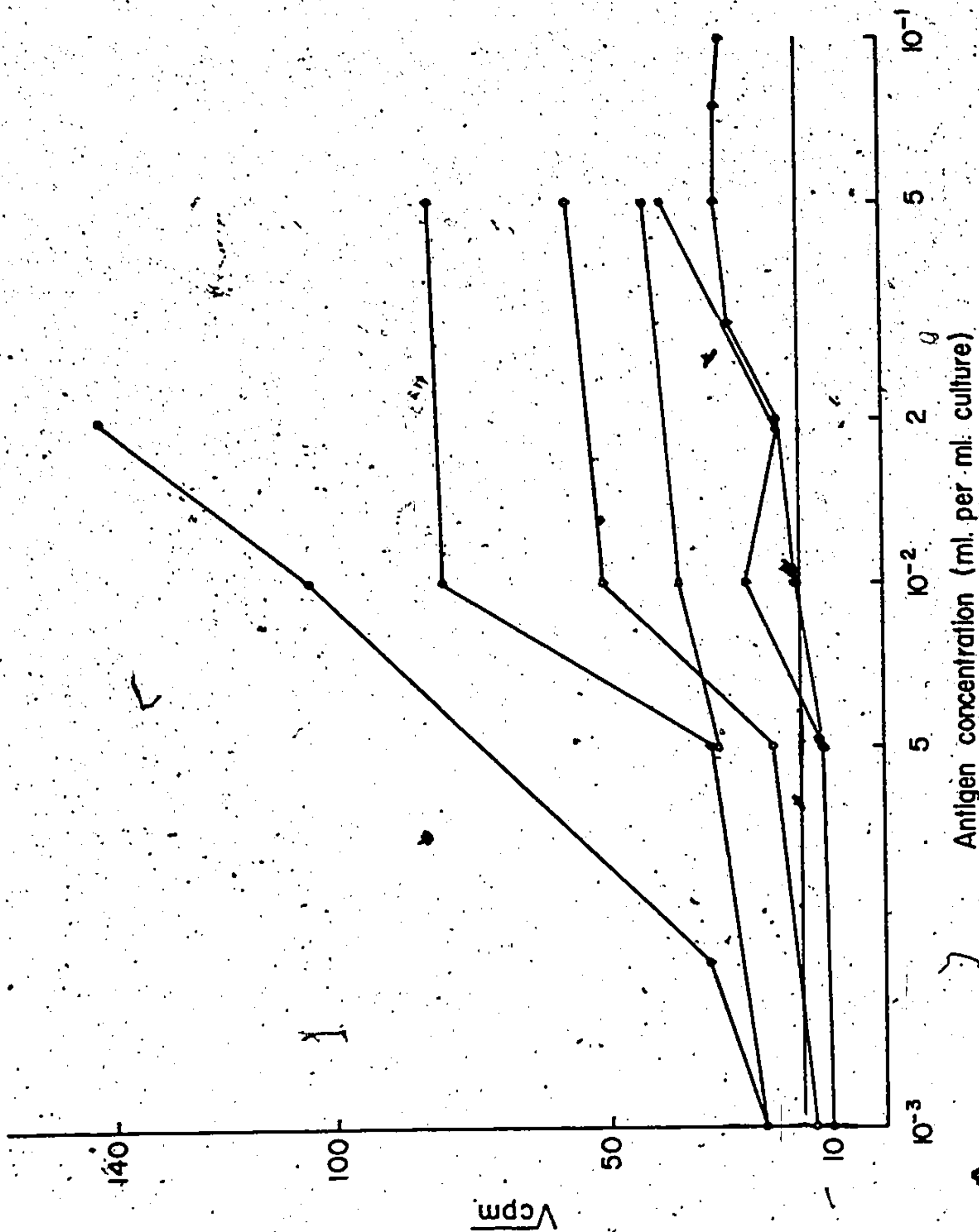
Figure 3: PHA dose response.

^{3}H-thymidine incorporation in cultures treated with different concentrations of PHA on day 0. Cultures were harvested day 5.



140
100
50
10
√cpm
10^{-3}
5
10^{-2}
2
5
10^{-1}
PHA concentration (ml. per ml. culture)

Figure 4: Antigen dose response.
^{3}H-thymidine incorporation in cultures treated with different concentrations of antigen. Antigens were added on day 0 and cultures harvested day 5, 6 or 7.



√cpm
140
100
50
10
10⁻³
5
10⁻²
2
5
10⁻¹
Antigen concentration (ml. per ml. culture)

3.2 Time Course and Activation Levels

3.2.1 Time Course

PHA response (as measured by incorporation of ^{3}H-thymidine) was found to peak on day 2 or 3, then to decline to a low level by day 7 or 8. It is noteworthy that although this response peaks much earlier than that to antigen, there is still a considerable increase over control incorporation up until at least day 6 (Fig. 3.5).

Antigen response usually reached its peak between day 5 and day 7, VAR usually at day 6 and/or 7, SLO at days 5 and 6 (Fig. 3.6a,b). With SLO, the increase over control incorporation was already noticeable by day 3 or 4, with VAR, by day 4 or 5.

Therefore, in designing experiments in which responses to PHA and to antigens were to be measured, it was necessary to reconcile days of peak activity of PHA-(2,3,4), SLO-(4,5,6), and VAR-(5,6,7) treated cells. Since PHA was used mainly as a control, to show lymphocyte activation, cultures were usually harvested on days 5 and/or 6.

3.2.2 Levels of Activation

PHA stimulation resulted in levels of thymidine incorporation ranging from about 100 times control in early days to about 5 times control in later days. There was, of course, a great deal of inter-individual variation, as well as some intra-individual variation over time. The same is true of antigen response, which varied from up to 100 times control in the case of the high response to streptococcal

Figure 5: Time course of PHA stimulation. ^{3}H-thymidine incorporation in cultures treated with PHA on day 0.

200

150

100

$\sqrt{cpm}$

50

0 1 2 3 4 5 6 7 8 9

DAYS IN CULTURE

Figure 6a: Time course of SLO stimulation.
^{3}H-thymidine incorporation in cultures treated with SLO on day 0.

200

150

100

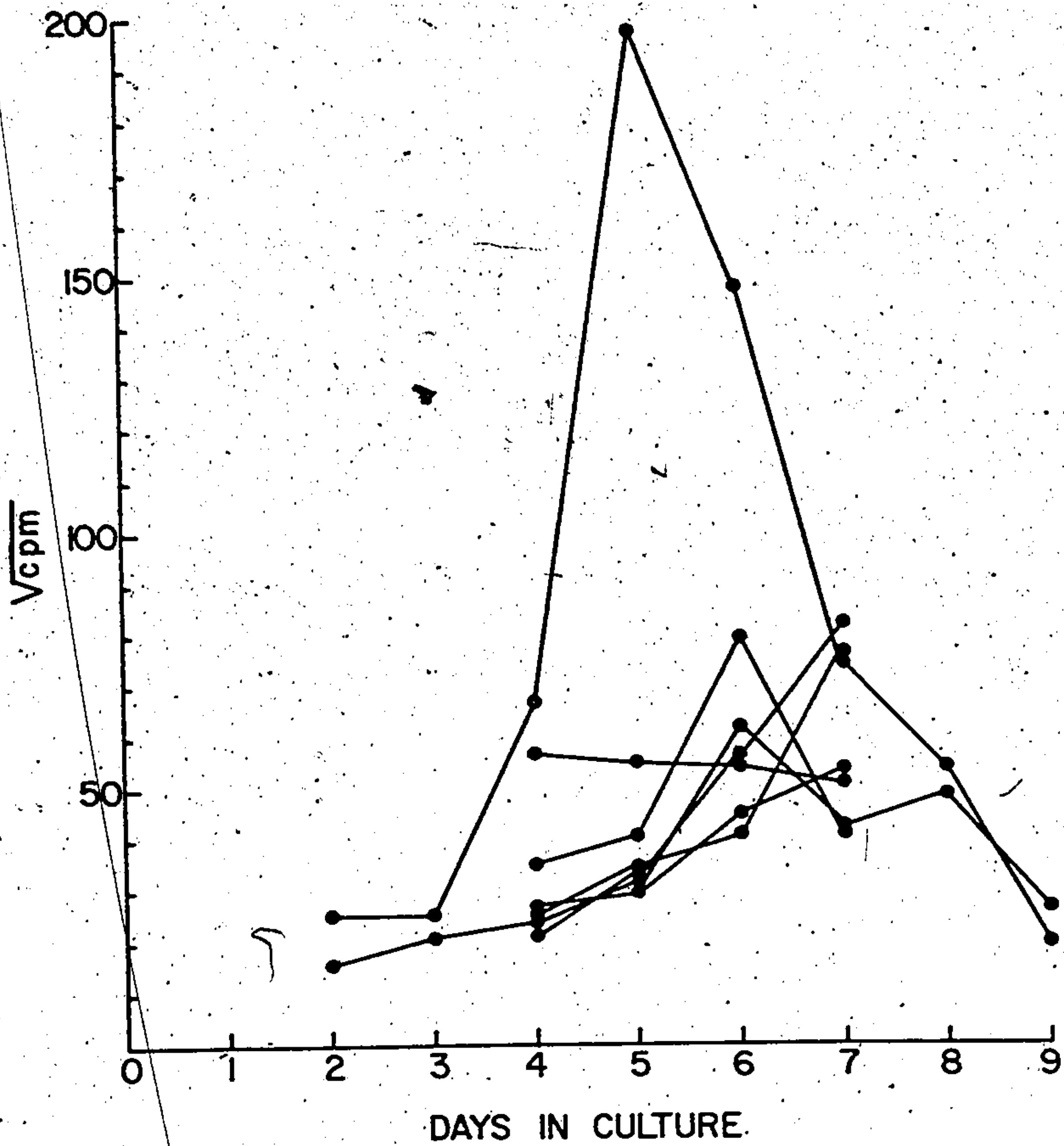
50

√cpm

0 1 2 3 4 5 6 7 8 9

DAYS IN CULTURE

Figure 6b: Time course of VAR stimulation. ^{3}H-thymidine incorporation of cultures treated with VAR on day 0.

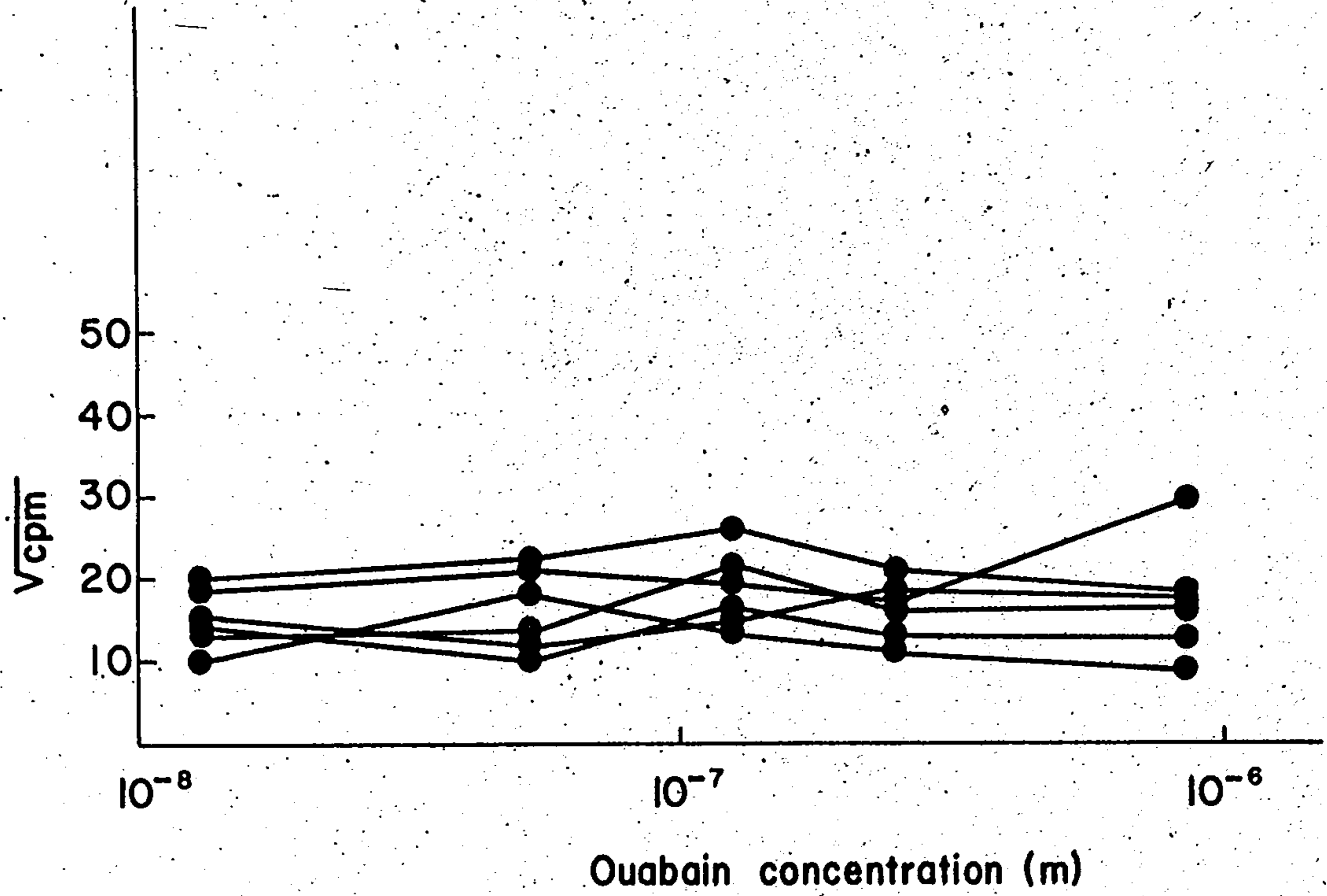

200
150
100
50
√cpm
0
1
2
3
4
5
6
7
8
9
DAYS IN CULTURE

antigens to the 2 to 10 times control characteristic of response to tetanus or typhoid antigens. The range of values of the cpm were usually of a magnitude which permitted clear distinction between stimulated and non-stimulated levels of incorporation. When variability among replicates was small, even low response appeared to be distinct from none, but in some cases, although the mean cpm of antigen-treated cultures was higher than that of the control, there was overlap in the range of values. In such cases the data were considered inconclusive and were ignored.

3.3 Inhibition of response by ouabain

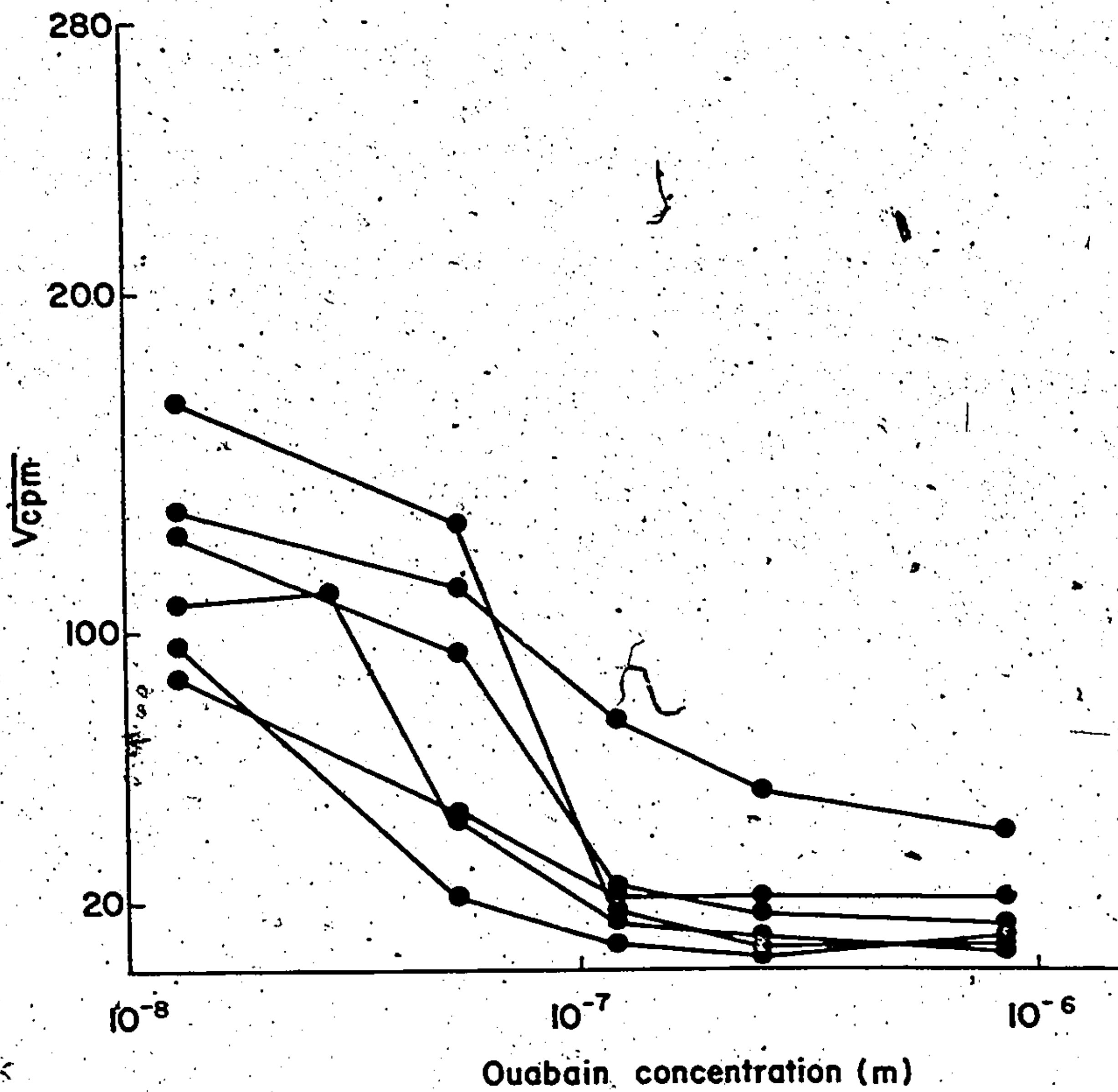
The protocol for this experiment was patterned directly on that of Quastel and Kaplan (1968), including the range of concentrations likely to be effective. Fig. 3.7 shows that ouabain had no effect on the low background incorporation in control cultures. Fig. 3.8 shows that in my culture system, PHA response is inhibited in a way directly comparable to that found by Quastel and Kaplan (1968, 1970). In parallel experiments, response to antigen (SLO or DTT) was found to be inhibited in a similar way, with complete inhibition of incorporation at 2×10^{-7} M ouabain in the culture medium (Fig. 3.9). Thus it seemed that response to antigen was sensitive to this inhibitor of the Na^{+}-K^{+}-ATPase in the same way as was response to mitogen.

Figure 7: Effect of ouabain on untreated cultures. ^{3}H-thymidine incorporation in control cultures (not treated with mitogen or antigen) harvested on day 5, 6 or 7. Ouabain was added for the final 24 hours.



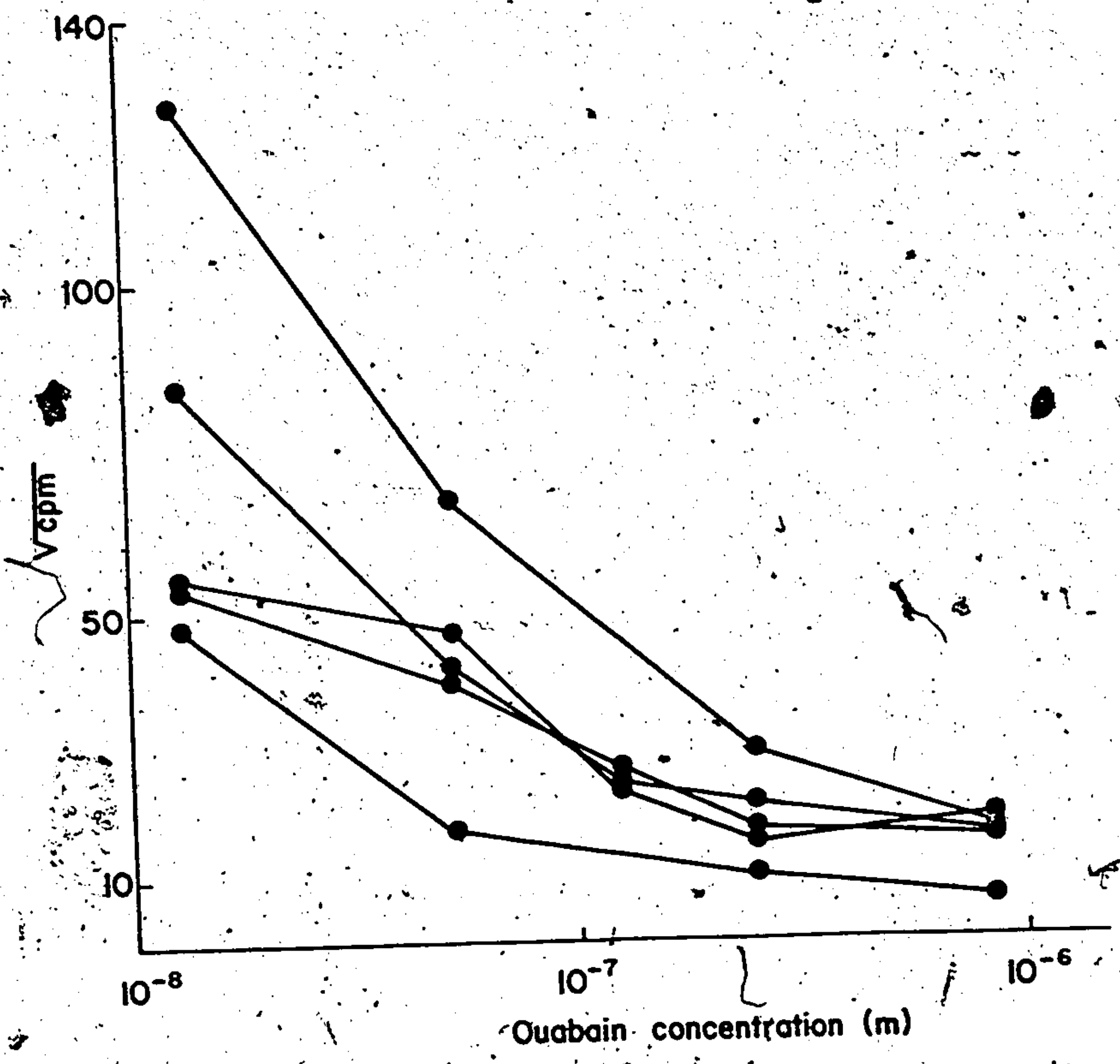
√cpm
50
40
30
20
10
10⁻⁸
10⁻⁷
10⁻⁶
Ouabain concentration (m)

Figure 8. Effect of ouabain on PHA stimulation. ^{3}H-thymidine incorporation in cultures treated with PHA and ouabain on day 0, harvested day 3.



280
200
100
20
√cpm
10⁻⁸
10⁻⁷
10⁻⁶
Ouabain concentration (m)

Figure 9: Effect of ouabain on antigen stimulation. ^{3}H-thymidine incorporation in cultures treated with antigen (SLO or DTT) on day 0, harvested day 5 or 7. Ouabain was added for the final 24 hours of culture.


140
100
50
10
√cpm
10⁻⁸
10⁻⁷
10⁻⁶
Ouabain concentration (m)

3.4 Prevention of ouabain inhibition by increased K^+ in the culture medium

Again, the protocol was the same as in experiments with PHA done by Quastel and Kaplan (1970). KCl was added to give different K^+ concentrations in the medium on day 0, and ouabain was added for the final 24 hours of culture, day 5 to day 6 in this case. Fig. 3.10 shows that increasing the K^+ concentration could partially or completely prevent any inhibition by ouabain of the PHA response. That the same was true for antigen response is shown by Fig. 3.11.

Again, these results indicated that the effects of ouabain on antigen response were similar to its effects on mitogen response. They were also evidence that the effect of the drug was a K^+-specific one, as was concluded by Quastel (1970). That is, the ouabain was apparently acting on the Na^+-K^+ ATPase to prevent cation transport, which action could be reversed or prevented by excess K^+.

3.5 Reversal of ouabain inhibition by washing and medium change

In the experiments of Quastel and Kaplan (1970) on the effect of ouabain on the PHA response, the inhibition could be reversed if the ouabain was removed by washing the cultures and adding fresh, ouabain-free medium. Fig. 3.12 shows that if cells cultured with PHA were also treated with ouabain for the initial 1,2 or 3 days of culture, the inhibition lasted only as long as the ouabain was present. There was no reduction of peak response and the delay in

Figure 10: Effect of extracellular K^+ concentration on ouabain inhibition of PHA response.
^3H-thymidine incorporation in cultures treated with PHA on day 0, harvested on day 5. K^+ was added as isotonic KCl on day 0; ouabain (2×10^{-7} M) was added for the final 24 hours of culture. Controls without ouabain are represented by open circles.

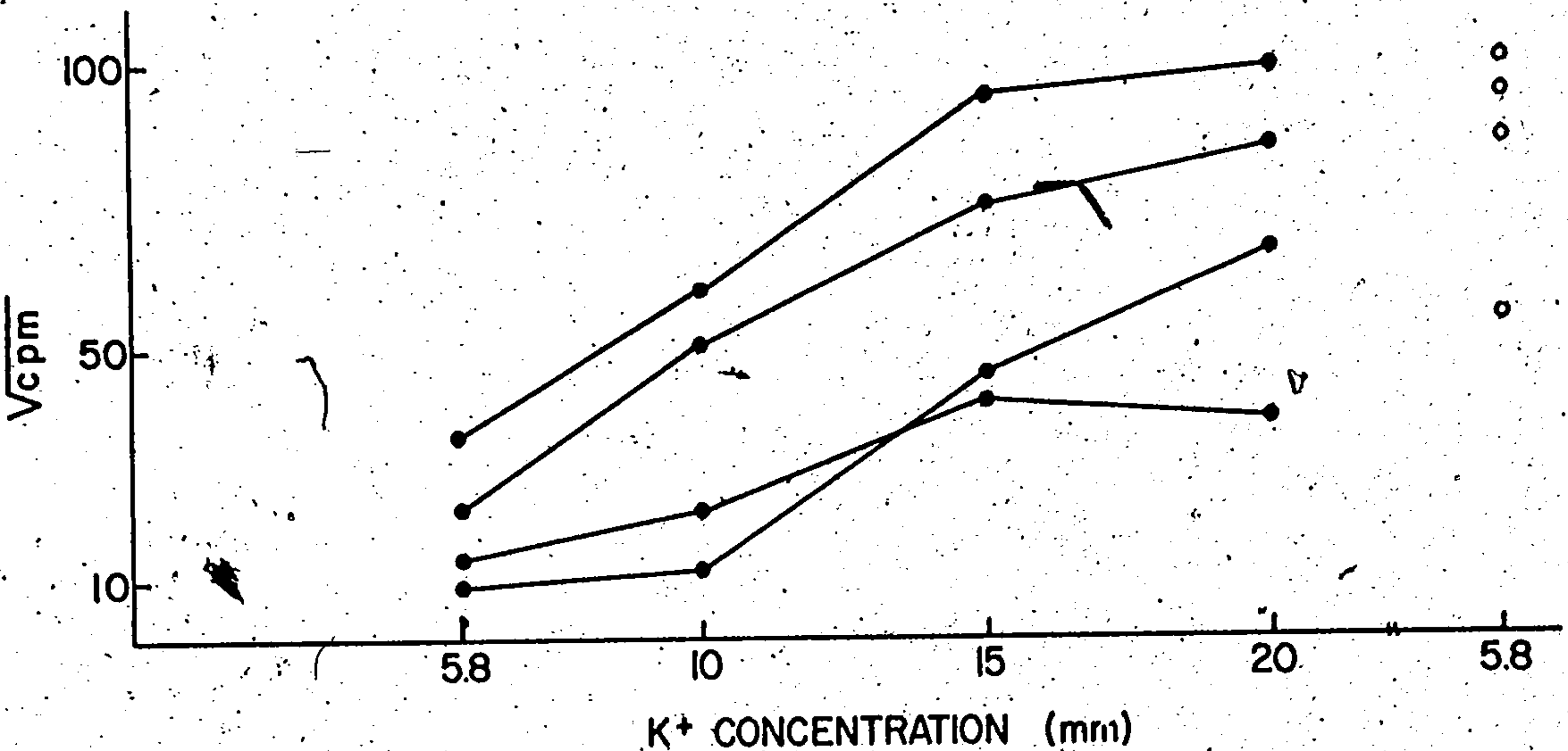
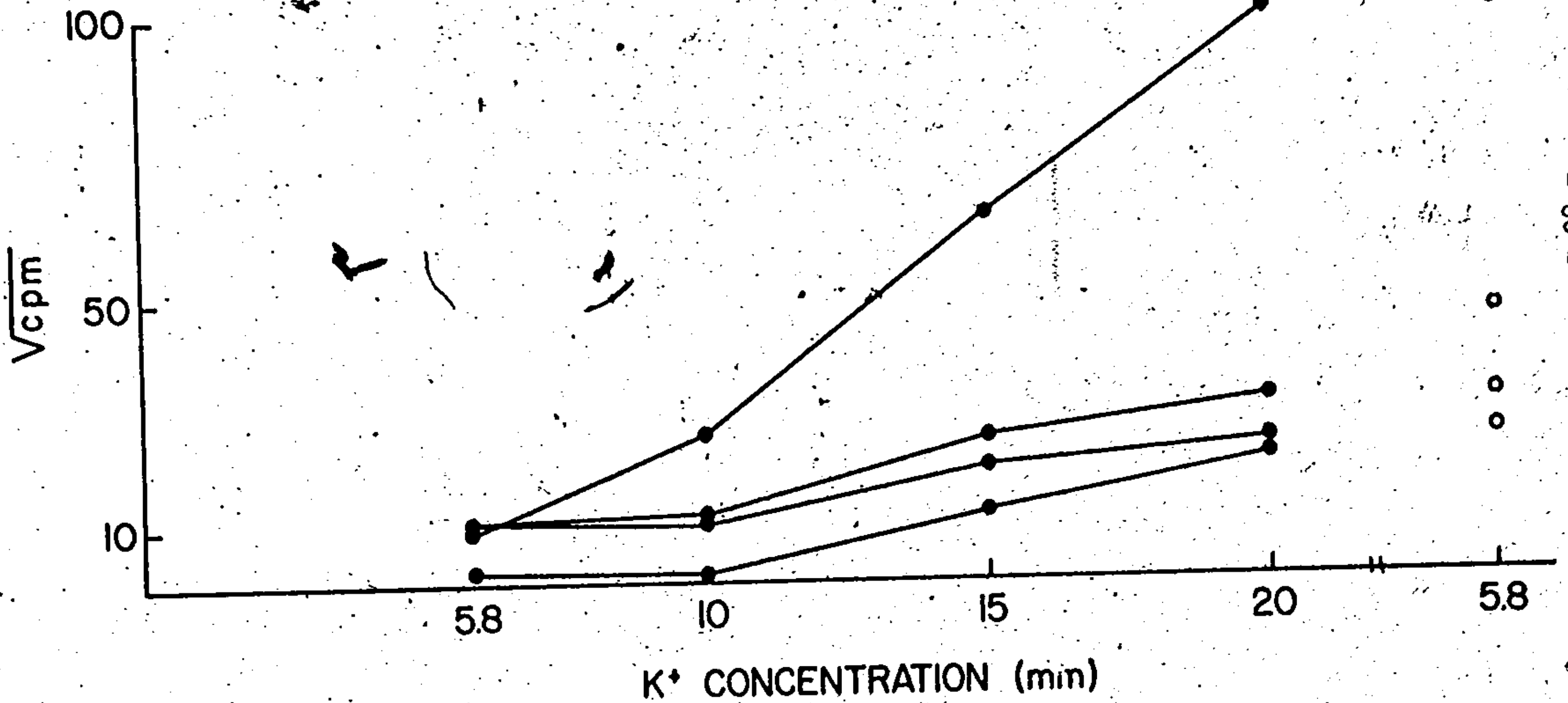

$\sqrt{cpm}$
100
50
10
5.8
10
15
20
5.8
K+ CONCENTRATION (mm)

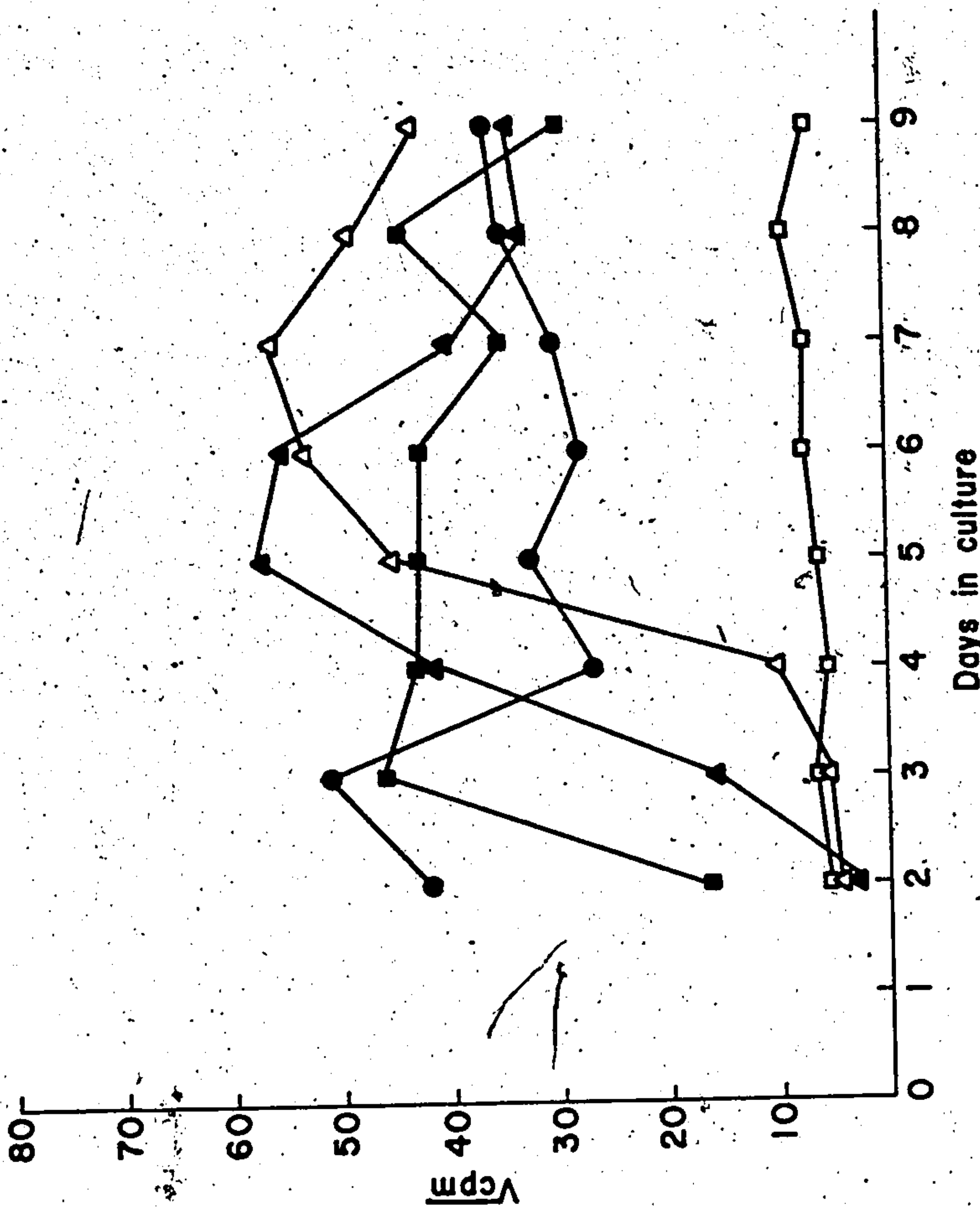
Figure 11: Effect of extracellular K^+ concentration on ouabain inhibition of antigen response.
^{3}H-thymidine incorporation in cultures treated with antigen (SLO, VAR, DTT, TABT) on day 0, harvested day 5 or 6. K^+ (as isotonic KCl) and ouabain (2×10^{-7} M) were added for the last 24 hours of culture. Controls without ouabain are represented by open circles.



$\sqrt{cpm}$
100
50
10
5.8
10
15
20
5.8
K+ CONCENTRATION (min)

Figure 12: Effect of incubation with ouabain for 1, 2 or 3 days on response to PHA.

^{3}H-thymidine incorporation in cultures treated with PHA and ouabain (2×10^{-7} M) on day 0, then washed on day 1, 2 or 3 and reincubated in ouabain-free medium. Cells were exposed to ouabain not at all: ●——● or for one (■——■), two (▲——▲) or three (△——△) days. Incorporation into control cultures, having had neither PHA nor ouabain, is also shown (□——□).


Vcpm
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
Days in culture

reaching the peak was equivalent to the length of time the ouabain had been in the culture.

However, as is clear from Fig. 3.13, response to antigen was not so easily recovered. Removal of the drug after two days in the medium seemed to cause an irreversible loss of antigen response. A one-day exposure seems not to have had this drastic effect. These experiments were performed using the lowest concentration of ouabain (2×10^{-7} M) that in previous experiments had resulted in complete inhibition of ^{3}H-thymidine incorporation when it was present during (and 24 hours before) the incubation with the isotope. This concentration was also that shown by Quastel and Kaplan (1970b) to completely inhibit transport of K^+ into control and PHA-treated cells.

It seemed, then, that there was a difference in the sensitivity of the PHA- and antigen-treated cells to prolonged incubation with this inhibitor.

3.6. Washing effects

The first obvious possibility to investigate was whether the difference was merely one of binding of antigen versus mitogen to the cell, such that the wash removed antigen but not mitogen. Table 3.1 shows that simply washing antigen and PHA treated cells didn't usually result in a decrease in response--PHA was usually increased, probably because of renewed nutrients. In some cases antigen response was decreased. However, this problem was easily circumvented by adding antigen anew after the washing operation. Thus, the differential

Figure 13: Effect of incubation with ouabain for 1 or 2 days on response to antigen.

^{3}H-thymidine incorporation into cultures treated with antigen (VAR) on day 0. Ouabain was added on day 0, cultures were washed on day 1 or 2 and reincubated with antigen, without ouabain. Shown are: control antigen-treated cultures (●——●), + ouabain, wash day 1 (■——■), + ouabain, wash day 2 (□——□), untreated control (▲——▲).

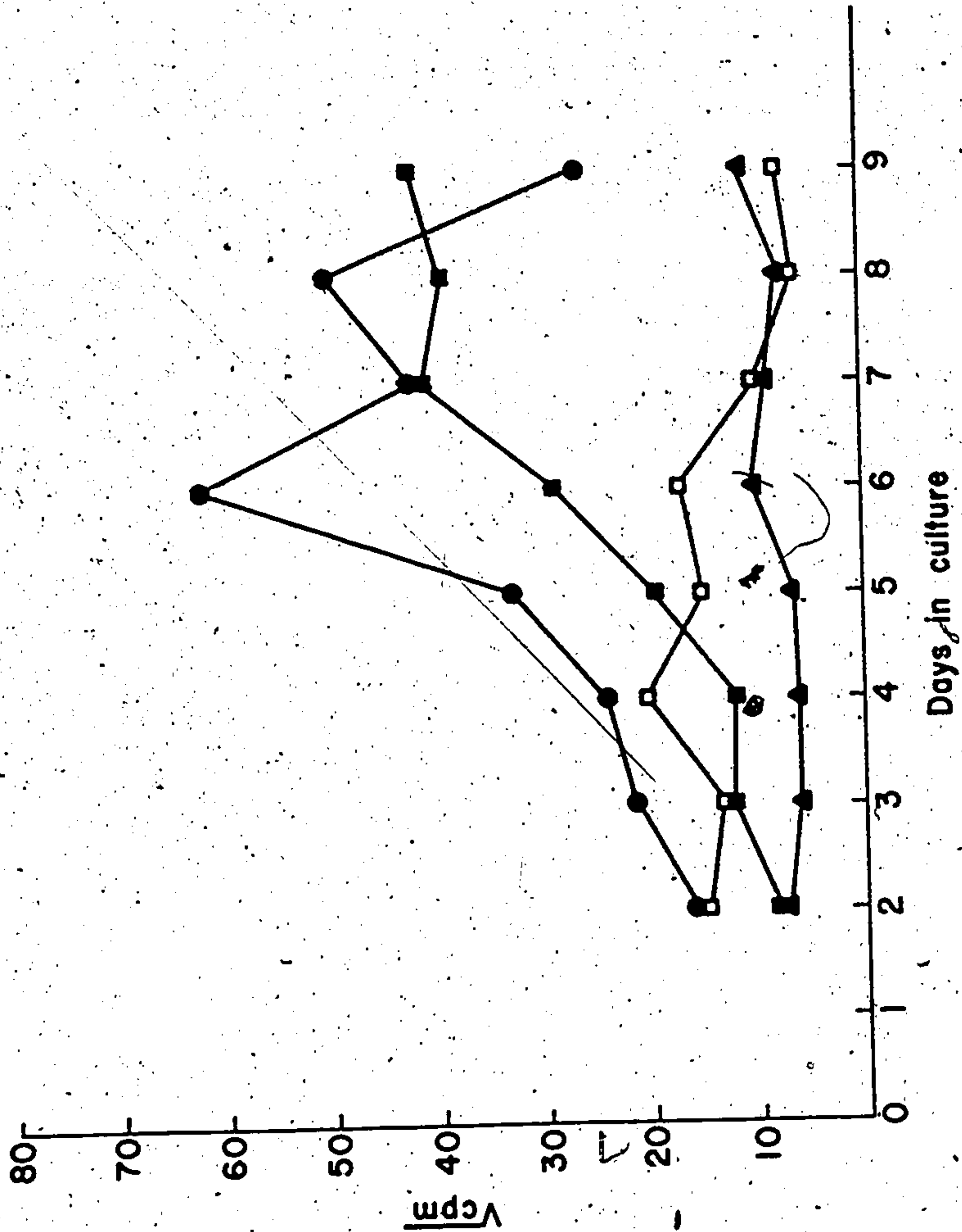

√cpm
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
Days in culture

Table 3.1: Effect of Washing and Medium Change on Lymphocyte Activation

	Unwashed			Washed day 2		
Expt.	Control	PHA[2]	Ag[2]	Control	PHA	Ag
A	9[1]	16	65	10	49	122
B	27	41	63	27	72	33
C	28	75	54	25	155	48
D	33	124	66	18	232	56
E	28	167	70	---	233	26
F	16	50	54	24	203	43

1: $\sqrt{cpm}$, incorporation of ^{3}H-thymidine/4 hrs/culture.

2: Mitogen or antigen added day 0, not replaced in the medium after washing.

sensitivity to washing was discounted as the cause of lost antigen response.

3.7 Zoschke-Bach effect?

The experiments of Zoschke and Bach (1970, 1971) involved activation of HPLs by one antigen, killing of stimulated cells by BUdR-light treatment, followed by re-stimulation with a different antigen. The observation that addition of the second, following antigen resulted in normal response was taken as evidence that response to different antigens involved different subpopulations, at least as far as the few tested antigens were concerned. The question, then, was whether the effect of the ouabain was to kill or inactivate the cells responding to the antigen, in which case there should remain cells able to respond to other antigens and to mitogens. Experiments were performed in which cells were cultured with ouabain and antigen for two days, washed, then cultured with the same or a different antigen or with mitogen for several more days. Table 3.2 shows that in such "sequential" experiments, addition of the second antigen still failed to elicit response, while addition of PHA resulted in the usual high level of thymidine incorporation. From this it was concluded that the inhibition was a nonspecific one, affecting the cells' ability to respond to any antigen.

3.8 Effect of ouabain alone day 0-day 2

Conclusive evidence of this lack of specificity came from experiments in which cells were cultured with or

Table 3.2: Response of ouabain + antigen-treated cells to antigen and to PHA

(a) Response to antigen

Expt.	Control	A_1	A_2	+ ouabain[2] 0-2 A_1	+ ouabain[2] 0-2 A_2	+ ouabain 0-2, then add other antigen A_1	+ ouabain 0-2, then add other antigen A_2
A	25 [1]	41 [3]	71 [4]	11	---	12	---
B	20	32 [3]	246 [4]	24	32	39	21
C	17	68 [3]	58 [4]	22	24	17	42
D	33	134 [4]	48 [3]	21	18	24	22

(b) Response to PHA

Expt.	Control	A_1	A_2	PHA	+ ouabain 0-2 A_1	+ ouabain 0-2 A_2	+ ouabain 0-2 then PHA A_1	+ ouabain 0-2 then PHA A_2
E	20	32 [3]	246 [4]	122	24	32	264	373
F	17	68 [3]	58 [4]	191	22	24	106	138
G	20	64 [4]	----	54	20	---	87	----
H	23	134 [4]	48 [3]	124	21	18	73	55

1: $\sqrt{cpm}$, incorporation of ^{3}H-thymidine/4 hrs/culture

2: 2×10^{-7} M

3: TAB

4: SLO

without ouabain for the first two days of culture, washed, and only then, after washing, challenged with antigen or mitogen. In such experiments, as Table 3.3 illustrates, mitogen but not antigen response was consistently observed. Thus it was apparent that ouabain was affecting some component of the system necessary for measurable response to antigen but not for mitogen response.

3.9 Intermission

Several possible approaches presented themselves at this point in time. One simple explanation was that the drug was killing a large fraction of the lymphocytes, so that mitogens, which activate a large proportion of cells, still came in contact with a number of sensitive cells sufficient to elicit a response, whereas too few cells reactive to any one antigen remained to permit response. A massive killing should be detectable by simple dye-exclusion measurements of viability and by cell counts. Since a major difference in the requirements for antigen and for PHA stimulation is the need for macrophages in the specific response, the effect of the inhibitor on these cells was also an obvious target for investigation. It was also of interest to check whether this differential effect was only between antigen and PHA, or could be extended to distinguish other sorts of specific (for example MLR) and nonspecific (other mitogens) responses.

3.10 Viability

The only easy way to measure viability in this system is to count the proportion of cells with membranes

Table 3.3: Effect of Ouabain alone day 0-day 2 on lymphocyte activation

Expt.	Control	PHA[3]	Ag[3]	+ ouabain[2] 0-2 PHA[3]	+ ouabain[2] 0-2 Ag[3]	control + ouabain 0-2, then add: PHA	control + ouabain 0-2, then add: Ag
A	28[1]	167	70	----	---	246	18
B	34	55	151	137	17	106	25

1: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hrs.

2: 2×10^{-7} M

3: added to the cultures on day 0; cultures were washed and reincubated with PHA or antigen on day 2.

damaged enough that they do not exclude dyes such as trypan blue. In several experiments, the fraction of cells excluding the dye was always over 90% at the start of the experiment and this did not decrease in the first two days, whether or not ouabain was included in the culture medium (Table 3.4). It had been obvious that since PHA response was intact, there was not complete killing; it now seemed that there was not even a significant per cent killing, since the lymphocyte concentration did not seem to decrease during this time. Dye exclusion techniques do not indicate the fraction of cells which have died and disintegrated, but if such cells were in large numbers, large enough to lose antigen response purely on the basis of low cell concentration (i.e. more than 50%, from section 3.1.1), this should be reflected in reduced numbers in counting.

The cells, then, seemed to be alive and well enough to respond to mitogen, but not to antigen.

3.11 Effect of ouabain 0-2 on uridine incorporation in antigen- and in PHA-treated cells

It was of interest to know whether only DNA synthesis was affected by the two-day ouabain treatment. Table 3.5 shows that incorporation of ^{3}H-uridine, presumably into RNA, was also differentially sensitive. In each case antigen-induced incorporation was reduced to control levels or less, while the effect on PHA-induced incorporation, while variable, was in no case drastically inhibitory. Thus, the drug seemed also to be affecting events earlier in the process of

Table 3.4: Viability of cells exposed to ouabain for two days

Expt.	day	ouabain concentration	cell number	% viable cells
A	0	--	0.85×10^6/ml	~100
	2	--	1.0	99
		8×10^{-7} M	0.84	99
		2×10^{-7} M	0.97	~100
B	0	--	1.0×10^6	~100
	2	--	0.95	99
		8×10^{-7} M	0.525	97
		2×10^{-7} M	0.925	98
C	0	--	1.0×10^6	~100
	2	--	1.0	99
		8×10^{-7} M	1.1	97
		2×10^{-7} M	1.1	98
D	0	--	1.0×10^6	98
	2	--	2.7	99
		2×10^{-7} M	1.9	99

Table 3.5: Effect of two day exposure to ouabain on uridine incorporation in PHA- and antigen-treated cultures.

Expt.	Control[1]	mitogen		+ ouabain[2] 0-2
A	47	PHA	389	110
		SLO	388	13
B	24	SLO	36	16
C	30	PHA	88	147
		SLO	92	14
		VAR	75	11
D	12	PHA	51	152
		SLO	16	11
E	13	PHA	69	97
		SLO	19	8
		VAR	29	7

1: $\sqrt{cpm}$, ^{3}H-uridine/4-6 hrs/culture
2: 2×10^{-7} M

transformation than DNA synthesis.

3.12 Macrophages

Could it be then that the macrophages were the cells affected? The concentration of macrophages in culture is too low to permit direct viability estimates, but experiments were performed to permit evaluation of their capacity to function after exposure to ouabain as accessory cells in lymphocyte response to soluble antigen. The experiments were performed as follows: cultures of "purified" lymphocytes and of macrophages were prepared as described in Sections 2.1.2.2 and 2.1.2.3; samples of each were cultured for two days with and without ouabain. They were then recombined, control lymphocytes + control macrophages; lymphocytes treated with ouabain for two days + control macrophages; control lymphocytes + macrophages treated for two days with ouabain. After further incubation, these cultures were assayed for response. Many difficulties arose with this system. The first was inability to collect enough macrophages to do the experiment in the tube culture system. The advent of the microsystem made the experiment possible, but the number of manipulations made the contamination rate for this experiment higher than normal. The other major problem was failure to purify the cells well enough, so that there was still antigen response in the "pure" control cultures. In such cases it was not possible to demonstrate that untreated macrophages were able to reconstitute response to control lymphocytes, so results from the treated population were made equivocal. Table 3.6 gives the data

from the macrophage reconstitution experiments that gave some kind of result. Although most of the experiments did not give definitive results in <u>all</u> treatment classes, in several of them, treatment of lymphocytes with ouabain did result in loss of antigen response, which was not regained after addition of untreated macrophages. Presumably, if the effect had been due to inhibition of macrophages only, addition of untreated cells should have enabled the lymphocytes to respond (assuming that in these experiments the macrophages as isolated <u>were</u> functional). In fact, in the two experiments (B and C) in which the macrophages were shown to reconstitute response in purified cells, the results with ouabain-treated cells supported the above interpretation.

Thus, considering the observations of Rosenthal et al. (1972), that high concentrations of ouabain did not affect the ability of guinea pig macrophages to present immunologically relevant antigen to lymphocytes, as well as the above results, it seems unlikely that the site of our differential sensitivity is the macrophage rather than the lymphocyte.

3.13 <u>Other mitogens and ouabain</u>

Figs. 3.14 and 3.15 show that lymphocytes stimulated by PWM or Con A and in the MLR had a sensitivity to ouabain comparable to those stimulated by PHA and antigen. Quastel (1970) also showed that the MLR was similarly inhibited. The effect of prolonged treatment with the drug (from initiation till day 2) is shown in Table 3.7 for the mitogens and in Table 3.8 for the MLR.

Table 3.6: Effect of macrophages on antigen response in ouabain-treated cultures

		UP[1] cells		P[2] cells		P + M[3]		UP + O[4] 0-2		(P+O 0-2) + M		P + (M+O 0-2)	
Expt.	Control	PHA	Ag	PHA	Ag	PHA	Ag	PHA	Ag	PHA	Ag	PHA	Ag
A	17[5]	51	27	73	9	71	33	50	9	71	16	66	21
B	22	59	63	117	17	118	209	73	12	88	23	89	181
C	9	79	38	116	16	133	34	68	7	108	6	138	39
D	13	105	33	165	35	144	24	120	10	131	8	----	----
E	16	----	158	----	138	----	180	----	8	----	15	----	185
		----	66	----	66	----	60	----	8	----	6	----	45

1: UP = unpurified leucocytes

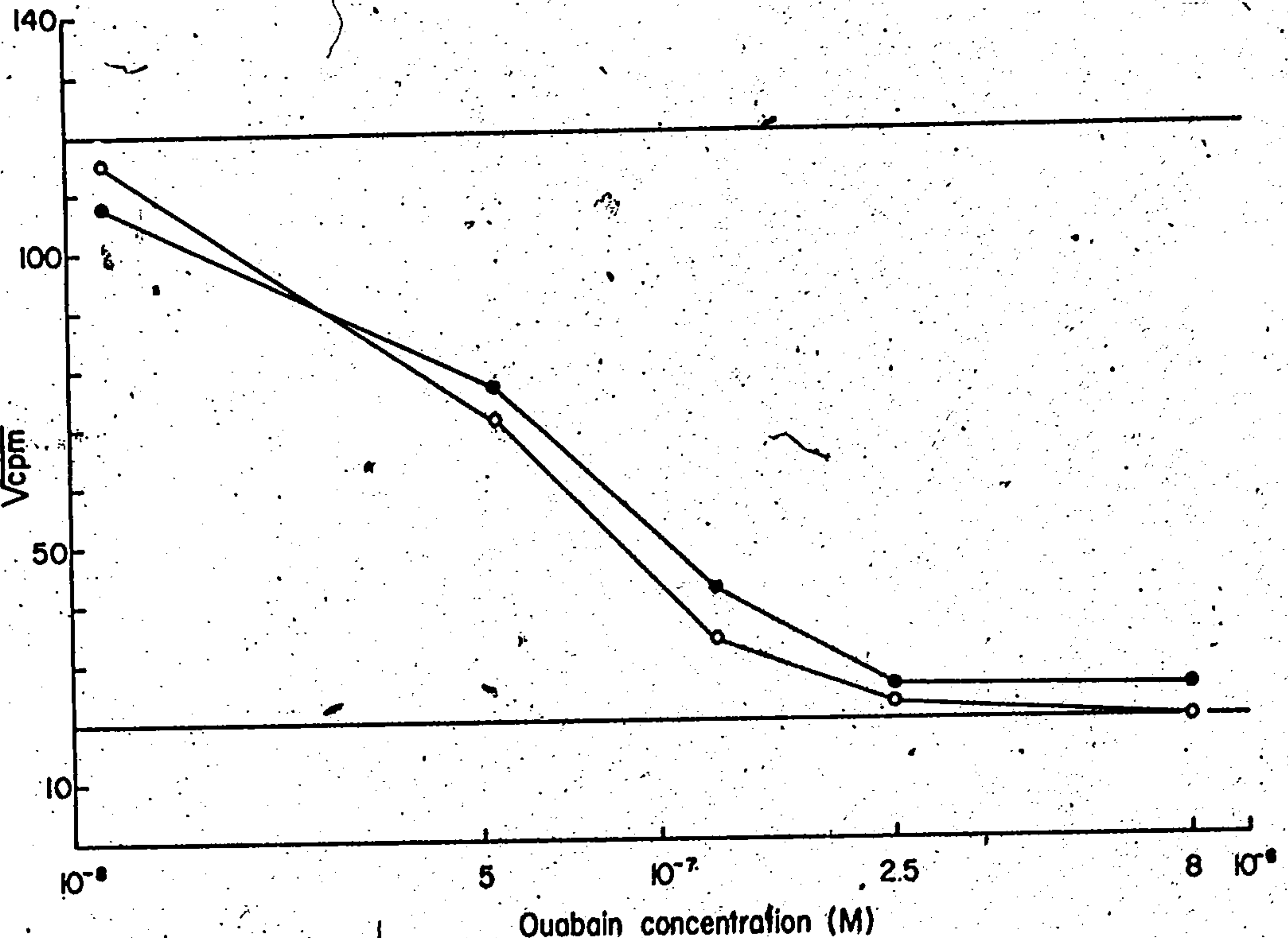
2: P = glass-wool-separated lymphocytes

3: M = macrophages separated as described in the text

4: O = ouabain (2×10^{-7} M), included in the culture medium day 0-day 2

5: $\sqrt{cpm}$ ^{3}H-thymidine/culture/4 hours.

Figure 14:—Effect of ouabain on PWM and Con A stimulation. ^{3}H-thymidine incorporation in cultures treated with ouabain and mitogen on day 0, harvested on day 3. PWM (●——●), Con A (O——O). The upper horizontal line represents the average incorporation in stimulated culture, the lower the average in untreated controls.



140
100
50
10
√cpm
10^{-8}
5
10^{-7}
2.5
8
10^{-6}
Ouabain concentration (M)

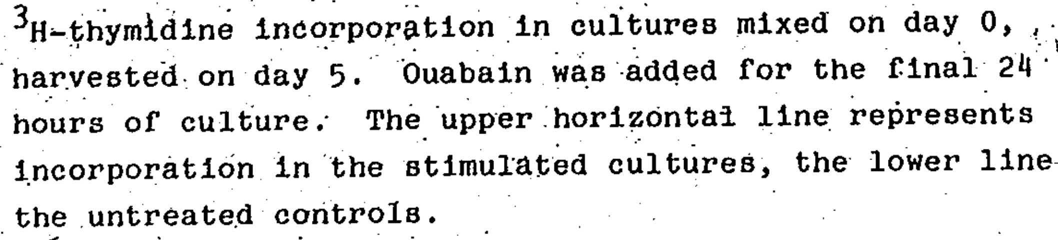
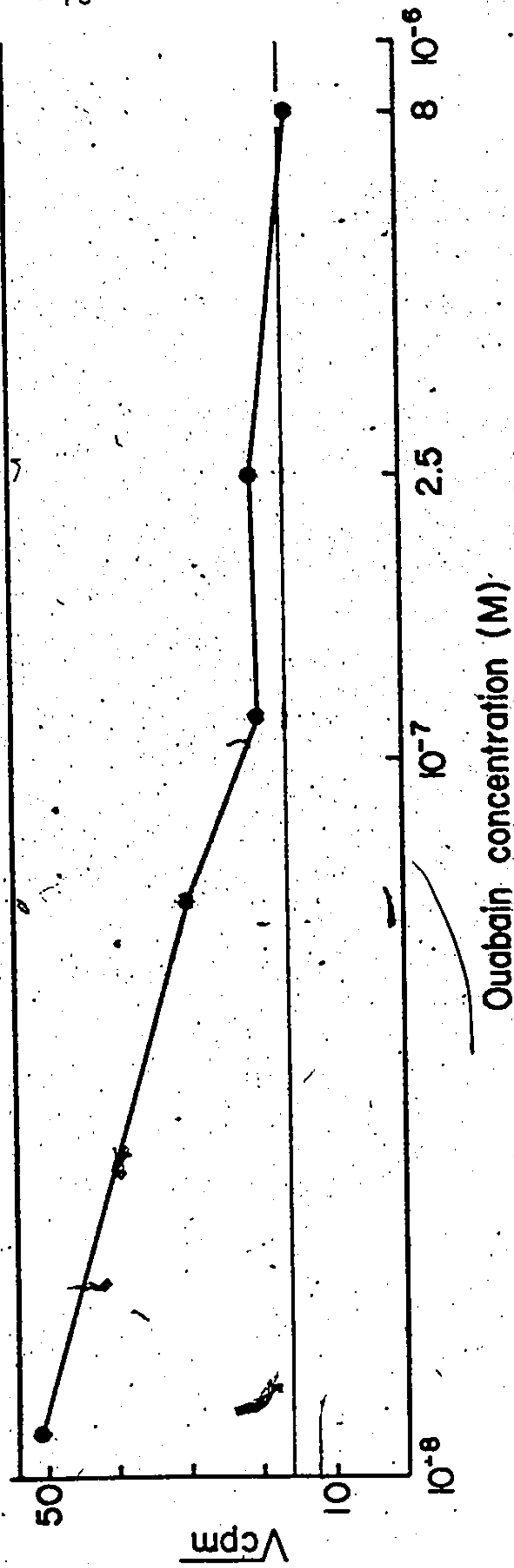


Figure 15: Effect of ouabain on stimulation in the mixed leukocyte reaction.
^{3}H-thymidine incorporation in cultures mixed on day 0, harvested on day 5. Ouabain was added for the final 24 hours of culture. The upper horizontal line represents incorporation in the stimulated cultures, the lower line the untreated controls.



√cpm
50
10
Ouabain concentration (M)
10⁻⁸
10⁻⁷
2.5
8
10⁻⁶

3.13:1 Other Mitogens

The effect of a two-day incubation with 2×10^{-7} M ouabain on mitogen response varied among the five experiments shown in Table 3.7. The PHA response was reduced in 3 of the experiments by 20 to 40%, was unchanged in one and in another was increased by 40%. Con A response was also reduced, in the same three experiments, by 20 to 50%, and in the others was unchanged. Response to PWM was decreased in four of the five experiments, by 40 to 70%; in the fifth it was increased by 40%.

Thus, Con A seems comparable to PHA in its resistance to ouabain inhibition, but PWM is apparently more sensitive. It was not, however, as sensitive as was antigen response; in none of the experiments was PWM-induced ^{3}H-thymidine incorporation reduced to the level of the controls.

3.13.2 MLR

It was of interest to determine whether the MLR, a somewhat different specific response, was affected like antigen or like mitogen response. A MLR in which the cells are mixed on day 0 and cultured with ouabain until day 2 was affected similarly to the antigen-treated cultures i.e. irreversibly inhibited. (Table 3.8). However, since the MLR involves two separate events, stimulation and response, it was of interest to know which of these was inhibited. Attempts to perform one-way MLRs after two-day preincubation, with or without ouabain (using mitomycin-C as inhibitor of thymidine incorporation in the stimulating cells) were unsuccessful, for reasons

Table 3.7: Effect of two-day exposure to ouabain on response to PHA, Con A and PWM

Expt.	control	mitogen[1]	no ouabain	+ ouabain[2] 0-2
A	11 [3]	PHA	99	58
		Con A	91	39
		PWM	91	26
B	14	PHA	179	141
		Con A	207	160
		PWM	223	116
C	20	PHA	83	115
		Con A	84	96
		PWM	51	77
D	35	PHA	93	109
		Con A	75	99
		PWM	97	53
E	23	PHA	115	89
		Con A	86	50
		PWM	54	33

1: mitogens present in culture medium for 3-4 days after washing to remove ouabain.

2: 2×10^{-7} $\underline{M}$

3: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours.

Table 3.8: Effect of two-day exposure to ouabain on the MLR

a) Two-way reactions mixed day 0

Expt.	control ($\frac{1}{2}A+\frac{1}{2}B$)	AB	AB + ouabain[1] 0-2
A	20[2]	129	20
B	37	138	43
C	17	39	12
D	17	112	18

b) Two-way reactions mixed day 2

Expt.	control	AB	(A + ouabain[1]) + B[3] 0-2
E	13	92	90
F	22	39	47
G	14	63	22

1: 2×10^{-7} M, in culture medium day 0-day 2 then removed by washing

2: $\sqrt{cpm}$ ^{3}H-thymidine/culture/4 hours, 5-8 days after medium change

3: B cells fresh on day 2, added to A cells, preincubated with or without ouabain day 0-day 2.

unknown. We were often unable to get even two-way reactions in cells mixed after two days in culture, with or without ouabain. The results of one variation of the MLR involving preincubation with ouabain are shown in Table 3.8b. In these experiments, cells from donor A were incubated with or without ouabain for two days then washed; fresh donor B cells were then added. This combination did result in some response; in two experiments, preincubation of A cells with ouabain did not diminish the response; in the third the incorporation is rather close to that of the control, although still higher. Thus, although the two-way (mixed day 0) reactions show that at least one facet of the MLR was irreversibly inhibited, the latter experiments show that at least one was not inhibited. In these, the response must represent either A's unaffected response to B, or A's unaffected ability to stimulate B.

Thus, although the MLR is a type of specific reaction, and was similarly sensitive to prolonged exposure to ouabain, the inhibition did not affect both the response to foreign cells and the ability to stimulate those cells. The experiments to distinguish between these were not done in this laboratory and will be included in the discussion.

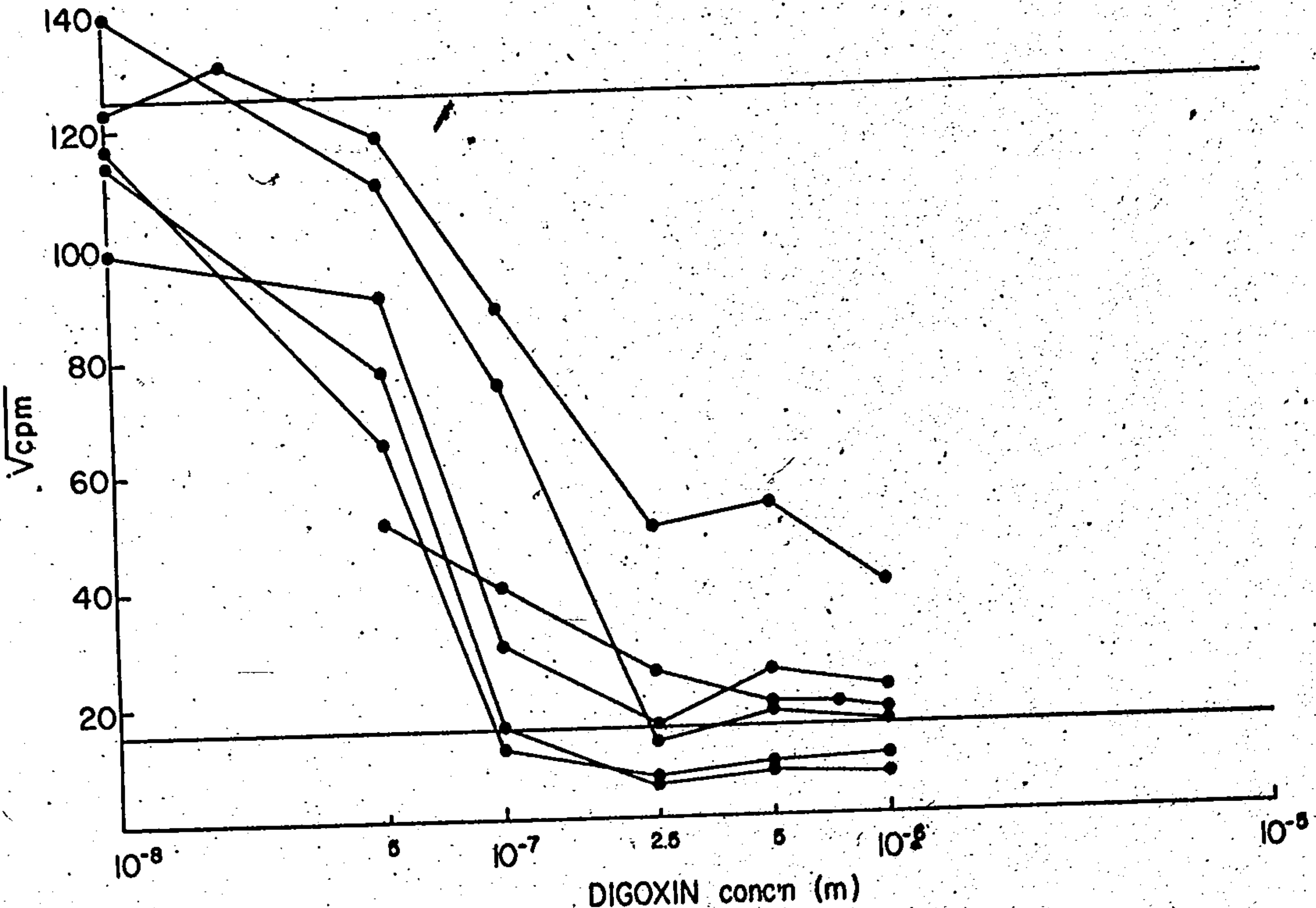
3.14 Other ATPase inhibitors--do they have the same effect?

It was of some interest to discover whether the effect of the ouabain was specific to that drug, or whether other drugs which inhibit the Na^+-K^+-ATPase had similar effects. Quastel (1970) had already found that digoxin but not digitoxin gave inhibition curves similar to that of ouabain for PHA

stimulated cells. Figs. 3.16a,b and 3.17a,b show the effects of digoxin and of acetylstrophanthidin on PHA- and antigen-treated cells. Digoxin again was effective at concentrations similar to ouabain, while somewhat higher concentrations of AcStro were required to give the same inhibition. Diphenylhydantoin was also tried, over a concentration range of 10^{-4} to 10^{-6} M, but had no inhibitory effect.

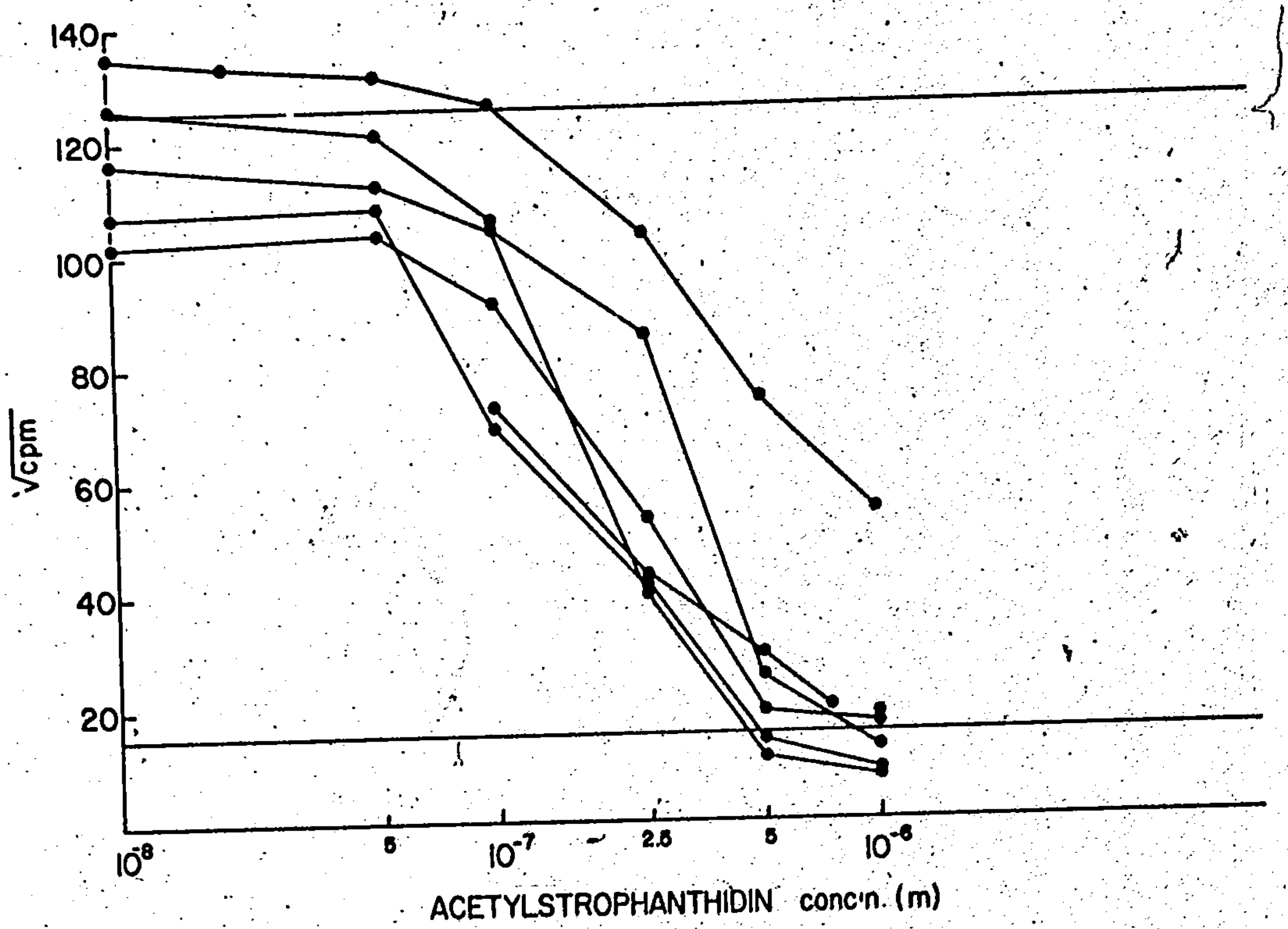
The effect of including the inhibitors in the culture medium for the first two days, then washing to remove them is shown in Table 3.9. The concentrations used were different in each experiment (for digoxin and AcStro), depending on the sensitivities of the donors. In experiment A, response to SLO was irreversibly lost, while response to PHA, although decreased, was still present. In experiment B, responses to SLO and VAR were eliminated, while PHA was if anything increased after ouabain and AcStro treatments and much decreased but still in evidence after digoxin treatment. In C, response to one of the antigens, VAR, was much reduced although not completely eliminated by digoxin and AcStro treatments, while SLO response was unaffected, as was PHA response, probably because the concentrations were not quite high enough. However, it did seem that with appropriate concentrations of these drugs, differential sensitivity of antigen and mitogen response could be demonstrated. This indicated that the ouabain effect was not specific to that drug (and therefore not a result of its particular structure or binding properties) and suggests that the important feature is inhibition of the

Figure 16a: Effect of digoxin on PHA-stimulated cultures. ^{3}H-thymidine incorporation in cultures treated with PHA and digoxin on day 0, harvested on day 3. Each line represents one experiment. The upper horizontal line represents the average PHA response without inhibitor, the lower line the untreated control.



140
120
100
80
60
40
20
$\sqrt{cpm}$
10^{-8}
5
10^{-7}
2.5
5
10^{-6}
10^{-5}
DIGOXIN conc'n (m)

Figure 16b: Effect of acetylstrophanthidin on PHA-stimulated cultures. ^{3}H-thymidine incorporation in cultures treated with PHA and acetylstrophanthidin on day 0, harvested on day 3. Each line represents one experiment. The upper horizontal line represents the average PHA response in the absence of inhibitor, the lower line the untreated control.



140
120
100
80
60
40
20
√cpm
10⁻⁸
5
10⁻⁷
2.5
5
10⁻⁶
ACETYLSTROPHANTHIDIN conc'n. (m)

Figure 17a: Effect of Digoxin on antigen stimulation. ^{3}H-thymidine incorporation in cultures treated with antigen (SLO) on day 0, harvested on day 5. Inhibitor was added on day 4. The upper horizontal line represents the average antigen response in the absence of inhibitor, the lower line the untreated control.

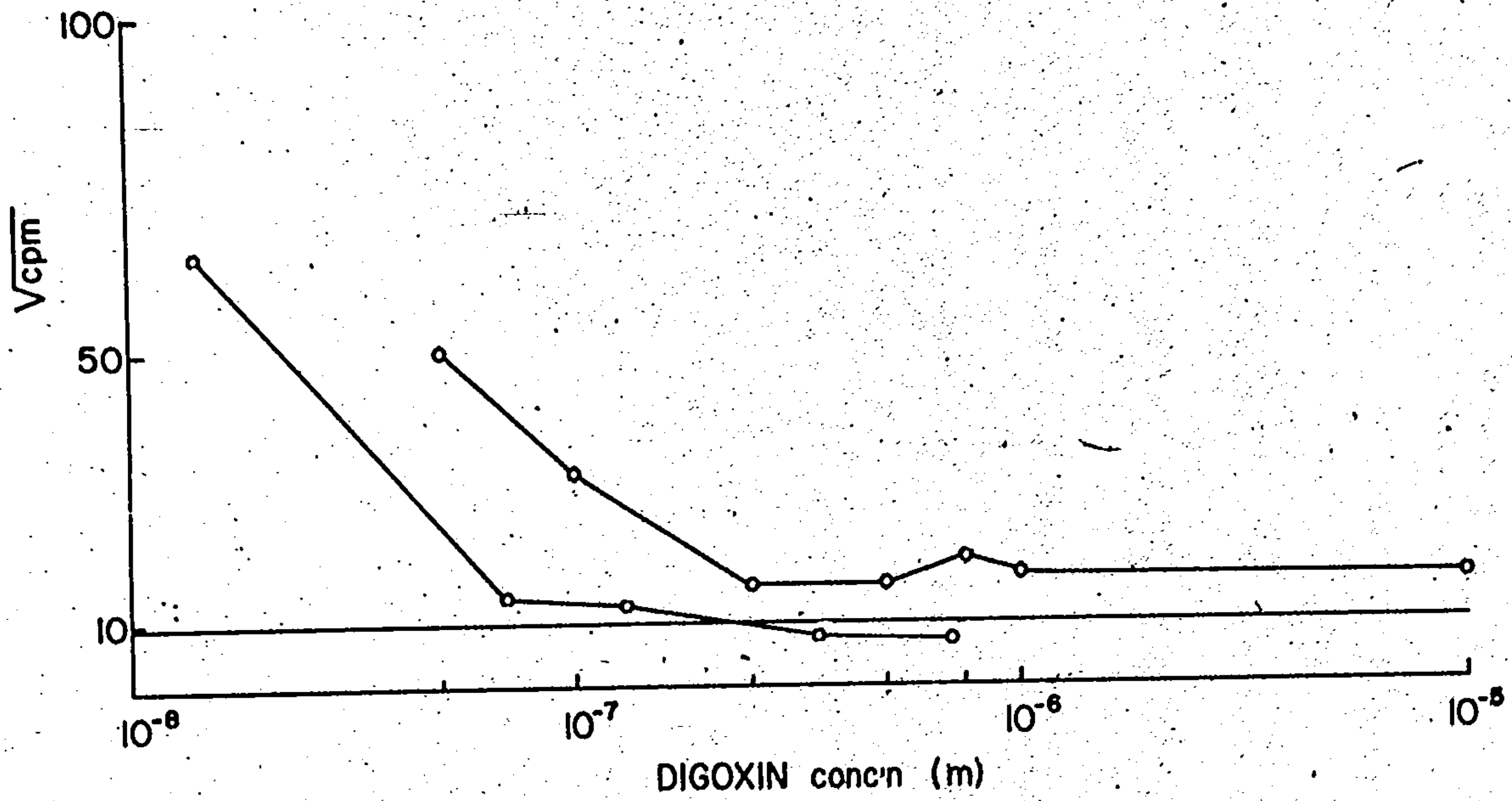

100
50
10
√cpm
10^{-8}
10^{-7}
10^{-6}
10^{-5}
DIGOXIN conc'n (m)

Figure 17b: Effect of acetylstrophanthidin on antigen stimulation. ^{3}H-thymidine incorporation in cultures treated with antigen (SLO) on day 0, harvested on day 5. Inhibitor was added on day 4. The upper horizontal line represents the average incorporation in the absence of inhibition in antigen-treated cultures, the lower line is the untreated control.

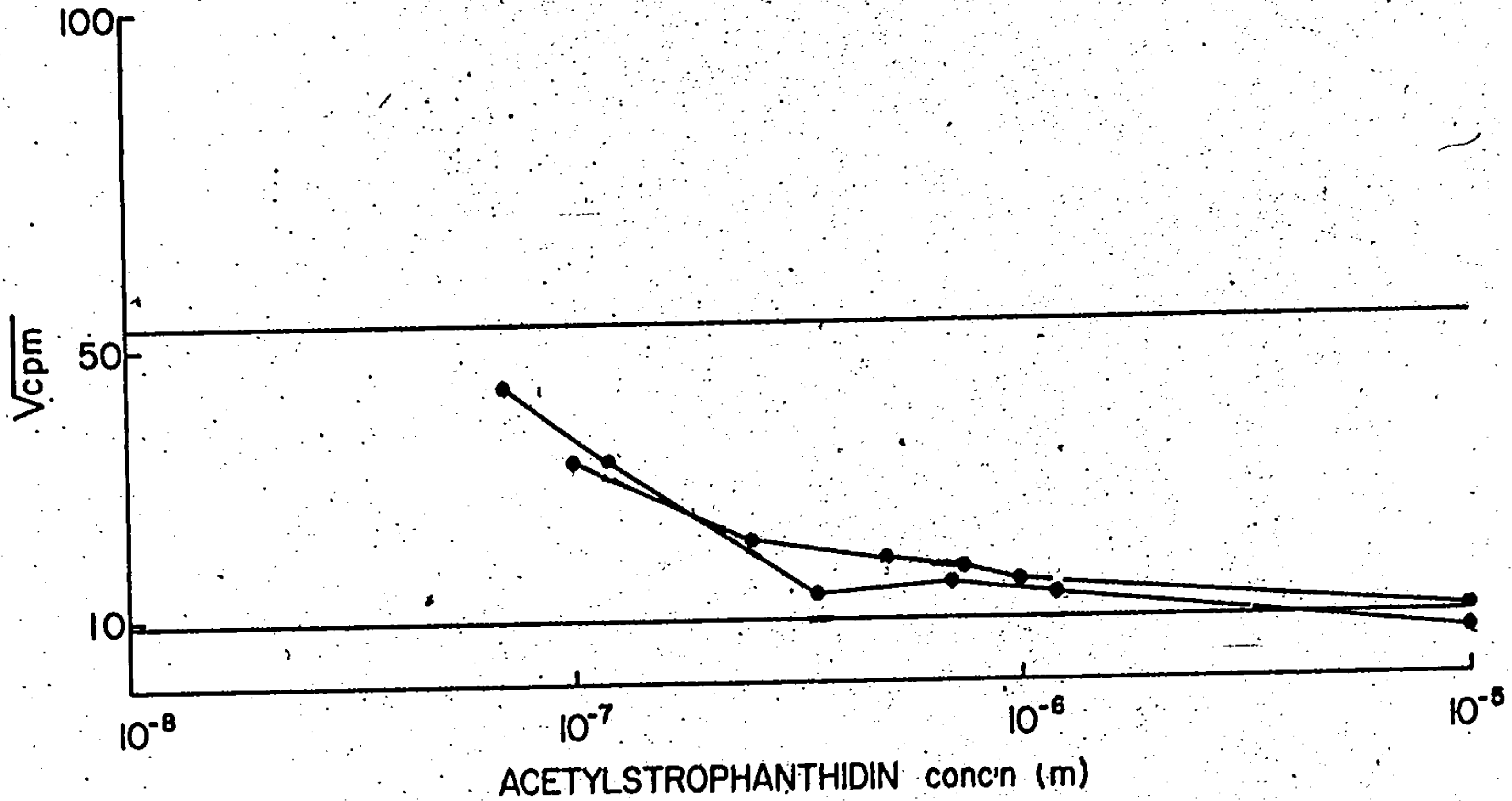

100
50
10
√cpm
10^-8
10^-7
10^-6
10^-5
ACETYLSTROPHANTHIDIN conc'n (m)

Table 3.9: Effect of two-day exposure to ATPase inhibitors on lymphocyte response

a) Response to PHA

Expt.	control		no inhibitor	ouabain[1] 0-2	digoxin 0-2	ac. stroph. 0-2
A	18 [2]	PHA	99	58	37	52
B	20		129	167	33	190
C	7		75	56	124	96

b) Response to antigen

Expt.	control		no inhibitor	ouabain[1] 0-2	digoxin 0-2	ac. stroph. 0-2
A	22	SLO	148	25	15	27
B	20	VAR	71	7	6	14
		SLO	32	10	7	11
C	7	SLO	162	12	184	207
	7	VAR	80	6	15	26

1: 2×10^{-7} M

2: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours

membrane Na^+-K^+-ATPase (since that is what these three drugs have in common).

3.15 Surface Effects

One explanation for the differential sensitivity could be that the drug affects surface components responsible for antigen response, while leaving relatively unaffected those components which bind and/or mediate response to PHA and other mitogens. Surface immunoglobulins are generally considered to be receptors for specific antigens, so it would be of obvious interest to see whether such a surface characteristic as binding of anti-immunoglobulin would be affected by the two-day incubation with ouabain.

As can be seen in Table 3.10, cultures exposed to ouabain, even at high concentrations (up to 4×10^{-6} M) exhibited proportions of cells binding fluorescent anti-immunoglobulin similar to the proportion in control cultures. In a few cases there appeared to be an increase in the fraction of cells binding the anti-immunoglobulin, but without a great deal more data no quantitative comparisons can be made. However, it can be seen clearly that there is, after ouabain preincubation, no significant loss of surface immunoglobulins, nor of cells from the population which bears surface immunoglobulin.

These experiments were not designed to reveal subtle changes in distribution or amount of surface immunoglobulins which could influence responses.

Table 3.10: Effect of two-day incubation with ouabain on binding of fluorescent anti-immunoglobulin by lymphocytes.

	Ouabain Concentration (M)			
Expt.	——	2×10^{-7}	8×10^{-7}	4×10^{-6}
A	27[1]	36.5	45	34
B	28.7	26.9	25.7	38.2
C	17	20	18.4	----
D	9.1	8.6	8.7	----

1. per cent of cells fluorescing

An experiment was also done to compare binding of cells to Sepharose beads with PHA, Con A or PWM coupled to them (as described by Greaves and Bauminger, 1972). Although the method was not quantitative, control cells were seen to bind profusely to the Con A and PHA beads, but hardly at all to the PWM beads. Cells incubated with or without ouabain (2×10^{-7} M) for two days bound equally well to the PHA and Con A beads. This was taken as further evidence that the treatment did not result in generalized disruption of cell surfaces.

Thus, although a subtle surface effect is not excluded by these experiments, it seemed that a general loss of surface receptors was unlikely as an explanation of the loss of response.

3.16 Effect of length of exposure and concentration of ouabain on responses to antigen and mitogen

3.16.1 Effect of one day exposure to ouabain on antigen response

Since two days is a long time to expose cells to an inhibitor we wanted to know whether a shorter incubation would have any effect. In 12 experiments, shown in Table 3.11, in which 1 and 2 day treatments were compared, the 2 day treatments reduced counts to control levels in all but two cases, which were reduced by 70 and 90%. The one day treatments showed great variability in effect: 4 were reduced to control level, 3 were unchanged, and the counts in the other 5 were reduced by 30 to 85%. Thus, there was

Table 3.11: One-day versus two-day exposure to ouabain: effect on antigen response

Expt.	control	antigen		+ ouabain[1] 0-1	+ ouabain[1] 0-2
A	17 [2]	VAR	62	29	10
B	18	SLO	155	194	25
C	13	SLO	31	32	11
D	34	SLO	151	67	25
E	27	TAB	39	33	11
F	10	SLO	24	9	8
G	12	SLO	170	13	12
H	7	SLO	152	47	13
		VAR	21	11	6
I	20	SLO	246	42	32

1: 2×10^{-7} M

2: $\sqrt{cpm}$, ^{3}H-thymidine/4 hours/culture

generally a diminution of response even after a one day exposure but the effect was not usually so drastic as was that of a two day treatment.

The above experiments were done using 2×10^{-7} M ouabain--would a higher concentration have a more consistent effect with the shorter incubation? In the 8 experiments in which both 2 and 8×10^{-7} M ouabain were used (Table 3.12), the higher concentration eliminated response in all cases, while the lower accomplished the same in 7 out of 8 with a two day exposure, but only 4 out of 8 after a one day exposure. However, in 5 out of 6 cases the one-day treatments with the higher concentration also eliminated PHA response, so this shorter harsher treatment could not be used to contrast antigen and mitogen responses.

3.16.2 Effects of two day exposure to different concentrations of ouabain on mitogen and antigen responses

The only experiments discussed here are those in which more than one concentration was tested:

PHA response (Table 3.13): Of seven experiments, 8 and 4×10^{-7} M reduced the incorporation to control levels in all but one case, which was unchanged from untreated levels. Two $\times 10^{-7}$ M had the usual range of effects, only one reduced to control, the others from 50 to 200% of untreated counts. In four experiments, 1.5 and 1×10^{-7} M had a range of effects similar to that of 2×10^{-7} M. Thus, concentrations slightly higher than the usual were inhibitory, while concentrations slightly lower had about the same effect.

Table 3.12: Effect of one-day exposure to 8×10^{-7} M ouabain on antigen response

Expt.	control	antigen		+ ouabain 0-2 2×10^{-7} M	+ ouabain 0-1 8×10^{-7} M
A	18 [1]	SLO	149	25	17
B	10	SLO	24	8	6
C	12	SLO	170	12	8
	12	VAR	38	11	9
D	17	SLO	149	45	6
	17	VAR	62	10	9
E	7	SLO	152	13	17
	7	VAR	21	6	14

1: $\sqrt{cpm}$ ^{3}H-thymidine incorporation/culture/4 hours, 5-7 days after washing.

Table 3.13: Effect of concentration of ouabain (O-2) on inhibition of PHA response

Expt.	control	untreated	8×10^{-7} $\underline{M}$	4×10^{-7} $\underline{M}$	2×10^{-7} $\underline{M}$	1.5×10^{-7} $\underline{M}$	1×10^{-7} $\underline{M}$
A	11[1]	99	20	-----	58	-----	-----
B	10	32	7	6	68	60	65
C	12	46	9	9	14	-----	-----
D	10	102	7	7	55	-----	-----
E	13	50	6	6	104	66	88
F	8	123	7	7	74	39	71
G	7	126	102	130	141	134	122
H	24	82	6	6	80	-------	125

1: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours

Antigen response (Table 3.14): In twelve and 6 experiments respectively, the two higher concentrations reduced incorporation in all cultures to control level. The usual concentration, 2×10^{-7} M, reduced all but one of the twelve to control; that one was reduced to less than 30% of its untreated value. The next concentration, 1.5×10^{-7}, in three experiments brought incorporation in all cultures down to the control level. The lowest concentration used in these experiments, 1×10^{-7} M, did show some variation, from no effect to complete inhibition. It seems, then, that there is still an inhibitory effect on the cells of some donors even down to 10^{-7} M, and with all tested even at 1.5×10^{-7} M.

3.16.3 PHA and 2×10^{-7} M ouabain -- variation in effect

In 66 experiments in which cells were incubated for two days with or without ouabain, then treated with PHA, there was quite a bit of variation in the occurrence and in the severity of the inhibitory effect. In 30 experiments, there was no significant difference between rates of thymidine incorporation in control and ouabain-treated cultures. In 20 experiments the ouabain treated cultures incorporated quite a bit more thymidine than did the controls. The increases ranged from 2 to several hundred times, the latter usually when the control counts were very low, particularly after six or seven days in culture. In 11 experiments, PHA-induced counts were decreased 50 to 80%, and in 6 of the 66 experiments, the incorporation was reduced to control level or less. Although the ouabain affected the response in 36 out of 66

Table 3.14: Effect of concentration of ouabain (0-2) on inhibition of antigen response

Expt.	control	antigen		8×10^{-7} M	4×10^{-7} M	2×10^{-7} M	1.5×10^{-7} M	1×10^{-7} M
A	8 [1]	SLO	61	7	---	14	---	----
B	50	SLO	180	37	---	68	---	165
C	50	SLO	100	38	---	55	---	62
D	18	SLO	149	19	---	25	---	----
E	10	SLO	24	6	7	8	11	35
F	17	SLO	149	9	----	45	---	----
		VAR	62	6	----	10	---	----
G	12	SLO	170	6	9	12	---	----
		VAR	38	7	9	11	---	----
H	9	VAR	37	6	7	6	---	----
I	7	SLO	152	7	7	13	11	17
		VAR	21	5	7	6	6	7
J	24	SLO	156	8	6	5	---	7
		VAR	131	5	12	7	---	7

1: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours

cases, it caused inhibition on the scale of that common to antigen response in only 6 cases. This variability may be due in part to different sensitivities of the donors to the drug, as well as to the varying levels of contaminating non-lymphoid, ouabain-binding cells in the cultures.

3.17 Effects of K^+ on ouabain inhibition

As was previously described (section 3.4), high K^+ concentrations in the culture medium served to antagonize the ouabain inhibition of precursor incorporation. It seemed logical to investigate the effect of increased K^+ concentration on the lingering inhibition of antigen response after prolonged exposure to ouabain.

Table 3.15 shows that for antigen activation, the presence of 26 mM K^+ in the culture medium during the two day exposure to ouabain did not protect against the lingering inhibition. If we were concerned with a simple competition between K^+ and ouabain for binding sites on the membrane enzyme this would not be the result one would have expected. However, Allen and Schwartz (1970) reported that although K^+ did antagonize ouabain binding to a calf heart enzyme preparation, the ion did not prevent binding of the glycoside but only slowed the rate at which the maximum was reached. Thus, the two day incubation may be long enough to permit maximum binding of ouabain regardless of the K^+ concentration.

Inclusion of K^+ had no effect on PHA response, which was generally not affected by the ouabain treatment.

In the next experiments, the cells were incubated

Table 3.15: Effect of inclusion of K^+ (26 mM) in the culture medium from day 0 to day 2 on ouabain (0-2) inhibition of antigen response

Expt.	control	antigen		+ ouabain[1] 0-2	+ ouabain 0-2 + K^+ 0-2
A	20[2]	SLO	246	32	32
B	31	SLO	64	31	29
C	9	VAR	53	7	24
D	6	VAR	73	9	43
E	20	SLO	173	7	11
F	18	VAR	35	7	9
G	7	SLO	74	8	6
H	12	VAR	38	12	11
		SLO	170	12	28

1: 2×10^{-7} M

2: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours

in the usual manner with ouabain for two days, then, in the replacement medium after washing, were given extra K^+ (20 mM). This treatment also failed to assist recovery of the response (Table 3.16). Such a result might be explained by the finding of Akera and Brody (1971) that K^+, when added after formation of the ouabain-enzyme complex, stabilized that complex and inhibited dissociation of the ouabain from the complex.

3.17.1 Effect of K^+ on ouabain inhibition of PHA response

As was previously mentioned (section 3.15.1), a two day preincubation with 8×10^{-7} M ouabain did serve to inhibit PHA response. Seeking to compare this inhibition with that of antigen response, the experiments described above were repeated using this high concentration of ouabain and PHA. Tables 3.17 and 3.18 show that although inclusion of high K^+ during exposure to ouabain did not prevent inhibition, addition of extra K^+ after washing out the ouabain did allow the response to be recovered. Thus it seemed that the effect on PHA response is a more easily reversible one than is that on antigen. This result is compatible with the above explanation for lack of recovery of antigen response under the same conditions if one assumes that PHA causes conformational changes associated with uncovering of cryptic K^+ transport sites as postulated by Quastel (1970).

This result also militates against the hypothesis that the inhibitory effect is exerted by cell killing.

Table 3.16: Effect of inclusion of K^+ (26 mM) in the culture medium from day 2 to end of culture on ouabain (0-2) inhibition of antigen response

Expt.	control	antigen	untreated	+ ouabain[1] 0-2	+ ouabain 0-2 + K^+ 2-end
A	19[2]	TAB	38	9	9
B	38	SLO	61	14	11
C	9	VAR	53	7	6
D	6	VAR	73	9	7
E	16	SLO	145	7	19
F	11	VAR	32	5	9
G	10	VAR	34	10	13

1: 2×10^{-7} M

2: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours

Table 3.17: Effect of inclusion of K^+ (26 mM) in the culture medium from day 0 to day 2 on ouabain (0-2) inhibition of PHA response

Expt.	control	PHA		
		no ouabain	ouabain[1] 0-2	ouabain 0-2 + K^+ 0-2
A	9[2]	62	8	77
B	8	143	8	12
C	15	110	6	22
D	16	65	15	11
E	17	108	5	15
F	10	91	5	5
G	7	47	5	5
H	10	90	7	16

1: 8×10^{-7} M

2: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours

Table 3.18: Effect of inclusion of K^+ (26 mM) in the culture medium from day 2 to end of culture on ouabain (0-2) inhibition of PHA response

Expt.	control	PHA no ouabain	PHA ouabain[1] 0-2	PHA + ouabain 0-2 K^+ 2-end
A	48[2]	308	77	307
B	9	62	8	106
C	8	143	8	74
D	15	110	6	81
E	17	108	5	102
F	10	90	7	88

1: 8×10^{-7} M

2: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours

3.18 The effect of pre-incubation in K^+-free medium on response to antigen and to PHA

If the effect of the ouabain were to deprive the lymphocytes of the needed level of intracellular K^+, it seemed worth checking whether the inhibition would also be observed following prolonged incubation in medium devoid of K^+. Table 3.19 shows the results of such experiments. The first experiments were done with autologous plasma in the culture medium, which could have contributed a small amount of K^+. Since this treatment did not seem to have the same effect as did the parallel ouabain treatment, the latter experiments were done with the two day incubation in plasma-free medium, for both control and K^+-free cultures. This made no difference. Of seven experiments, in only one was response to antigen lost after incubation in K^+-free medium. Thus, it appeared that the effect of K^+-free medium was not, experimentally, comparable to that of ouabain. This was tested by setting up control cultures, with antigen or mitogen, washing them, two days before harvest, with K^+-free medium, and reincubating them in K^+-free medium for the last two days. Table 3.20 shows that this treatment did not affect the PHA response at all, while a parallel ouabain treatment did. In one of the experiments, the antigen counts, which had been fairly low, were reduced to control levels. In the other, antigen response was not reduced at all. Thus incubation in K^+-free (even when plasma-free) medium was not equivalent to incubation in a standard medium with ouabain. It is possible that the cells

Table 3.19: Effect of incubation in K^+-free culture medium day 0-day 2 on response to PHA and to antigen

a)

Expt.	control	PHA	no ouabain	ouabain[1] 0-2	K^+-free 0-2
A	15 [2]		110	50	143
B	14		63	15	79
C	17		131	92	107
D	14		70	94	64
E	10		91	----	77
F	10		32	68	40
G	7		47	97	69

b)

Expt.	control	PHA	no ouabain	ouabain[1] 0-2	K^+-free 0-2
A	15	VAR	51	4	8
B	14	SLO	130	7	129
C	11	VAR	32	5	54
D	14	SLO	129	15	140
		VAR	47	6	40
E	18	VAR	35	7	40
F	10	SLO	24	8	21
G	7	SLO	74	8	66

1: 2×10^{-7} M

2: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours.

Table 3.20: Effect of incubation in K^+-free medium for last 48 hours of culture on PHA and antigen response

	Control			+ ouabain 8×10^{-7} M day 4-6			+ ouabain 2×10^{-7} M day 4-6			K^+-free medium day 4-6		
Expt.	control	PHA	Ag	control	PHA	Ag	control	PHA	Ag	control	PHA	Ag
A	15	53	75	6	16	8	7	9	6	6	141	95
B	15	92	35	8	19	7	7	14	8	13	152	16
C	18	59	---	--	20	--	--	7	--	---	122	-----

1: $\sqrt{cpm}$, ^{3}H-thymidine/culture/4 hours.

were able to maintain their intracellular K^+ by continuous pumping, especially since the rate of leak is relatively slow (Quastel, 1970). In this case, the intracellular K^+ would not be much reduced, while in the presence of ouabain, it would be reduced to the concentration in the culture medium (about 6 mM).

Discussion

This work was begun with the intent to extend to antigen stimulated cells the observations of Quastel (1970) on the effects of ouabain on PHA-treated lymphocytes. These observations were, essentially, that the specific inhibition of the membrane Na^+-K^+-ATPase resulted in a profound but reversible inhibition of all parameters of PHA-induced blast transformation. Quastel (1970) proposed the following hypothesis to account for these results. An early effect of PHA could be induction of conformational changes of proteins in the cell membrane, leading to activation of K^+ transport and thus to increased levels of K^+ in the cell, which would be necessary for cell enlargement and for activation of metabolic processes during transformation. Ouabain, then, would simply prevent the increased K^+ transport, thereby preventing subsequent developments.

Antigen binding to the cell surface would be expected to cause perturbations in the membrane different from those induced by binding of lectin. Such perturbations might or might not lead to conformational changes associated

with an increase in the number of available K^+ transport sites, although the activated cell would be expected to need additional K^+ in order to increase in size and metabolic activity. Due to the low number of responding cells in antigen-treated leukocyte cultures, at least in the critical initial stages, it would have been difficult to measure changes in K^+ transport directly. However, it was possible to study the consequences of inhibition of K^+ transport by ouabain, especially when response was assayed at later stages (such as at DNA synthesis).

The first experiments suggested that ouabain affected antigen response in much the same way as it did response to PHA. Equivalent inhibition of DNA synthesis was achieved at similar concentrations and the inhibition was antagonized by increased extracellular K^+. However, in one aspect of the inhibition the response to antigen differed markedly from that to mitogen. This was in the reversing of inhibition by washing the cultures to remove the drug. When this was done after a prolonged incubation (two days), mitogen but not antigen response was recovered. Although there was some variability in the effect of this treatment on leukocyte reactivity, generally there remained virtually no response to antigen in cultures preincubated for two days with the drug (at 2×10^{-7} M), whereas response to PHA was nearly always in evidence, although sometimes reduced or increased.

This finding indicated a difference, either in the requirements for stimulation or in the events following

stimulation, between responses to antigen and to PHA. It was then shown that this differential sensitivity was applicable to the MLR (which was as inhibited as antigen response) and to other mitogens, PWM and Con A (response to which was demonstrable after ouabain pretreatment).

Effect of K^+ on the inhibition

The most likely reason for the effect of ouabain is the depletion of intracellular K^+ which results from inactivation of the Na^+-K^+ pump. It was not possible to reverse or to antagonize the effect of preincubation with ouabain by including up to 26 mM K^+ in the culture medium, during or following the ouabain treatment. This K^+ concentration was sufficient to prevent ouabain inhibition during the last 24 hours of response, in the early experiments. However, this may not be surprising in view of the reports that in isolated membrane preparations, K^+ antagonism cannot completely prevent ouabain binding, but can only decrease its rate (Allen and Schwartz, 1970). Thus, over the long incubation period in these experiments, sufficient ouabain might, even in the presence of high K^+, bind to the membrane sites to cause the effect responsible for loss of response to antigen. Quastel (1974) found that excess K^+ could prevent binding of ^{3}H-ouabain to PHA-treated lymphocytes but could not reverse it, over periods of one to two days. However, even the prevention of binding was incomplete, and the experiments were done with a ouabain concentration of only 3×10^{-8} M and a K^+ concentration of about 24 mM. Failure of K^+ to

accelerate dissociation of bound ouabain may be explained by certain results from isolated membrane preparations. Akera and Brody (1971) have shown that K^+, when added after the ouabain is bound to the enzyme, serves to stabilize the complex, thereby slowing the rate of dissociation of the drug.

Unfortunately, the relevant biochemical studies have been carried out over time periods of minutes or hours, not days, so there is no detailed information on drug-enzyme interactions over the time period that is important in this work. Even the few tissue culture studies (such as Lamb and McCall, 1972; Vaughn and Cook, 1972) did not involve exposure to ouabain for more than 24 hours. It is therefore difficult to speculate as to the interactions that might have occurred during the lengthy treatment used here.

There is of course the possibility that the effect of ouabain is not mediated by depletion of cell K^+. It is known that the drug remains largely on the outside of the cell, bound to a component of the membrane. The inhibition could therefore be caused by the mere presence of the molecule, perhaps interfering with the binding of antigens to their receptors, or, in the case of the MLR, masking surface characteristics by which the cell is recognized as foreign. Or it could by its binding prevent rearrangement of surface molecules following contact of antigen and cell. Although the ouabain pretreatment did not affect binding of immunoglobulins, nor binding of cells to sepharose-PHA, this

kind of effect could be more subtle, not detected by studies of this nature.

In addition, Leu et al. (1973) had evidence that ouabain could affect migration of guinea-pig macrophages in a manner which was unrelated to Na^{+}-K^{+} ion exchange; they postulated that in this case the drug might directly stimulate actinomyosin contraction mechanisms. Information on effects of ouabain not directly resulting from its inhibition of the Na^{+}-K^{+} pump is scant; the drug has been implicated in transport of Mg^{++} (Beauchamp et al., 1971) and of glucose (Bronstead, 1970; Ward and Becker, 1970) but these are not well-defined phenomena.

Clearly, it would be difficult, with the present amount of information about ouabain interactions with mammalian cells, to hypothesize a particular mode of action not related to its effect on K^{+} transport.

Is the macrophage function affected by the ouabain treatment?

One of the important differences between specific and non-specific response is the requirement in the former for macrophages (section 1.1.3.1.). The data obtained suggested that these cells were not the target for the lingering inhibitory effect, since ouabain-treated macrophages were able to restore antigen reactivity to macrophage-depleted lymphocyte cultures, while untreated macrophages were unable to restore reactivity in ouabain-treated lymphocyte cultures. This conclusion is supported by the finding of Rosenthal et al. (1972) that ouabain, at concentrations which inhibited

K^+ transport, had no effect on the ability of guinea pig macrophages to present immunologically relevant antigen to lymphocytes. Thus it seems we must look to the lymphocyte as the site of the differential sensitivity.

Does the ouabain affect only T cells or only B cells?

One obvious possibility was that one could attribute this difference to the activities of one or the other sub-population of lymphocytes. The sum of recent evidence suggests that in the MLR it is predominantly the B cell which stimulates and the T cell which responds (Lohrman et al., 1974 a,b; Lohrman and Whang-Peng, 1974). Since preincubation with ouabain affects stimulation but not response in the MLR (Sasportes et al., 1974), one is led to suspect that the B cell might be the specific target of the ouabain effect. This hypothesis is fortified by the observation that PWM response was rather more sensitive than were PHA and Con A response to this treatment, since PWM response includes a B cell component as well as T cell activation. Proliferation induced by PHA and Con A is apparently almost entirely of T cells (Greaves et al., 1974 a,b; Geha and Merler, 1974). However, such evidence as exists suggests that T cells are also prominent in the proliferative response of human lymphocytes to soluble antigens (Geha et al., 1973; Geha and Merler, 1974; Smith and Haegert, 1974). Thus, if only B cells were irreversibly affected by the drug, one would expect at worst an effect on antigen response similar to that on response to PWM, i.e. if B cell proliferation were eliminated, total

response might be reduced, but there would be a residual response, due to the unaffected T cells, which also respond to mitogens. But, although PWM response was reduced in some cases by two-day preincubation with 2×10^{-7} M ouabain, antigen response was eliminated in nearly all cases by this treatment. It therefore seems unlikely that the cause of loss of response is due solely to a selective suppressive effect on B cells.

Is there inhibition of synthesis of a secreted molecule?

An alternative hypothesis to account for both loss of response to antigens and loss of stimulatory capacity in leukocyte cultures is that the metabolic shutdown effected by long exposure to ouabain prevented the synthesis, immediately upon contact of cells with antigen, of an essential "message" required for stimulation in the MLR or for "recruitment" in the response to antigens. Parsa and Kountz (1974) have shown that a number of newly synthesized proteins are released into the culture medium in the first few hours of an MLR and that this early synthesis must take place for stimulation to occur. Many authors (beginning with Kasakura, 1970) have presented evidence of the release of "blastogenic factors" into culture medium by lymphocytes activated by various means (section 1.2.2.1.). Schellekens and Eijsvoogel (1971) had evidence that recruitment by soluble factors during the first days of culture with antigens was a critical factor in elicitation of a measurable response. Valentine (1972) was able to dissociate proliferative response in cultures of antigen-treated human

leukocytes, from production of "lymphocyte transforming factor". Perhaps in these important early hours of response the cell might not have recovered enough of its faculties to produce these proteins for release into the medium. In this case, the lymphocytes could be capable of developing response to antigen as cell K^+ was restored, but the low number of cells involved would not be detected in this culture system. Some attempts were made to study this possibility, but we were not able consistently to obtain a "recruitment" factor from antigen-treated cells. Supernatants from PHA-treated cells did cause stimulation of DNA synthesis in homologous cultures, but neither the production of nor the response to these supernatant factors was affected by two-day preincubation with ouabain (2×10^{-7} M). This result suggests that failure to produce or to respond to recruitment factors was not the cause of the differential sensitivity. It does not exclude a difference in production of recruitment factors between antigen-treated and mitogen-treated cells.

Is this an effect on a surface component?

One tempting hypothesis is that the ouabain may be affecting synthesis of a surface receptor for antigen while having less effect on synthesis of the surface components which bind plant lectins. Unstimulated lymphocytes have a constant low rate of turnover of membrane components (Cone et al., 1971); some of these, including immunoglobulins, are shed into the medium and must be replaced (Vitetta and Uhr, 1973). Since K^+ is important in protein synthesis its absence

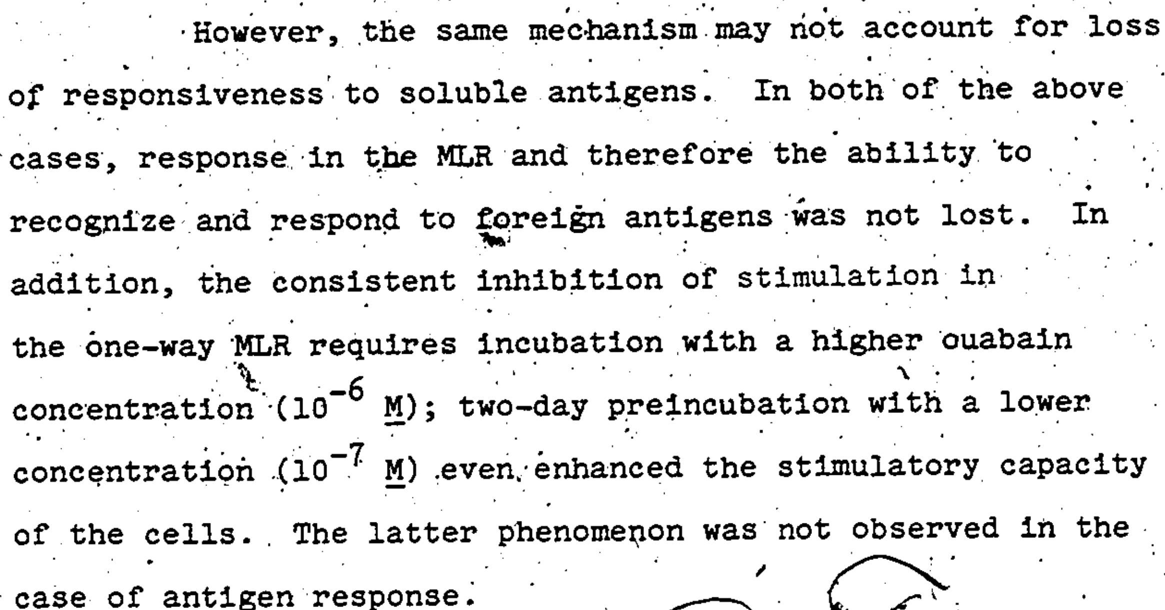
could affect replacement of shed receptors, thus affecting ability to bind specific antigens. On the other hand, a cell might be able to suffer the loss of many of its lectin receptor sites and still be able to bind sufficient mitogen to induce activation. For example, Andersson et al. (1972) measured the binding of radiolabelled Con A to mouse thymus lymphocytes and found that although maximum binding was 10^7-10^8 molecules per cell, only 3-10% of the receptors needed to be occupied for optimal stimulation.

An effect on surface receptors might be expected to be reversible, so that when the ouabain left on the cell after the washing has dissociated and the intracellular K^+ has been restored, new receptors should be synthesized and inserted into the membrane and responsiveness restored. This kind of effect was demonstrated by Milton and Mowbray (1972) using Fraction C, a component of bovine serum, to suppress reversibly, responses of human lymphocytes. This substance had a rapid inhibitory effect on secretion of antibody by mouse spleen cells (2 hours) a slower effect on binding of anti-immunoglobulin or PPD to human cells (2 days) and a much slower effect on PHA binding and response (4-5 days preincubation with inhibitor required). They suggested that Fraction C was acting at the level of mRNA synthesis and that the different rates of disappearance of the different functions were related to differences in turnover time of either the molecules themselves or of their mRNAs. Unlike the case of Fraction C, a two-day preincubation with ouabain did not

result in a general loss of anti-immunoglobulin-binding capacity in the cells, nor was the effect freely reversible.

This kind of hypothesis could account for the observations of Desportes et al. (1974) concerning the effect of preincubation with ouabain on the MLR. They were able to extend my work (showing that the two-way MLR was irreversibly inhibited by this treatment) to show in addition that, in one-way reactions, stimulation but not response was affected. This was in contrast to the effect on response to soluble antigens, but could be explained by a differential loss of surface components, in this case loss of the molecules which are recognized as foreign by heterologous lymphocytes. This was the explanation offered by Opelz and Terasaki (1974) for their finding that mere preincubation for long periods (4 or more days) resulted in loss of stimulatory abilities but not of response in the MLR. Thus failure to replace shed membrane components ~~may~~ account for selective loss of stimulatory capacity in both cases.

However, the same mechanism may not account for loss of responsiveness to soluble antigens. In both of the above cases, response in the MLR and therefore the ability to recognize and respond to foreign antigens was not lost. In addition, the consistent inhibition of stimulation in the one-way MLR requires incubation with a higher ouabain concentration (10^{-6} M); two-day preincubation with a lower concentration (10^{-7} M) even enhanced the stimulatory capacity of the cells. The latter phenomenon was not observed in the case of antigen response.



The effect of ouabain which results in enhancement of stimulation in the MLR may or may not be similar to its inhibitory effect at higher concentrations. For example, the irreversible inhibition might be due to irreversible inactivation of a critical enzyme sensitive to intracellular K^+ levels. At the lower concentration of ouabain, which would not completely inhibit K^+ transport, the enzyme may be inactive only as long as the bound ouabain keeps intracellular K^+ suboptimal. After removal of the ouabain and restoration of normal cell K^+, there might be an overshoot in production in the affected pathway, due to accumulation of intermediates prior to the site of the block or of enzyme subunits, which might result in enhanced stimulation. On the other hand, although treatment with the high concentration might result in a depression of cell activity, the lower concentration, not being completely inhibitory, might act as a localized injury to the membrane (cf. Vaughn and Cook, 1972). The repair of this injury might involve synthesis of new segments and their insertion into the membrane. Such additions might result in increased stimulation via, for example, an increase or rearrangement of the surface components that act as signals.

We have discovered a simple treatment, using a drug with well known properties, which distinguishes between antigen and mitogen stimulation of human leukocyte cultures. A two-day incubation with ouabain, a specific inhibitor of the membrane Na^+-K^+ ATPase, results in virtually total loss

of antigen but not mitogen response. We have shown that the irreversible inhibition is not due to cell killing, nor to inhibition of macrophage function, nor to a generalized destruction of cell surface characteristics. The inhibition was neither prevented nor reversed by increasing the external K^+ concentration up to 26 mM (added during or after the ouabain treatment), but since this was a long incubation this does not necessarily exclude an inhibition mediated by depletion of cell K^+.

This differential sensitivity to an established drug may have potential for exploitation in clinical situations. It has already been extended by Sasportes et al. (1974) to show that in the one-way MLR the capacity to stimulate is irreversibly lost after pretreatment with ouabain, while the capacity to respond is unaffected.

In the following section, I shall discuss some of the possible approaches to be taken if one were to continue this study.

Directions

Since an effect on surface structures could be both subtle and pleotropic, approaches which would detect slight changes in, for example, antigen binding would be informative. Recently, Hersh and Dyre (1974) have been able to study binding of keyhole limpet hemocyanin to human lymphocytes, by coating RBC with the antigen and counting rosettes. Such an experiment could perhaps detect any change in ability to bind specific antigens caused by the ouabain

treatment. One might also check rosette formation in ouabain pretreated cells, as the complementary study to that on anti-immunoglobulin binding. And a microscopic study of, for example, cap formation would indicate whether the bound inhibitor exerted its effect on movement of membrane components.

Since Sasportes et al. (1974) found that the sensitivity to prolonged incubation with ouabain lay in the stimulating cells and since the macrophage is very important in the MLR (Alter and Bach, 1970) one wonders whether the ability of the macrophage to participate in the MLR is affected by the inhibitor. Marshall et al. (1966) and Rode and Gordon (1974) have reported that cultured human macrophages can be used as stimulating cells in the MLR; this would seem to be a convenient one-way reaction system in which to assess macrophage function after long exposure to ouabain.

Separation and selective stimulation of T and B cells would clarify whether one or the other was especially sensitive to the effects of ouabain. These experiments might also shed some light on the interactions of these cell types in response to antigens and to heterologous cells in culture.

The effect of K^+ on this inhibition might be further investigated using a variety of home-made media including those with very low and very high (up to 100 mM) K^+ concentrations.

The specific effects of the treatment on cellular activities could be assessed. The possible enzymes affected may be too numerous to study, though perhaps some of those

References

Akera, T. and T.M. Brody. (1971). Membrane adenosine triphosphatase: the effect of potassium on the formation and dissociation of the ouabain-enzyme complex. J. Pharmacol. Exp. Therapeut. 176, 545-557.

Albers, R.W., G.J. Koval and G.J. Siegel. (1968). Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. Molec. Pharmacol. 4, 324-336.

Allen, J.C. and A. Schwartz. (1970). Effects of potassium, temperature and time on ouabain interaction with the cardiac Na^+, K^+-ATPase: further evidence supporting an allosteric site. J. Molec. Cell. Cardiol. 1, 39-45.

Allwood, G., G.L. Asherson, M.J. Davey and P.J. Goodford. (1971). The early uptake of radioactive calcium by human lymphocytes treated with phytohaemagglutinin. Immunology 21, 509-516.

Almendares, J.A. and A. Kleinzeller. (1971). The Ouabain Inhibition of Sugar Transport in Kidney Cortex Cells. Arch. Biochem. Biophys. 145, 511-19.

Alter, B.J. and F.H. Bach. (1970). Lymphocyte reactivity in vitro 1. Cellular reconstitution of purified lymphocyte response. Cell. Immunol. 1, 207-218.

Andersson, J., G. Möller and O. Sjoberg. (1972). B lymphocytes can be stimulated by Concanavalin A in the presence of humoral factors released by T cells. Eur. J. Immunol. 2, 99-101.

Andersson, J., G.M. Edelman, G. Möller and O. Sjoberg. (1972b). Activation of B lymphocytes by locally concentrated concanavalin A. Eur. J. Immunol. 2, 233-235.

Averdunk, R. (1972). The effect of phytohaemogglutinin and antilymphocyte serum on the transport of potassium ions, glucose and amino acids in human lymphocytes. Hoppe-Seyler's Z. Physiol. Chem. 353, 79-87.

Bach, F.H., H. Bock, K. Graupner, E. Day, and H. Klosterman. (1969). Cell kinetic studies in mixed leukocyte cultures: an in vitro model of homograft reactivity. Proc. Natl. Acad. Sciences U.S.A. 62, 377-384.

Bach, F.H., B.J. Alter, S. Solliday, D.C. Zoschke and M. Janis. (1970). Lymphocyte reactivity in vitro 2 soluble reconstituting factor permitting response of purified lymphocytes. Cell. Immunol. 1, 219-227.

Bach, M.L., B. Alter, D. Zoschke and F.H. Bach. (1971). Soluble adherent cell activity permitting purified lymphocyte response. Transplant. Proc. III, 844-847.

Baker, P.F. and J.S. Willis. (1970). Potassium ions and the binding of cardiac glycosides to mammalian cells. Nature 226, 521-523.

Beauchamp, R.S., S. Silver and J.W. Hopkins. (1971). Uptake of Mg^{2+} by KB cells. Biochim. Biophys. Acta 225, 71-76.

Benacerraf, B. and H.O. McDevitt. (1972). Histocompatibility-linked immune response genes. Science 175, 273-279.

Bender, M.A. and D.M. Prescott. (1962). DNA synthesis and mitosis in cultures of human peripheral leukocytes. Exptl. Cell Res. 27, 221-229.

Berendes, H.D., F.M.A. van Breugel and Th.K.H. Holt. (1965). Experimental puffs in salivary gland chromosomes of Drosophila hydei. Chromosoma (Berlin) 16, 35-46.

Bianco, C. and V. Nussenzweig. (1971). Theta-bearing and complement receptor lymphocytes are distinct populations of cells. Science 173, 154-156.

Biberfeld, P. (1971). Morphogenesis in blood lymphocytes stimulated with phytohaemagglutinin (PHA). Acta Path. Microbiol. Scand. Section A, Suppl. 223.

Boardman, L.J., J.F. Lamb, D. McCall. (1972). Uptake of ^{3}H-ouabain and sodium pump turnover rates in cells cultured in ouabain. J. Physiol. 225, 619-635.

Bona, C., R. Robineaux, A. Anterenis, C. Heuclin and A. Astesano. (1973). Transfer of antigen from macrophages to lymphocytes. II Immunological significance of transfer of lipopolysaccharide. Immunology 24, 831-840.

Bonting, S.L., L.L. Caravaggio, and W.M. Hawkins. (1962). Studies on sodium-potassium activated adenosine triphosphatase. IV Correlation with cation transport sensitive to cardiac glycosides. Arch. Biochem. Biophys. 98, 413-419.

Bonting, S.L., N.M. Hawkins and M.R. Canaday. (1964). Studies of sodium-potassium activated adenosine triphosphatase. VII Inhibition by erythrophleum alkaloids. Biochem. Pharmacol. 13, 13-22.

Brondsted, H.E. (1970). Ouabain-sensitive carrier-mediated transport of glucose from the cerebral ventricles to surrounding tissues in the cat. J. Physiol. 208, 187-201.

Buckton, K.E. and M.C. Pike. (1964). Chromosome investigations on lymphocytes from irradiated patients: effect of time in culture. Nature 202, 714-715.

Burke, G. (1970). Effects of cations and ouabain on thyroid adenyl cyclase. Biochim. Biophys. Acta 220, 30-41.

Byrnes, J.J., K.M. Downey and A.G. So. (1973). Bone marrow cytoplasmic deoxribonucleic acid polymerase. Variation in pH and ionic environment as a possible control mechanism. Biochemistry 12, 4378-4384.

Caldwell, P.C. and R.D. Keynes. (1959). The effect of ouabain on the efflux of sodium from a squid giant axon. J. Physiol. 148, 8P-9P.

Caron, G.A., S. Poutala and T.T. Provost. (1970). Lymphocyte transformation induced by inorganic and organic mercury. Int. Arch. Allergy 37, 76-87.

Catovsky, D., P.J.L. Holt and D.A.G. Galton. (1972). Lymphocyte transformation in immunoproliferative disorders. Brit. J. Cancer 26, 154-163.

Chalmers, D.G., E.H. Cooper, C. Evans and N. Topping. (1967). Quantitation of the response of lymphocytes in culture to specific and nonspecific stimulation. Int. Arch. Allergy 32, 117-130.

Claman, H.N. and E.A. Chaperon. (1969). Immunologic complementation between thymus and marrow cells - A model for the two-cell theory of immuno competence. Transplant.

Rev. 1, 92-113.

Cline, M.J. and V.C. Swett. (1968). The interaction of human monocytes and lymphocytes. J. Exp. Med. 128, 1309-1325.

Cone, R.E., J.J. Marchalonis and R.T. Rolley. (1971). Lymphocyte membrane dynamics. Metabolic release of cell surface proteins. J. Exp. Med. 134, 1373-1384.

Cooper, H.L. and A.D. Rubin. (1965). Lymphocyte RNA metabolism. Lancet 21, 723-724.

Cooper, H.L. (1972). Studies on RNA metabolism during lymphocyte activation. Transplant. Rev. 11, 3-38.

Cowling, D.C. and D. Quaglino. (1965). Effect of some antigens on leucocyte cultures. J. Path. Bacteriol. 89, 63-70.

Crane, R.K., D. Miller, and I. Bihler. (1961). The restrictions on possible mechanisms of intestinal transport of sugars. Membrane Transport and Metabolism eds. A. Kleinzeller and A. Kotyk. Academic Press, New York, pp. 439-449.

Cross, M.E. and M.G. Ord. (1971). Changes in histone phosphorylation and associated early metabolic events in pig lymphocyte cultures transformed by phytohaemagglutinin or 6N,2'-O-dibutyladenosine 3'-5'-cyclic monophosphate. Biochem. J., 124, 241-248.

Csaky, T.Z., H.G. Hartzog and G.W. Fernald. (1961). Effect of digitalis on active intestinal sugar transport. Amer. J. Physiol. 200, 459-460.

Curtis, J.E. and E.M. Hersh. (1973). Cellular Immunology in Man:Correlation of Leukocyte Migration Inhibitory Factor Formation and Delayed Hypersensititivy. Cell. Immunol. 8, 55-61.

Das, K.C. and A.V. Hoffbrand. (1970). Studies of folate uptake by phytohaemagglutinin-stimulated lymphocytes. Brit. J. Haematol. 19, 203-221.

Davies, A.J.S., E. Leuchars, V. Wallis and M.J. Doenhoff. (1971). A system for lymphocytes in the mouse. Proc. Roy. Soc. London B 176, 369-384.

de Petris, S. and M. Raff. (1973). Normal distribution, patching and capping of lymphocyte surface immunoglobulin studied by electron microscopy. Nature (New Biol.), 241, 257-259.

Douglas, S.D. (1972). Electron microscopic and functional aspects of human lymphocyte response to mitogens. Transplant. Rev. 11, 39-59.

Dumonde, D.C. (1970). 'Lymphokines': molecular mediators of cellular immune responses in animals and man. Proc. Roy. Soc. Med. 63, 899-902.

Dunham, E.T. and S.M. Glynn. (1961). Adenosinetriphosphatase activity and the active movements of alkali metal ions. J. Physiol. 156, 274-293.

Dunham, P.B. and J.F. Hoffman. (1971). Active cation transport and ouabain binding in high potassium and low potassium red blood cells of sheep. J. Gen. Physiol. 58, 94-116.

Epstein, L.B., M.J. Cline and T.C. Merigan. (1971). PPD-

stimulated interferon:in vitro macrophage-lymphocyte interaction in the production of a mediator of cellular immunity. Cell. Immunol. 2, 602-613.

Evans, J.V. and J.W.B. King. (1955). Genetic control of sodium and potassium concentrations in the red blood cells of sheep. Nature 176, 171.

Feldmann, M. (1972). Cell interactions in the immune response in vitro. II. The requirement for macrophages in lymphoid cell collaboration. J. Exp. Med. 135, 1049-1058.

Feldmann, M. and G.J.V. Nossal. (1972). Tolerance and Enhancement and the Regulation of Interactions between T cells, B cells and Macrophages. Transplant. Rev. 13, 3-34.

Fellner, M.J., P.L. Baer, C.S. Ripps and K. Hirschhorn. (1967). Response of lymphocytes to penicillin:Comparison with skin tests and circulating antibodies in man. Nature 216, 803-804.

Fisher, D.B. and G.C. Mueller. (1968). An early alteration in the phospholipid metabolism of lymphocytes by phytohaemagglutinin. Proc. Natl. Acad. Sci. U.S.A. 60, 1396-1402.

Fisher, D.B. and G.C. Mueller. (1971). Studies on the mechanism by which phytohaemagglutinin rapidly stimulates phospholipid metabolism of human lymphocytes. Biochim. Biophys. Acta 248, 434-448.

Gajl-Peczalska, K., H.J. Meuwissen and R.A. Good. (1969). Pokeweed mitogen-induced blastoid transformation in purified and non-purified leukocyte cultures. Int. Arch. Allergy 36, 546-53.

Geha, R.S., E. Schneeberger, F.S. Rosen and E. Merler. (1973). Interactions of human thymus-derived and non-thymus-derived lymphocytes in vitro. Induction of proliferation and antibody synthesis in B lymphocytes by a soluble factor released from antigen-stimulated T lymphocytes. J. Exp. Med. 138, 1230-1247.

Geha, R.S. and E. Merler. (1974). Response of human thymus-derived (T) and non-thymus-derived (B) lymphocytes to mitogenic stimulation in vitro. Eur. J. Immunol. 4, 193-199.

Geha, R.S., F.S. Rosen and E. Merler. (1974). Unresponsiveness of human B lymphocytes to phytohaemagglutinin. Nature 248, 426-428.

Glynn, I.M. (1957). The action of cardiac glycosides on sodium and potassium movements in human red cells. J. Physiol. 136, 148-173.

Golstein, P., H. Wigzell, H. Blomgren and E.A.J. Svedmyr. (1972). Cells mediating specific in vitro cytotoxicity II. Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic target cells. J. Exp. Med. 135:890-906.

Gowans, J.L. (1967). Life span, recirculation and transformation of lymphocytes. Rev. Exper. Path. 5:1-24.

Gräsbeck, R., C.T. Nordman and A. De LaChapelle. (1964). The leucocyte-mitogenic effect of serum from rabbits immunized with human leucocytes. Acta Med. Scand. 175, Suppl. 412, 39.

Greaves, M.F. and N.M. Hogg. (1971). Immunoglobulin determinants on the surface of antigen binding T- and B-lymphocytes in mice. In: Progress in Immunology ed. B. Amos Academic Press, New York, pp. 111-126.

Greaves, M. and G. Janossy. (1972). Elicitation of selective T and B-lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11, 87-130.

Greaves, M.F. and I.M. Roitt. (1968). The effect of phytohaemagglutinin and other lymphocyte mitogens on immunoglobulin synthesis by human peripheral blood lymphocytes in vitro. Clin. Exp. Immunol. 3, 393-412.

Greaves, M.F. and S. Bauminger. (1972). Activation of T and B lymphocytes by insoluble phytomitogens. Nature (New Biol.) 235, 67-70.

Greaves, M.F., G. Janossy and M. Doenhoff. (1974a). Activation of human B and T lymphocytes with polyclonal mitogens. Nature 248, 698-701.

Greaves, M.F., G. Janossy and M. Doenhoff. (1974b). Selective triggering of human T and B lymphocytes in vitro by polyclonal mitogens. J. Exp. Med. 140, 1-18.

Hadden, J.W., E.M. Hadden, M.K. Haddox and N.D. Goldberg. (1972). Guanosine 3':5'-cyclic monophosphate: a possible intracellular mediator of mitogenic influences in lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 69, 3024-3027.

A.W. Ham. (1969). Histology, 6th Edition. J.B. Lippincott Company, Philadelphia, p. 273.

Hartzmann, R.J., M.L. Bach, F.H. Bach, G.B. Thurman and K.W. Sell. (1972). Precipitation of radioactively labelled samples: a semi-automatic multiple-sample processor. Cell. Immunol. 4, 182-186.

Haskill, J.S., B.E. Elliott, R. Kerbel, A. Axelrod and D. Eidinger. (1972). Classification of thymus-derived and marrow-derived lymphocytes by demonstration of their antigen binding characteristics. J. Exp. Med. 135, 1410-1415.

Hayden, G.A., G.M. Crowley and G.A. Jamieson. (1970). Studies on glycoproteins, 5. Incorporation of glucosamine intermembrane glycoproteins of phytohaemagglutinin-stimulated lymphocytes. J. Biol. Chem. 245, 5827-5832.

Hayry, P., L.C. Andersson, S. Nordling. (1972). Allograft response in vitro. Transplant. Rev. 12, 91-140.

Hersh, E.M. and S.E. Dyre. (1974). Cells binding the antigen keyhole limpet and haemocyanin in the peripheral blood and in the lymphocyte cultures of non-immune and immunized human subjects. Clin. Exp. Immunol. 17, 299-309.

Hersh, E.M. and J.E. Harris. (1968). Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral leukocytes. J. Immunol. 100, 1184-1194.

Hirschhorn, K. F. Bach, R.L. Kolodny, I.L. Firschein and N. Hashem. (1963). Immune response and mitosis of human peripheral blood lymphocytes in vitro. Science 142, 1185-1187.

Hokin, L.E. and J.L. Dahl. (1972). The sodium-potassium adenosine triphosphatase. Metabolic Pathways, volume VI:Metabolic Transport, ed. L.E. Hokin, Academic Press, N.Y., pp. 269-315.

Hülser, D.P. and J.H. Peters. (1971). Intercellular communication in phytohaemagglutinin-induced lymphocyte agglutinates. Eur. J. Immunol. 1, 494-495.

Hungerford, D.A., A.J. Donnelly, P.C. Nowell and S. Beck. (1959). The chromosome constitution of a human phenotype intersex. Amer. J. Human Genet. 11, 215-236.

Jimenez, L., B.R. Bloom, M.R. Blume, H.F. Oettgen. (1971). On the number and nature of antigen-sensitive lymphocytes in the blood of delayed-hypersensitive human donors. J. Exp. Med. 133, 740-751.

Jondal, M., G. Holm and H. Wigzell. (1972). Surface markers on human B and T lymphocytes. I. A large population of lymphocytes forming non-immune rosettes with SRBC. J. Exp. Med. 136, 207-215.

Jondal, M. and G. Klein. (1973). Surface markers on human B and T Lymphocytes. II. Presence of Epstein-Barr virus receptors on B lymphocytes. J. Exp. Med. 138, 1365-1378.

Jung, C. and A. Rothstein. (1967). Cation metabolism in relation to cell size in synchronously grown tissue culture cells. J. Gen. Physiol. 50, 917-932.

Kasakura, S. (1970). Heterogeneity of blastogenic factors produced in vitro by antigenically stimulated and unstimulated leukocytes. J. Immunol. 105, 1162-1167.

Kay, J.E. (1968). Phytohaemagglutinin:an early effect on lymphocyte lipid metabolism. Nature, 219, 172-173.

Kay, J.E. (1972). Lymphocyte stimulation by phytohaemagglutinin: Role of the early stimulation of potassium uptake. Exptl. Cell. Res. 71, 245-247.

Kay, J.E., T. Ahern and M. Atkins. (1971). Control of protein synthesis during the activation of lymphocytes by phytohaemagglutinin. Biochim. Biophys. Acta, 247, 322-334.

Kernan, R.P. (1965). Cell Potassium. Butterworth, Inc. Washington, D.C.

Kinsolving, C.R., R.L. Post and D.L. Beaver. (1963). Sodium plus potassium transport adenosine triphosphatase activity in kidney. J. Cell. Comp. Physiol. 62, 85-93.

Kleinzeller, A. and A. Kotyk. (1961). Cations and transport of galactose in kidney-cortex slices. Biochim. Biophys. Acta 54, 367-369.

Kreuger, H. (1963). Chemical nature of the system controlling gene activities in insect cells. Nature 200, 1234-1235.

Lanotte, M. and J. Panijel. (1973). DNA replication in mixed lymphocyte culture. Nature (New Biol.) 245, 41-44.

Lamb, F.J. and D. McCall. (1972). Effect of prolonged ouabain treatment on Na^+, K^+, Cl^- and Ca^{++} concentrations and fluxes in cultured human cells. J. Physiol. 225, 599-617.

Larralde, C. (1970). In vitro mitotic activity of unsensitized lymphocytes in the presence of antigen and sensitized lymphocytes. Proc. Soc. Exp. Biol. Med. 133, 1175-1177.

Lauf, P.K., B.A. Rasmusen, P.G. Hoffman, P.B. Dunham, P. Cook, M.L. Parmelee and D.C. Tosteson. Stimulation of active potassium transport in LK sheep red cells by blood group-L-antiserum. J. Membrane Biol. 3, 1-13.

Leu, R.W., A.W.L.F. Eddleston, R.A. Good and J.W. Hadden. (1973). Paradoxical effects of ouabain on the migration of peritoneal and alveolar macrophages. Exptl. Cell. Res. 76, 458-461.

Lichtman, M.A., A.H. Jackson and W.A. Peck. (1972). Lymphocyte monvalent cation metabolism:cell volume, cation content and cation transport. J. Cell. Physiol. 80, 383-396.

Lindahl-Kiessling, K. and J.A. Böök. (1964). Effects of phytohaemagglutinin on leucocytes. Lancet 2, 591.

Lindahl-Kiessling, K. and J. Säfwenberg. (1972). Mechanism of stimulation in the mixed lymphocyte culture. Proceedings of the Sixth Leucocyte Culture Conference ed. M.R. Schwarz, Academic Press, New York, N.Y. p. 623-638.

Lischner, H.W., M.A. Valdes-Dapena, J. Biggin, S. Hann and N. Amoni. (1973). Proliferative responsiveness of human B and T lymphocytes to phytohaemagglutinin and pokeweed mitogen. Proceedings of the Seventh Leucocyte Culture Conference, ed. F. Daguillard, Academic Press, New York, pp. 547-560.

Loeb, L.A. and S.S. Agarwal. (1971). DNA polymerase:Correlation with DNA replication during transformation of human lymphocytes. Exptl. Cell Res. 66, 299-304.

Lohrman, H.-P., L. Novikovs and R.G. Grav, jun. (1974). Stimulatory capacity of human B and T lymphocytes in the mixed leukocyte culture. Nature 250, 144-145.

Lohrman, H.-P. and J. Whang-Peng. (1974). Human mixed leukocyte culture:identification of the proliferating lymphocyte subpopulation by sex chromosome markers. J. Exp. Med. 140, 54-60.

Loor, F. (1974). Binding and redistribution of lectins on lymphocyte membranes. Eur. J. Immunol. 4, 210-220.

Loos, J.A. and D. Roos. (1973). Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. III. Stimulation by tuberculin and allogenic cells. Exptl. Cell Res. 79, 136-142.

Lubin, M. (1964). Cell potassium and the regulation of protein synthesis. The Cellular Functions of Membrane Transport, ed. J.F. Hoffman, Prentice Hall, Inc., Englewood Cliffs, N.J. pp. 193-211.

Lubin, M. (1967). Intracellular potassium and macromolecular synthesis in mammalian cells. Nature 213, 451-453.

Lucas, D.O. (1970). Non-uniformity of phospholipid changes occurring with antigenic stimulation of lymphocytes in culture. Proceedings of the Fifth Leukocyte Culture Conference, ed. J. Harris, Academic Press, New York, N.Y., pp. 43-58.

Lundholm, L., T. Rall and N. Vamos. (1967). Influence of K-ions and adrenaline on the adenosine 3'-5'-monophosphate content in rat diaphragm. Acta Physiol. Scand. 70, 127-128.

MacKinney, A.A. Jr., P. Stohlman, Jr., G. Brecher. (1962). The kinetics of cell proliferation in cultures of human peripheral blood. Blood 19, 349-358.

Marshall, W.H., F.T. Valentine and H.S. Lawrence. (1969). Cellular immunity in vitro. Clonal proliferation of antigen-stimulated lymphocytes. J. Exp. Med. 130, 327-343.

Matsui, H. and A. Schwartz. (1968). Mechanism of cardiac glycoside inhibition of the Na^{+}-K^{+}-dependent ATPase from cardiac tissue. Biochim. Biophys. Acta 151, 655-663.

Mendelsohn, J., A. Skinner and S. Kornfeld. (1971). The rapid induction by phytohaemagglutinin of increased alpha-aminoisobutyric acid uptake in lymphocytes. J. Clin. Invest. 50, 818-826.

Meuwissen, H.J., O. Stutman and R.A. Good. (1969). Functions of the lymphocytes. Sem. Haemat. 6, 28-66.

Michalowski, A. (1963). Time-course of DNA synthesis in human leukocyte cultures. Exptl. Cell Res. 32, 609-612.

Miller, J.F.A.P., A. Basten, J. Sprent and C. Cheers. (1971). Interaction between lymphocytes in immune responses. Cell. Immunol. 2, 469-495.

Milton, J.D. and J.F. Mowbray. (1972). Reversible loss of surface receptors on lymphocytes. Immunology 23, 599-608.

Moorhead, J.F., J.J. Connolly and W. McFarland. (1967). Factors affecting the reactivity of human lymphocytes in vitro. I. Cell number, duration of culture, and surface area. J. Immunol. 99, 413-419.

Mosinger, B. and V. Kujalova. (1966). Potassium-dependent lipomobilizing effect of adrenaline on incubated adipose tissue. Biochim. Biophys. Acta 116, 174-177.

Näslund, P.H. and T. Hultin. (1970). Effects of potassium deficiency on mammalian ribosomes. Biochim. Biophys. Acta 204, 237-247.

Näslund, P.H. and T. Hultin. (1971). Structural and functional defects in mammalian ribosomes after potassium deficiency. Biochim. Biophys. Acta 254, 104-116.

Newlin, C.M. (1970). Response of rat peripheral blood lymphocytes fractionated by density gradient centrifugation to phytohaemagglutinin and in the mixed leucocyte interaction. Proceedings of the Fifth Leukocyte Culture Conference, ed. J. Harris, Academic Press, New York, N.Y. pp. 441-453.

Novogrodsky, A. (1972). Concanavalin A stimulation of rat lymphocyte ATPase. Biochim. Biophys. Acta 226, 343-349.

Novogrodsky, A. and E. Katchalski. (1971). Induction of lymphocyte transformation by periodate. FEBS Letters 12, 297-300.

Opelz, G. and P.I. Terasaki. (1974). Lymphocyte antigenicity loss with retention of responsiveness. Science 184, 464-466.

Palmer, R.F., K.C. Lanseter and S.G. Melvin. (1966). Stimulation of Na^+ and K^+ dependent adenosine triphosphatase by ouabain. Arch. Biochem. Biophys. 113, 629-633.

Parker, J.W., R.L. O'Brien, R.J. Lukes and J. Steiner. (1972). Transformation of human lymphocytes by sodium periodate. Lancet 1, 103-104.

Parsa, I. and S.L. Kountz. (1974). Rapid mixed lymphocyte culture test based on relative increase in protein synthesis. Nature 250, 675-678.

Pearmain, G., R.R. Lycette and P.H. Fitzgerald. (1963). Tuberculin-induced mitosis in peripheral blood leukocytes. Lancet 1, 637-638.

Pena, A., B. Cinco, A.G. Puyou and M. Tuena. (1969). Studies on the mechanism of the stimulation of glycolysis and respiration by K^{+} in Saccharomyces cerevisiae. Biochim. Biophys. Acta, 180, 1-8.

Peters, J.H. and P. Hausen. (1971a). Effect of phytohaemagglutinin on lymphocyte membrane transport. 1. Stimulation of uridine uptake. Eur. J. Biochem. 19, 502-508.

Peters, J.H. and P. Hausen. (1971b). Effect of Phyto-haemagglutinin on lymphocyte membrane transport. 2. Stimulation of "facilitated diffusion" of 3-O-methyl-glucose. Eur. J. Biochem. 19, 509-513.

Phillips, B. and I.M. Roitt. (1973). Evidence for transformation of human B lymphocytes by PHA. Nature (New Biol.) 241, 254-256.

Phillips, B. and E. Weisrose. (1974). The mitogenic response of human B lymphocytes to phytohaemagglutinin. Clin. Exp. Immunol. 16, 383-392.

Pick, E. and J.L. Turk. (1972). The biological activities of soluble lymphocyte products: a review. Clin. Exp. Immunol. 10, 1-24.

Polgar, P.R. and S. Kibrick. (1970). Origin of small lymphocytes following blastogenesis induced by short-term PHA stimulation. Nature 225, 857-858.

Post, R.L. and C.D. Albright. (1961). Membrane adenosine triphosphatase as a part of a system for active sodium and potassium transport. Membrane Transport and Metabolism eds. A. Kleinzeller and A. Kotyk, Academic Press, New York, pp. 219-227.

Quastel, M.R. (1970). Lymphocyte transformation and potassium transport. Ph.D. Thesis, University of Ottawa.

Quastel, M.R. and J.G. Kaplan. (1968). Inhibition by ouabain of human lymphocyte transformation induced by phytohaemagglutinin in vitro. Nature 219, 198-200.

Quastel, M.R. and J.G. Kaplan. (1970). Early stimulation of potassium uptake in lymphocytes treated with PHA. Exptl. Cell Res. 63, 230-233.

Quastel, M.R., P. Milthorp, J.G. Kaplan and I.J. Vogelfanger. (1974). Further studies on M-ATPase in lymphocytes and plaque-forming cells: possible species and functional differences between lymphocyte subclasses. Lymphocyte Recognition and Effector Mechanisms, eds. K. Lindahl-Kiessling and D. Osoba, Academic Press, New York, pp. 493-500.

Rabinowitz, Y. (1964). Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. Blood 23, 811-828.

Raff, M. (1973). T and B lymphocytes and immune responses. Nature 242, 19-23.

Resch, K. and E. Ferber. (1972). Phospholipid metabolism of stimulated lymphocytes: Effects of PHA, ConA and anti-immunoglobulin serum. Eur. J. Biochem. 27, 153-161.

Resch, K., E. Ferber and E.W. Gelfand. (1973). Phospholipid metabolism in plasma membranes of stimulated lymphocytes. Proceedings of the Seventh Leucocyte Culture Conference, ed. F. Daguillard, Academic Press, New York, pp. 75-86.

Riggs, T.R., L.M. Walker and H.N. Christensen. (1958). Potassium migration and amino acid transport. J. Biol. Chem. 233, 1479-1484.

Robinson, J.D. (1968). Regulating ion pumps to control cell volume. J. Theoret. Biol. 19, 90-96.

Rode, H.N. and J. Gordon. (1974). Macrophages in the mixed leukocyte culture reaction. J. Cell. Immunol. 13, 87-94.

Rogers, J.C., D. Boldt, S. Kornfeld, S.A. Skinner and C.R. Valeri. (1972). Excretion of DNA by lymphocytes stimulated by PHA or antigen. Proc. Nat. Acad. Sci. U.S.A. 69, 1685-1689.

Roitt, I.M., M.F. Greaves, G. Torrigiani, J. Brostoff and J.H.L. Playfair. (1969). The cellular basis of immunological responses. Lancet 2, 367-371.

Roos, D. and J.A. Loos. (1970). Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral Lymphocytes. I. Stimulation by phytohaemagglutinin. Biochim. Biophys. Acta 222, 565-582.

Roos, D. and J.A. Loos. (1973). Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. II. Relative importance of glycolysis and oxidative phosphorylation on phytohaemagglutinin stimulation. Exptl. Cell Res. 77, 127-135.

Rosenthal, A.S., D.L. Rosenstreich, J.T. Blake, P.E. Lipsky and J.A. Waldron. (1973). Mechanisms of antigen recognition by T lymphocytes. Proceedings of the Seventh Leucocyte Culture Conference, ed. F. Daguillard, Academic Press, New York, pp. 201-216.

Rubin, A.D., K. Havemann and W. Dameshet. (1969). Studies on CLL: further studies of the proliferative abnormality of the blood lymphocyte. Blood 33, 313-328.

Rühl, H., H. Kirchner and G. Bochert. (1971). Kinetics of the Zn^{2+}-stimulation of human peripheral lymphocytes. Proc. Soc. Exp. Biol. Med. 137, 1089-1092.

Sarma, D.S.R. and J. Zubroff. (1973). Synthesis and fragmentation of DNA in phytohaemagglutinin-stimulated human peripheral blood lymphocytes. Immunol. Commun. 2, 277-285.

Sasaki, M.S. and A. Norman. (1966). Proliferation of human lymphocytes in culture. Nature 210, 913-914.

Sasportes, M., Y. Christen, C. Mawas, J.G. Kaplan and J. Dausset. (1974). "Differential sensitivity in mixed lymphocyte reaction of stimulating and responding human lymphocytes to ouabain." Ninth Leukocyte Culture Conference abstr. #167, Williamsburg, Va. Dec. 1974.

Schatzmann, H.-J. (1953). Herzglykoside als Hemmstoffe für den aktiven Kalium-und Natriumtransport durch die Erythrocytenmembran. Helv. Physiol. Pharmacol. Acta 11, 346-354.

Schellekens, P.Th.A. and V.P. Eijsvoogel. (1971). Lymphocyte transformation in vitro. IV. Recruitment in antigen-stimulated cultures. Clin. Exp. Immunol. 8, 187-194.

Schlossman, S. (1972). Antigen recognition: the specificity of T cells involved in the cellular immune response. Transplant. Rev. 10, 97-111.

Schrek, R. (1963). Cell transformation and mitosis produced in vitro by tuberculin purified protein derivatives in human blood cells. Amer. Rev. Resp. Dis. 87, 734-738.

Schwartz, R.S., R.J.W. Ryder and A.A. Gottlieb. (1970). Macrophages and antibody synthesis. Progr. Allergy 14, 81-144.

Sheldon, P.J., E.H. Hemsted, M. Papamichail and E.J. Holborrow. (1973). Thymic origin of atypical lymphoid cells in infectious mononucleosis. Lancet 1, 1153-1155.

Simons, M.J. and M.G. Fitzgerald. (1969). Apparent dissociation of antigen responsiveness and antibody production by human small lymphocytes in culture. Clin. Exp. Immunol. 4, 55-63.

Singer, S.J. and G.L. Nicolson. (1972). The fluid mosaic model of the structure of cell membranes. Science 175, 720-731.

Skou, J.C. (1973). The relationship of the sodium-potassium activated enzyme system to transport of sodium and potassium across the cell membrane. J. Bioenergetics 4, 1-30.

Sjoberg, O., J. Andersson and G. Möller. (1972). Requirement for adherent cells in the primary and secondary immune response in vitro. Eur. J. Immunol. 2, 123-126.

Smith, J.L. and D. Haegert. (1974). B- and T-lymphocyte markers on transformed lymphocytes from mitogen-stimulated cultures of normal and CLL lymphocytes and on tonsil blasts. Clin. Exp. Immunol. 17, 547-560.

Smith, J.W., A.L. Steiner, W.M. Newberry, Jr., and C.W. Parker. (1971). Cyclic adenosine 3',5'-monophosphate in human lymphocytes. Alterations after phytohaemagglutinin stimulation. J. Clin. Invest. 50, 432-441.

Sokal, R.R. and F.J. Rohlf. (1969). Biometry. The principles and practice of statistics in biological research. W.H. Freeman and Company, San Francisco.

Sören, L. (1973). Variability of the time at which PHA-stimulated lymphocytes initiate DNA synthesis. Exptl. Cell Res. 78, 201-208.

Souleil, C. and J. Panijel. (1972). Studies on a bromouracil-labelled DNA fraction induced by antigenic stimulation. Eur. J. Biochem. 30, 234-241.

Souleil, C. and J. Panijel. (1973). Effect of antigen dose on the presence of a particular BrdU-labelled DNA fraction in lymph node cells. Exptl. Cell Res. 78, 462-466.

Stobo, J.D., A.S. Rosenthal and W.E. Paul. (1972). Functional heterogeneity of murine lymphoid cells. 1. Responsiveness to and surface binding of concanavalin A and phytohaemagglutinin. J. Immunol. 108, 1-17.

Suelter, C.H. (1970). Enzymes activated by monovalent cations. Science 168, 789-795.

Tärnvik, A. (1970). A role for red cells in phytohaemagglutinin-induced lymphocyte stimulation. Acta path. microbiol. scand. Section B, 78, 733-740.

Tormey, D.C. and G.C. Meuller. (1965). An assay for the mitogenic activity of phytohaemagglutinin preparations. Blood 26, 569-578.

Twomey, J.J., O. Sharkey, J.A. Brown, A.H. Laughter and P.H. Jordan, Jr. (1970). Cellular requirements for the mitotic response in allogeneic mixed leukocyte cultures. J. Immunol. 104, 845-853.

Valentine, F.T. (1972). The dissociation of 95% oxygen of the production of lymphocyte transforming factor and the proliferation of lymphocytes stimulated with antigen. Proceedings of the Sixth Leucocyte Culture Conference, ed. M.R. Schwarz, Academic Press, New York, pp. 337-356.

van den Burg, K.J. and I. Betel. (1973). Increased transport of 2-aminoisobutyric acid in rat lymphocytes stimulated with concanavalin A. Exptl. Cell Res. 76, 63-72.

van den Burg, K.J. and I. Betel. (1974). Regulation of amino acid uptake in lymphocytes stimulated by mitogens. I. increase in aminoisobutyric acid transport dependent on cell metabolism. Exptl. Cell Res. 84, 412-418.

Vaughn, G.L. and J.S. Cook. (1972). Regeneration of cation-transport capacity in HeLa cell membranes after specific blockade by ouabain. Proc. Natl. Acad. Sci. U.S.A. 69, 2627-2631.

Vitetta, E.S. and J.W. Uhr. (1973). Synthesis, transport, dynamics and fate of cell surface immunoglobulin and alloantigens in murine lymphocytes. Transplant. Rev. 14, 50-75.

Ward, P.A. and E.L. Becker. (1970). Potassium reversible inhibition of leukotaxis by ouabain. Life Sci. 9, 355-360.

Whitney, R.B. and R.M. Sutherland. (1973). Kinetics of calcium transport in lymphocytes before and after stimulation by phytohaemagglutinin. Proceedings of the Seventh Leukocyte Culture Conference, ed. F. Daguillard, Academic Press, New York, N.Y., pp. 63-74.

Whittam, R. and D.M. Blond. (1964). Respiratory control by an adenosine triphosphatase involved in active transport in brain cortex. Biochem. J. 92, 147-158.

Wigzell, H. and B. Andersson. (1971). Isolation of lymphoid cells with active surface receptor sites. Ann. Rev. Microbiol. 25, 291-308.

Wofsy, L., J. Kimura and P. Truffa-Bachi. (1971). Cell separation on affinity columns: the preparation of pure populations of anti-hapten specific lymphocytes. J. Immunol. 107, 725-729.

Woodruff, M.F.A., B. Reid and K. James. (1967). Effect of antilymphocytic antibody and antibody fragments on human lymphocytes in vitro. Nature 215, 591-594.

Wu, L.Y.F., A.R. Lawton, M.F. Greaves and M.D. Cooper. (1973). Evaluation of human B lymphocyte differentiation using pokeweed mitogen (PWM) stimulation: in vitro studies in various antibody deficiency syndromes. Proceedings of the Seventh Leukocyte Culture Conference, ed. F. Daguillard, Academic Press, New York, pp. 485-500.

Yachnin, S., L.W. Allen, J.M. Baron and R.H. Svenson. (1972). The potentiation of phytohaemagglutinin-induced lymphocyte transformation by cell-cell interaction; a matrix hypothesis. Cell. Immunol. 3, 569-589.

Yachnin, S. (1972). The potentiation and inhibition by autologous red cells and platelets of human lymphocyte transformation induced by pokeweed mitogen, concanavalin A, mercuric chloride, antigen, and mixed leukocyte culture. Clin. Exp. Immunol. 11, 109-124.

Zoschke, D. and F.H. Bach. (1970). Specificity of antigen recognition by human lymphocytes in vitro. Science 170, 1404-1406.

Zoschke, D.C. and F.H. Bach. (1972). Lymphocyte reactivity in vitro. VIII. Specificity of allogeneic cell recognition in mixed leucocyte cultures. Proceedings of the Sixth Leukocyte Culture Conference, ed. M.R. Schwarz, Academic Press, New York, p. 639-656.

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA