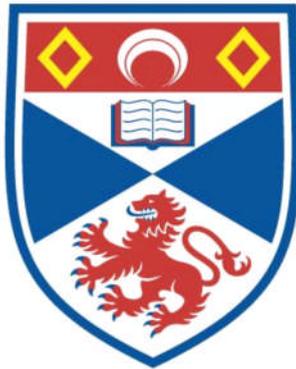


**AN INVESTIGATION USING CULTURED HUMAN CELL
LINES, OF THE INVOLVEMENT OF VANADIUM,
CATION TRANSPORT AND PHOSPHATIDYLINOSITOL
IN THE AETIOLOGY OF BIPOLAR MANIC-DEPRESSIVE
PSYCHOSIS**

ROSAMONDE ELIZABETH BANKS

**A Thesis Submitted for the Degree of PhD
at the
University of St Andrews**



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OF THE INVOLVEMENT OF VANADIUM, CATION TRANSPORT
AND PHOSPHATIDYLINOSITOL IN THE AETIOLOGY OF
BIPOLAR MANIC-DEPRESSIVE PSYCHOSIS

A thesis submitted to the University
of St. Andrews for the degree of
Doctor of Philosophy

by

ROSAMONDE ELIZABETH BANKS

Department of Physiology and Pharmacology,
University of St. Andrews.

December, 1986



QUESTIONS ANSWERED AND ANSWERS WITHOUT QUESTIONS

If the human brain was so simple
that we could understand it
we would be so simple
that we couldn't.

EMERSON PUGH

(Cited by Von Scheele and Nordgren, 1986, in "The mind-body problem in medicine", Lancet, February 1st., 258-261.)

DECLARATION

a). I, Rosamonde Elizabeth Banks, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

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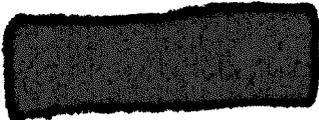


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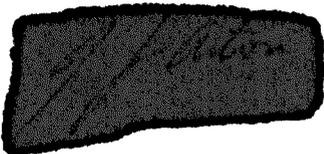
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ACADEMIC RECORD

I graduated from the University of St. Andrews in October 1982, with a B.Sc. (Hons.) in Physiology, 1st class. In October 1983 I was awarded an M.Sc. (by instruction) in Toxicology, by the University of Surrey.

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Lastly, but by no means least, I would like to thank my parents for their support over the years, and my husband David for giving me the support and encouragement needed to finish this thesis.

AIMS OF THE THESIS

This thesis is aimed at examining the intracellular effects of chronic vanadate treatment in a cultured human cervical epithelial cell line (HeLa), with particular regard to cation transport. Following this the possible involvement of vanadate, cation transport, and phosphatidylinositol in the aetiology of bipolar manic-depressive psychosis is investigated using cultured lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects.

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ABSTRACT

The symptoms, classification, occurrence and possible aetiologies of bipolar manic-depressive psychosis have been reviewed, with particular emphasis on the possible role of the vanadate ion (V^{5+}) and cation transport in the illness. The effect of vanadate on cation transport in intact cells has been determined using the well-characterised HeLa cell line. Cation transport in virally transformed lymphoblastoid cell lines from 13 bipolar manic-depressive patients and 13 control subjects has been examined, under normal conditions and after treatment (24 hours) with lithium, ouabain or vanadate. The phosphatidylinositol system has also been examined in these cell lines, in view of the therapeutic effect of lithium, and its known inhibitory actions on inositol 1-phosphatase.

In HeLa cells, no effects of vanadate on cation transport were seen until concentrations greater than $3.2 \times 10^{-6}M$. This was attributed to the intracellular reduction of V^{5+} to V^{4+} shown to occur using ESR. Similar decreases were seen in all the K^+ influx pathways, with maximum decreases of approximately 30% at $10^{-4}M$ vanadate extracellularly. Significant toxicity was also seen at these concentrations, with a maximum decrease in cell number of 40% at $10^{-4}M$ vanadate. No change in the energy charge was seen and changes in ATP levels occurred subsequently to the changes in cell number, with a decrease of 40% at $10^{-4}M$ vanadate.

Using the lymphoblastoid cell lines, no significant differences were seen in any of the cation transport parameters examined, with the exception of mean sodium pump number which was

30% greater in the bipolar group compared with the control group. Lithium or vanadate treatment produced either no effect or inconsistent changes in cation transport. Ouabain treatment produced similar decreases in sodium pump number in both groups.

Inositol uptake was similar in both groups, but the percentage incorporation into phosphoinositides was reduced in bipolar cell lines compared with controls.

LIST OF ABBREVIATIONS

Abbreviations are as illustrated in the text. In addition, the more extensively-used abbreviations are listed below. Abbreviations of measurements follow the S.I. system of nomenclature.

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
AUFS	Absorbance units full scale
B_{\max}	Maximum binding
CDP	Cytidine 5'-diphosphate
CMP	Cytidine 5'-monophosphate
cpm	Counts per minute
CSF	Cerebrospinal fluid
CTP	Cytidine 5'-triphosphate
DAG	Diacylglycerol
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid
DOPAC	Dehydroxyphenylacetic acid
DST	Dexamethasone suppression test
E_{Cl}	Cl^- equilibrium potential
E_{K}	K^+ equilibrium potential
E_{M}	Equilibrium membrane potential
E_{Na}	Na^+ equilibrium potential
EGF	Epidermal growth factor
E.R.	Endoplasmic reticulum
ESR	Electron spin resonance
GDP	Guanosine 5'-diphosphate
GFR	Glomerular filtration rate

GMP	Guanosine 5'-monophosphate
Gpp(NH)p	Guanyl imidodiphosphate
GSH	Glutathione (reduced)
GTP	Guanosine 5'-triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HVA	Homovanillic acid
IBMX	Isobutylmethylxanthine
i.d.	Internal diameter
IP	Inositol 1-phosphate
IP ₂	Inositol 1,4-bisphosphate
IP ₃	Inositol 1,4,5-trisphosphate or the 1,3,4 isomer
IP ₄	Inositol 1,3,4,5-tetrakisphosphate
IP ₅	Inositol pentakisphosphate
IP ₆	Inositol hexakisphosphate
K _{aff}	Affinity constant
K _d	Dissociation constant
K _i	Inhibitory constant
K _m	Michaelis-Menten constant
lcw	Litre of cell water
MAOI	Monoamine oxidase inhibitor
MHPG	3-methoxy-4-hydroxyphenylglycol
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PCA	Perchloric acid

PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol-4-phosphate
PtdIns(4,5)P ₂	Phosphatidylinositol-4,5-bisphosphate
rpm	Revolutions per minute
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
S.D.	Standard deviation
S.E.M.	Standard error of the mean
TBA	Tetrabutylammonium hydroxide
TLC	Thin layer chromatography
UV	Ultra-violet
VMA	Vanillylmandelic acid
5-HT	5-Hydroxytryptamine
5-HIAA	5-Hydroxyindoleacetic acid
5-HTP	5-Hydroxytryptophan
[] _o	Extracellular concentration
[] _i	Intracellular concentration

CHAPTER 1. GENERAL INTRODUCTION

In this general introduction an overall review of bipolar manic-depressive psychosis is presented - the symptoms, classification, genetic background and possible aetiologies. The chemistry and biological importance of vanadium is then illustrated followed by a discussion of the possible involvement of the vanadate ion in the aetiology of bipolar manic-depressive psychosis.

1.I. Symptoms and classification of bipolar manic-depressive psychosis

The typical central symptoms of mania can be grouped according to whether mood, activity or thought is affected. They are, respectively: a). humour, euphoria, irritability, anger when thwarted, poor impulse control, heightened sensory impressions and loss of inhibition, b). increased energy, decreased sleep, pressure of speech, increased social contact, sexual overactivity and excessive spending of money, and, c). poor judgement, unrealistic planning, distractability, flight of ideas, and grandiose ideas and delusions (Tyrer and Shopsin 1982).

In depression, mood can range from one of sadness to a profound melancholia, with a loss of interest in food, sex and work. There is generally a lack in mental/physical energy and initiative, together with poor concentration and decision-making. Characteristically the person feels worst in the morning, improving as the day progresses, and their sleep pattern may be disturbed. Psychological symptoms may include feelings of worthlessness and guilt, delusions of catastrophe, hypochondriasis and paranoid ideas (Shaw et al. 1982). Suicide

is not uncommon.

The concept of manic-depressive illness as a unification of mania and depression into one illness was originally formulated in the early 1900's by the German psychiatrist Kraepelin. This was a broad categorisation embracing all severe depressive syndromes, irrespective of whether mania occurred (Andreasen 1982). The primary feature of this disorder was defined as being a change in mood - an elevation in the case of mania and a lowering in depression. Any other symptoms were regarded as being secondary to this change. The disorder was defined as being relatively severe with recurrent bouts of illness interspaced with periods of full remission. As the main feature of the "anxiety states" was also a change in mood, these disorders were sometimes grouped together under the broader heading of "affective disorders", the classification of which varies, as demonstrated later in this section.

Subsequent research indicated that biological changes occurring in the affective disorders played a more central role in the disease, and that the changes in mood were not such a central feature. This led to an extension of the definition of the disorder so that the central feature became "a profound constitutional disturbance" with "changes in the cognitive, affective and physiological functioning" (Hamilton 1982).

There are a large number of classifications of psychiatric illness currently in existence. One of the two major systems used clinically is that contained within the ninth "International Classification of Diseases" (W.H.O. 1978, ICD-9). Section 296 pertaining to "affective psychoses" is summarised in Table 1.1..

Table 1.1. Classification of affective psychoses in ICD-9

296 Affective Psychoses

- 296.0 Manic-depressive psychosis, manic type:- mental disorders characterised by states of elation or excitement out of keeping with the patients circumstances and varying from enhanced liveliness (hypomania) to violent, almost uncontrollable excitement.
- 296.1 Manic-depressive psychosis, depressed type:- an affective psychosis in which there is a widespread depressed mood of gloom and wretchedness with some degree of anxiety. There is often reduced activity but there may be restlessness and agitation. There is a marked tendency to recurrence.
- 296.2 Manic-depressive psychosis, circular type but currently manic:- an affective psychosis which has appeared in both the depressive and the manic form, either alternating or separated by a period of normality, but in which the manic form is currently present. (The manic phase is far less frequent than the depressive).
- 296.3 Manic-depressive psychosis, circular type but currently depressed:- circular type as above but in which the depressive phase is currently present.
- 296.4 Manic-depressive psychosis, circular type, mixed:- an affective psychosis in which both manic and depressive symptoms are present at the same time.
- 296.5 Manic-depressive psychosis, circular type, current condition not specified
- 296.6 Manic-depressive psychosis, other and unspecified
- 296.8 Other
- 296.9 Unspecified

Here affective psychoses, which are subdivided into nine types of manic-depressive psychosis, are defined as, "Mental disorders, usually recurrent, in which there is a severe disturbance of mood (mostly compounded of depression and anxiety but also manifested as elation and excitement) which is accompanied by one or more of the following: delusions, perplexity, disturbed attitude to self, disorder of perception and behaviour; these are all in keeping with the patient's prevailing mood (as are hallucinations when they occur). There is a strong tendency to suicide".

A more compact classification of affective disorders is given in the third "Diagnostic and Statistical Manual of the American Psychiatric Association" (1980, DSM-III). In this latter classification, major affective disorders are subdivided into bipolar disorder where both mania and depression have occurred (equivalent to circular types of manic-depressive psychosis in the ICD-9), and major depression (or unipolar depression) where no manic episodes have been seen. Bipolar disorder is further subdivided into 3 groups, currently manic, currently depressed, and mixed (for those people who cycle within a single episode). There is no separate category for unipolar mania which is generally classified under "bipolar disorder currently manic" as many (but not all) manics eventually experience depression.

From the original broad grouping by Kraepelin of all severe forms of affective disorder under the heading "manic-depressive illness", current classifications such as those above now separate the disorders on the basis of the occurrence of mania. The widely accepted bipolar-unipolar distinction as opposed to a

continuum, was originally proposed by Leonhard et al. in the late 1950's and has been validated by genetic studies, treatment response studies and course of illness studies (reviewed by Perris 1982). However the evidence for this is by no means unequivocal and is discussed further in section 1.III.. This thesis will be confined to examining bipolar manic-depressive psychosis i.e. the illness where both mania and depression occur.

1.II. Occurrence and course of bipolar manic-depressive psychosis

Much work on this aspect of the disorder has been carried out by Perris, by Faris and Dunham, by Krauthammer and Klerman, and by a European team headed by Angst. The data presented in this section is a summary based on reviews by Boyd and Weissman (1982), Hamilton (1979) and Shaw et al. (1982), which cover many such studies. One main methodological problem emphasised in these studies is that the data is from hospitalised patients and that the course of the illness may be modified by drugs.

The morbid risk of bipolar manic-depressive psychosis for both sexes has been found to range from 0.6 to 0.88% in industrialised nations, with the annual incidence of new cases varying from 0.009 to 0.015% for men, and 0.007 to 0.03% for women. The ratio of women to men affected by the disease ranges from 1.3:1 to 2:1 (Boyd and Weissman 1982). Estimates of peak age of onset vary from a range of 24 to 31.5 years with a modal age of 30 (Boyd and Weissman 1982) to a range of 20 to 39 years with a median of 30 years (Hamilton 1979), whilst a median age of onset of 34.7 years is reported by Shaw et al. (1982).

The range of age of onset extends from adolescence to old age and is log-normally distributed.

The length of the cycles i.e. the interval between the onset of one illness and the beginning of the next, tends to shorten with the number of episodes and increasing age, whereas the length of the manic or depressive episode increases. The mean cycle length (or the mean first cycle length in the case of the review by Hamilton), is approximately 33 months, with a mean episodic duration of 4.4 months (Shaw et al. 1982, Hamilton 1979), and 50% of the episodes lying between 2.5 and 7.6 months (Shaw et al. 1982). The chances of spontaneous remission from recurrences are low although each episode of depression or mania does tend to remit spontaneously, as seen before the advent of effective treatments (Naylor - personal communication).

1.III. Life events and genetic influences in bipolar manic-depressive psychosis

There have been many studies regarding the involvement of life events in affective disorders. For the most part these studies have been concerned with affective disorders in general, and distinctions are not made between the different types. Paykel (1982) has reviewed many such studies, the majority of which seem to show an excess of life events prior to the first onset of depression, when compared with either the general population, medical or psychiatric patients. The events are often loss, separation or threatening events but it would appear that if they do play a precipitating role, it is likely to be as a result of summation - a cumulative effect rather than an isolated incident.

Other events which have also been implicated include viral infections, serious medical illnesses, and hormonal upsets but the relative contributions of such factors are difficult to evaluate (Shaw et al. 1982).

There is strong evidence for a genetic component in affective disorders, based on family, twin and adoption studies, many of which are covered in reviews by Gershon et al. (1976, 1977). When the results from six studies were examined concerning the morbid risk for first-degree relatives of bipolar and unipolar probands, an increased familial prevalence of affective disorder was found. The morbid risk of bipolar illness in first-degree relatives of bipolar probands ranged from 2.8% to 10.2% with a mean value of 6.9%, whereas that of unipolar illness ranged from 0.5% to 20.4% with a mean value of 7.6%. In contrast the mean morbid risk for bipolar illness in first-degree relatives of unipolar probands was 0.4% whilst that for unipolar illness was 6.0% (Gershon et al. 1976). Comparable morbid risk figures for the general population with respect to affective disorder (unipolar and bipolar) are approximately 2.4% (Nurnberger and Gershon 1982). This data strongly suggests a common genetic diathesis for unipolar and bipolar illnesses although one of the studies included indicated a separate mode of inheritance, showing few bipolar relatives of unipolar probands and vice versa (Perris 1966).

More recently, as part of the "Yale University-National Institute of Mental Health Collaborative Family Study of Depression", Weissman et al. (1984) published results similar in trend to the main body of results reviewed above i.e. bipolar

probands having both bipolar and unipolar relatives whilst unipolar probands show few bipolar relatives. This is further support to the hypothesis of a common genetic pathway. Tsuang et al. (1985) also reached similar conclusions after analysing the families of 100 bipolar probands and 225 unipolar probands using a 2-threshold multifactorial polygenic model of familial transmission as described by Reich et al. (1979). This model is discussed further below. The results also suggested genetic heterogeneity within affective disorders.

Data reviewed from 6 studies involving twins, also supports the possibility of affective illnesses being hereditary. The overall concordance rate for monozygotic twins was 69.2% compared with 13.3% for dizygotic twins (Gershon et al. 1976). A concordance rate of 67% for affective illness in monozygotic twins reared apart was also found. In 83 monozygotic twin pairs reviewed, 25.3% were concordant for bipolar illness, 26.5% were concordant for unipolar illness, 39.8% were not clearly discordant and 8.4% had one member unipolar and the other bipolar (Gershon et al. 1976). This again is consistent with the idea of a genetic transmission of manic-depressive illness. The presence of monozygotic twins discordant for either presence of affective illness or polarity of the illness suggests the involvement of an environmental factor as well as the genetic one suggested above, and/or a genotype which may be phenotypically manifest as either unipolar illness or bipolar illness or no illness at all in a monozygotic co-twin (Gershon et al. 1977).

In adoption studies, which enable the separation of genetic and environmental factors, Mendlewicz and Rainer (1977) have

reported that out of 29 bipolar adoptees, 28% of the biological parents suffered from either bipolar or unipolar disorder compared with 12% of the adoptive parents. In parents of non-adopted bipolars the morbid risk was 21% which is comparable to the 28% above, and the morbidity risk in biological or adoptive parents of normal adoptees was 2% and 7% respectively, again figures which are consistent with a genetic influence in affective disorders. No explanation is given for the high morbid risk values of the adoptive parents of the bipolar adoptees and the adoptive and biological parents of normal adoptees. These are much higher than the 0.6 to 0.88% reported for the general population (previous section). No father-to-son transmission of manic-depressive illness was seen in this study, indicating a possible X-linkage.

The mode of transmission of affective disorders has not been determined. Several genetic models have been put forward proposing a single dominant gene with reduced penetrance (e.g. Winokur and Tanna 1969, Gershon and Bunney, Jr. 1976) although controversy reigns as to whether the gene is autosomal or X-linked. The possibility of X-linkage was initially based on the finding that in some studies there were high rates of mother-son transmission and virtually no father-son transmission (reviewed by Nurnberger and Gershon 1982). However other studies have not supported this. Studies such as those by Bucher and Elston (1981) and Bucher et al. (1981) have reanalysed data from previous studies using segregation analysis. The model used allowed for factors such as variable age of onset and incomplete penetrance but neither an autosomal or X-linked mode of

transmission fitted the data.

The concept of bipolar and unipolar illnesses representing a continuum of illness rather than distinct entities has resulted in the use of multifactorial and single major locus threshold models for affective disorder (Gershon et al. 1975, Reich et al. 1979, Baron et al. 1981a,b, Gershon et al. 1982). In the multifactorial models, all environmental and genetic factors involved in the genesis of the illness are regarded as being additive and are grouped into one variable termed the "liability" of the individual. This is distributed normally in the general population, with thresholds which the liability must exceed before the illness is manifest. In the version of the model developed by Reich et al. (1979), there are three alternatives:- the "Independent" model, the "Isocorrelational" model and the "Environmental" model. The first of these corresponds to the hypothesis that bipolar and unipolar illnesses have separate aetiologies, with those individuals exceeding a threshold on the unipolar liability distribution developing a unipolar disorder and those individuals exceeding a threshold on the bipolar liability distribution developing bipolar disorder. The "Isocorrelational" model assumes that bipolar and unipolar disorders share the same liability distribution i.e. they share the same aetiological factors. There are two thresholds on this distribution; those individuals exceeding the less extreme threshold develop unipolar disorder whereas those exceeding the more extreme threshold develop bipolar disorder. The "Environmental" model proposes that bipolar and unipolar illnesses are non-familial environmental variants of the same

process.

No tested model of genetic transmission consistently fits all the data in the different studies (Gershon et al. 1975, Gershon et al. 1976, 1977, Sachar and Baron 1979, Baron et al. 1981a,b, Gershon et al. 1982, Tsuang et al. 1985) although the majority of the studies do seem to indicate a common genetic diathesis for unipolar and bipolar illnesses. The suggested presence of genetic heterogeneity and reduced penetrance, together with the methodological difficulties involved in the diagnosis and categorisation of the disorders possibly account for some of the difficulties in modelling the data.

Even if the mode of transmission is not known it should still be possible to predict the relative susceptibility of individuals if a genetic marker were identified. Autosomal linkage studies have produced conflicting reports regarding the ABO blood grouping system and overall this seems an unlikely candidate when reviewed (Gershon et al. 1977). Similar studies have been made on the HLA tissue antigen system, again with conflicting results which make it seem unlikely to be of any value as a genetic marker. More recent studies have also not supported such a linkage (Johnson et al. 1981, Suarez and Reich 1984).

Studies have also been carried out on possible linkage to X-chromosome markers following the previously discussed hypothesis that affective disorders may be X-linked. In a study by Mendlewicz and Fleiss (1974), it was concluded that there was close linkage with bipolar illness between both protan/deutan colour-blindness and the Xg blood group. This was disputed however due to the large known chromosomal map distance between

the Xg locus and the protan-deutan region which made it unlikely that a linkage with both loci could be present (Gershon and Bunney, Jr. 1976, Baron et al. 1981b). In a review by Gershon and Bunney (1976) they concluded that the relationship between colour-blindness and bipolar disorder may be association rather than true linkage. Close linkage of bipolar disorder with protan/deutan colour-blindness was reported however by Baron et al. (1981b). Genetic heterogeneity was seen with two homogeneous groups of close-linkage and non-linkage, a finding similar to that of Gershon et al. (1980). Mendlewicz et al. (1980) have reported the possibility of a linkage with glucose-6-phosphate dehydrogenase deficiency. Such a linkage would be expected if the postulated linkage between bipolar illness and colour-blindness was correct as this enzyme deficiency has been shown to be linked to colour-blindness on the X-chromosome (Mendlewicz et al. 1980).

A mutant protein, Pc 1 Duarte, has been found with increased frequency in brain specimens taken at autopsy from individuals with affective disorder (Comings 1979). The protein was most common in the caudate, putamen, thalamus, pons and brainstem, with a frequency of 72.7% in bipolar patients compared with 31.6% in controls. Comings suggested that this protein may be a product of a single major gene, combining aetiologically with environmental factors. It was also found with increased frequency in multiple sclerosis patients, an association that has prompted the suggestion that Duarte protein may alter the host response to a CNS viral infection.

1.IV. Biological aetiology of bipolar manic-depressive psychosis

Although there is evidence that bipolar manic-depressive psychosis is genetic in origin, the resultant biological changes which are responsible for the manifestation of the illness are not known with certainty. Biological and pharmacological abnormalities seen during the course of the illness have implicated:- 1). monoaminergic neurotransmission; 2). the neuro-endocrine system; and 3). ion transport, as being the main areas where the changes involved in the genesis of bipolar disorder may possibly occur, not necessarily mutually exclusively. Many studies have been carried out regarding each of these areas and it is beyond the scope of this chapter to detail them all. A synopsis of the research findings of these studies is presented here, based on reviews and selected original articles as cited. Note that the terms rubidium influx and potassium influx are used interchangeably in this chapter - the reasons for this are detailed in Chapter 3.

1). Aminergic neurotransmission:- The "amine hypothesis" was put forward in the 1950's following the serendipitous observation that some patients taking reserpine as an anti-hypertensive, became severely depressed. This, together with the knowledge that reserpine inhibited amine storage in the nervous system, and the observation that amphetamines potentiated the action of catecholaminergic neurones and improved mood, led to the model: reduced noradrenaline levels \longrightarrow reduced noradrenergic activity \longrightarrow depression, and conversely, increased noradrenergic activity \longrightarrow mania (Schildkraut 1965, Bunney, Jr.

and Davis 1965, Schildkraut and Kety 1967). Later an involvement of the serotonergic pathways was proposed after the discovery that tryptophan, the precursor of 5-hydroxytryptamine (5-HT), accelerated the recovery of depressed patients when given with monoamine oxidase inhibitors (MAOI's), suggesting the presence of reduced 5-HT activity in depression (from Shaw et al. 1982). The involvement of the 5-HT pathway was further validated by a study which showed combined treatment with imipramine and alphanethylparatyrosine (AMPT - inhibits noradrenaline synthesis) led to recovery, whereas treatment with imipramine and parachlorophenylalanine (PCPA - an inhibitor of tryptophan hydroxylase) led to a deterioration of the illness (Shopsin et al. 1975).

The main support for the involvement of cerebral monoamines in affective disorders is pharmacological e.g. tricyclic antidepressants such as imipramine, desipramine and amitriptyline inhibit neuronal uptake of neurotransmitters to varying extents, monoamine oxidase inhibitors (effective as antidepressants for a few patients) such as phenelzine prevent the breakdown of neurotransmitters, ECT is thought to act by increasing the levels of monoamines in the brain but the mode of action is not clear, and lithium (mainly prophylactic and antimanic but also antidepressant) facilitates the neuronal uptake of noradrenaline (for references see Bowman and Rand 1980). However many of the treatments show a spectrum of effects and affect more than one neurotransmission pathway e.g. tricyclics show anticholinergic type side-effects, block alpha-receptors, and are phosphodiesterase inhibitors, making the actions of such drugs

more difficult to interpret (Bowman and Rand 1980).

Factors difficult to reconcile with the "amine hypothesis" include the discrepancy between the rapidity of action of the tricyclics in inhibiting neuronal uptake mechanisms and the slow onset of therapeutic effects (approx. 2-3 weeks), the lack of correlation between the relative potencies of the tricyclics and related drugs as antidepressants and their relative abilities at inhibiting neuronal uptake (e.g. iprindole is effective but does not block amine uptake), the potentiation of catecholaminergic neuronal activity by cocaine but the failure of cocaine to reverse depressive symptoms, the failure of PCPA or reserpine (depletes stored amines) in mimicking classical depressive symptoms in normal subjects, and the effectiveness of lithium and electroconvulsive therapy as antimanic and antidepressant treatments respectively, despite having few or ill-defined effects on aminergic neurotransmission (from Shaw et al. 1982, Zis and Goodwin 1982). In addition, specific blockers of noradrenaline uptake e.g. maprotiline, and of 5-HT e.g. zimelidine, are both antidepressants and there is little or no evidence that different patients respond to drugs affecting different amine systems (Naylor - personal communication). Difficulties in defining the mode of action of therapeutic drugs largely arise from the diverse effects seen in acute or chronic studies coupled with the fact that many of the actions reported have not been shown clinically and caution must be exerted in extrapolating to man.

Many studies have been carried out analysing the levels of 3-methoxy-4-hydroxyphenylglycol and vanillylmandelic acid (MHPG and

VMA, major and minor metabolites respectively of noradrenaline), homovanillic acid and dehydroxyphenylacetic acid (HVA and DOPAC, major and minor metabolites respectively of dopamine) and 5-hydroxyindoleacetic acid (5-HIAA, metabolite of 5-HT) in CSF and urine of manic and depressed patients. Results of such studies are largely inconsistent due to factors such as different age and sex distribution of patients and controls within and between studies, different diagnostic procedures, patient heterogeneity with respect to bipolar and unipolar illness, sample sizes, diet, phase differences and sample timing (Koslow et al. 1983). There is also doubt about the validity of the above metabolites as being representative of central or peripheral amine turnover (Zis and Goodwin 1982). It must also be appreciated that if the defect is limited to a very small part of the brain, studies such as those above may not pick up such a small localised change. Similarly changes that are seen may be secondary to the main pathology.

In general, however, low levels of urinary MHPG have been shown in some studies of depressed patients, with increasing levels upon recovery or in mania (Gershon et al. 1977, Shaw et al. 1982, Zis and Goodwin 1982). In depressed patients, decreased CSF levels of 5-HIAA have been reported (Gershon et al. 1977, Bowman and Rand 1980, Zis and Goodwin 1982). Probenecid treatment (blocks transport of HVA and 5-HIAA out of the CSF) has indicated a decrease in both 5-HT and dopamine turnover in depressed patients and in manics (Sachar and Baron 1979, Zis and Goodwin 1982). More recently, a study involving 85 unipolar, 19 manic and 47 bipolar patients showed significantly increased CSF

MHPG levels in mania, and an increased 24 hour urinary excretion of VMA in both mania and depression (Koslow et al. 1983). Significant increases were also seen in urinary noradrenaline and adrenaline in unipolar patients, in adrenaline in bipolar patients, and in noradrenaline in manics. The results of this last study tend to suggest an excess of neurotransmitter rather than a deficit.

Studies involving the use of amine precursors are also equivocal. L-tryptophan has proved to be a useful antidepressant in some studies but not others (Mindham 1982) and potentiates MAOI's as previously mentioned. Some success in treatment-resistant patients has been reported with 5-hydroxytryptophan (Shaw et al. 1982) but L-Dopa seems to produce rather than ameliorate depressive symptoms (Mindham 1982).

With the realisation that many of the drugs effective in either depression or mania do not appear to share a common mode of action at the gross level, studies tended to be directed more into looking at receptors, second messengers, and imbalances between systems.

The main problem with receptor studies in clinical situations is that they are peripheral and may not be representative of central events. Extein et al. (1979) have reported a decreased number of beta-adrenergic receptors in lymphocytes of manic and depressed patients when compared with controls or euthymics, with a concomitant decrease in isoproterenol-stimulated but not prostaglandin E1-stimulated cAMP production. Lower values for beta-receptor numbers have also been reported in virally transformed lymphoblastoid cell lines of bipolar manic-depressive

families (Wright et al. 1984). Binding was decreased to less than 50% of the mean family control values in 4 out of 6 manic-depressive subjects and only 1 out of 18 non-psychiatric controls. The subjects showing these decreases were confined to 3 families out of the 5 examined, suggesting genetic heterogeneity. Generally however, results of studies involving beta-receptors are inconsistent, with possible explanations for this including the variable half-life among lymphocyte populations and the effect of physical activity on beta-receptor number (Wood and Coppen 1985).

Variable findings have been reported concerning α_2 -adrenergic receptors in platelets, possibly due to methodological differences (Wood and Coppen 1985). No difference has been reported for the K_d (the equilibrium dissociation constant for an agonist or antagonist with its receptor sites i.e. the free concentration of agonist or antagonist at which half the receptor sites are bound) or B_{max} (the maximum number of receptor sites bound i.e. the total number of receptors) values of 3H -yohimbine binding in depressives compared with controls (Stahl et al. 1983) but increases in B_{max} have also been reported (Garcia-Sevilla et al. 1981, Cameron et al. 1984), decreasing upon treatment with tricyclics (Garcia-Sevilla et al. 1981). This latter finding would support the hypothesis of a supersensitivity of α_2 -adrenergic receptors in depression, leading, in the case of presynaptic α_2 -receptors to a decreased availability of noradrenaline. Further support for the idea that tricyclics act by decreasing either the sensitivity or number of α_2 -receptors is given by the findings of Charney et al. (1983) where

the decrease in plasma MHPG following clonidine stimulation of pre-synaptic α_2 -receptors, is attenuated by long-term amitriptyline treatment. A sub-sensitivity of α_2 -receptors however is suggested by the finding of Siever et al. (1984) where the magnitude of the plasma MHPG response to clonidine is significantly less in depressives than in controls.

Binding studies have shown a decrease in 5-HT₂ receptors in platelets after chronic antidepressant treatment, and a significantly greater number of 5-HT₂ receptors in the frontal cortex of suicide victims compared with controls (Coppen and Wood 1985). A supersensitivity of 5-HT receptors in depression and mania has also been proposed following findings that oral administration of 5-HTP produces a larger increase in serum cortisol levels in depressives and manics compared with controls; a response augmented by lithium or MAOI's in manics and depressives respectively but attenuated by tricyclics in depressives (Meltzer et al. 1984a,b,c). This is consistent with the idea that lithium acts by enhancing 5-HT₂ receptor sensitivity and tricyclics decrease the sensitivity. Uptake of 5-HT into platelets has also consistently been reported as being decreased in depression, even following recovery (Wood and Coppen 1985). In several studies the tricyclic antidepressants amitriptyline and desipramine have been shown to actually stimulate, rather than inhibit, uptake of 5-HT in some depressed patients (Coppen and Wood 1985), with recovery being more evident in the group of patients showing the stimulatory response. If this was the mechanism by which tricyclics exerted their therapeutic effect then the time lag between administration and

therapeutic effect would be expected whilst the necessary build-up of transmitter occurred within the nerve terminal.

A possible sub-grouping of depression based on the relationship between drug response and pretreatment levels of noradrenaline and metabolites has been proposed by Maas et al. (1984). They found that in bipolar patients, normal to low levels of noradrenaline and metabolites in urine were associated with a high level of response to tricyclics. In unipolar patients, low 5-HIAA levels and high normetanephrine levels were associated with a good response to tricyclics. These findings suggest that in one group of unipolar patients, a low noradrenergic function is secondary to a primary change in the 5-HT system whereas in a group of bipolar patients, the primary change is in the noradrenergic system. However some patients failed to fit into either category, implying that such an analysis is either incomplete or incorrect.

Interest in the possible involvement of second messengers has recently focused on the role of the phosphatidylinositol system following the discovery that lithium interferes with the agonist-dependent metabolism of inositol via inhibition of the hydrolysis of inositol-1-phosphate (Allison et al. 1976, Berridge et al. 1982). If the stimulus were prolonged and further supplies of inositol were not readily available, this could have the effect of decreasing the response to, for example, alpha-adrenergic agonists. This is examined further in Chapter 7.

Certainly from the evidence available, coupled with the methodological differences between studies and the difficulty of deciding which abnormalities are primary or secondary, it would

appear that if the primary defect lies in the aminergic neurotransmission systems, the biological abnormalities which are manifest vary from patient to patient. This is possibly due to genetic heterogeneity within the illness, possible sub-groups within the illness having slightly different aetiologies, and the complex interactions between the various neurotransmitter systems. Alternatively it may be concluded that since so much variation in amine metabolism is seen from patient to patient, there is no common factor in this area which distinguishes bipolars from normal, and that individual variations from normal may be nothing more than random variations in a normal population.

ii). Neuroendocrine function:- Many of the symptoms of mania and depression are suggestive of hypothalamic-pituitary dysfunction and this has been investigated in several studies looking at neuroendocrine function. Results are difficult to compare in many such tests due to the involvement of several neurotransmitters and releasing factors in the regulation of secretion of each hormone, the influence of variables such as stress, age, sex, diet, and the circadian rhythms. Much work needs to be done however before any possible underlying neurotransmitter dysfunction can be elucidated.

The most commonly used test is the dexamethasone suppression test (DST). Dexamethasone is a potent synthetic glucocorticoid which suppresses plasma cortisol levels in normal individuals by means of a negative feedback inhibition. Both abnormal and normal dexamethasone suppression tests have been found in mania (e.g. Godwin et al. 1984) whilst results for depression have been

more consistent with approximately 40-50% of patients with major depression showing abnormal DST results, either non-suppression or an early escape from suppression (Sachar 1982, Lowy et al. 1984). This is generally associated with cortisol hypersecretion which appears to be secondary to ACTH hypersecretion (Sachar 1982), but the physical signs of Cushing's syndrome are not present, possibly due to a generalised decrease in glucocorticoid receptor sensitivity and/or function. Non-suppression with dexamethasone has also been correlated with a decreased sensitivity of lymphocytes to glucocorticoid-induced immunosuppression (Lowy et al. 1984).

Mitogen-induced lymphocyte proliferation has also been found to be decreased in patients with major depression (Schleifer et al. 1984), together with a generalised reduction in peripheral lymphocyte numbers but the percentage of T and B-cells did not differ compared with the controls. However no alteration in lymphocyte function has also been reported (Sengar et al. 1982) although medication was not withheld in this study and may have had some effect upon the results.

Many studies have reported a deficient release of TSH in response to TRH infusions (Sachar 1982) in depressed patients. This response was not seen in all depressed patients however and was more predominant amongst unipolar rather than bipolar patients.

iii). Ion transport:- The unequal distribution of sodium and potassium ions across cell membranes, i.e. high intracellular potassium and extracellular sodium, and low extracellular potassium and intracellular sodium, is achieved through a

combination of selective membrane permeability and the electrogenic sodium pump (Na^+/K^+ -ATPase molecule) which in most cell types is thought to extrude 3 sodium ions for every 2 potassium ions taken up (from Robinson 1975, Harrison and Lunt 1980). The greater permeability of the cell membrane to potassium ions results in a negatively-charged membrane potential (intracellular relative to extracellular). The maintenance of these ion gradients is necessary for the generation and propagation of impulses in excitable tissue.

Early observations of an increase in residual or intracellular sodium during mania and depression, returning to normal upon recovery, suggested a possible perturbation of ion transport in manic-depressive psychosis (Coppen and Shaw 1963, Coppen et al. 1966). Similar findings were later reported by Naylor et al. (1971) but Rybakowski et al. (1981b, 1983) found lower intracellular levels of potassium and sodium during depression with a decrease in plasma sodium upon recovery, and Frazer et al. (1983) reported no significant differences in intracellular or extracellular sodium levels during mania or depression. Whether the alterations in ion concentrations are of aetiological importance or are secondary to a change in mood is not clear. The majority of such ion studies have been carried out using erythrocytes as an easily accessible peripheral tissue, and whether any abnormalities seen here reflect the situation in nervous tissue would have to be further investigated.

Later studies involving the measurement of Na^+/K^+ -ATPase activity and ouabain-sensitive K^+ influx (i.e. that proportion of the potassium influx which is mediated by the sodium pump),

largely using erythrocytes, are reviewed in detail in Chapter 6. In summary, although results vary from study to study, the majority of the findings are consistent with a lower total sodium pump flux in mania and depression compared with remission (Naylor 1986), although whether remission values are also less than controls is less clear. This lower activity appears to be due to a lower activity per sodium pump molecule rather than to a change in number of transport sites per cell, in both mania and depression (Naylor et al. 1980b). Many studies have investigated the effects of lithium on Na^+/K^+ -ATPase activity but results are inconsistent and it has not been conclusively determined whether any effect is seen (reviewed in Chapter 6).

Following the work by Cantley, Jr. et al. (1977) who had shown that in vitro the vanadate ion is a powerful inhibitor of Na^+/K^+ -ATPase with a K_i (concentration producing half the maximal inhibition) in the region of normal plasma vanadium concentrations (although more recent measurements of plasma vanadium are considerably lower - see later), Dick et al. (1980) proposed that the factor in the plasma responsible for the changes in sodium pump activity may be vanadium. Plasma vanadium concentration as measured by atomic absorption spectrometry was found to be increased significantly in manics, and non-significantly in depressed patients when compared with controls. A negative correlation with erythrocyte Na^+/K^+ -ATPase activity was seen (Dick et al. 1980, Dick et al. 1982). In a later study, manic patients had significantly raised levels of vanadium in hair which fell towards control levels upon recovery but had no significant differences in the blood or serum levels

of vanadium. In contrast, depressed patients had raised levels of vanadium in blood and serum which fell with recovery (Naylor et al. 1984). These results probably reflect different aspects of vanadium metabolism and in addition the measurements are those of total vanadium, irrespective of oxidation state. A more recent study has also supported the increased serum levels of vanadium during depression, with means + S.D. of $0.061 + 0.025 \mu\text{M}$ for depressed patients and $0.013 + 0.006 \mu\text{M}$ for healthy controls. There was no correlation between vanadium level and type of depression (i.e. neurotic or psychotic) within the depressed group.

As vanadium given to normal subjects does not invariably produce depression of mood (Naylor 1983), it was proposed that if vanadium is an aetiological factor, manic-depressive patients must have some abnormality, probably genetic, which would increase their susceptibility to the actions of vanadium. The production of new pump sites in response to increasing intracellular sodium concentration has been shown to be dependent on de novo protein synthesis in HeLa cells. Whether this was as a result of alterations in transcription or translation was not clear (Boardman et al. 1974, 1975b). To this end the ability of lymphocytes from bipolar patients to increase their pump site numbers in response to an increase in intracellular sodium concentration was examined. The results showed that lymphocytes from bipolar patients had a significantly reduced ability to synthesise new pump sites when compared with controls (Naylor and Smith 1981a). Naylor suggested that this was indicative of a defect in pump site control which renders the patient more

vulnerable to vanadium and may predispose to manic-depressive psychosis. However this theory does not explain the difference between mania and depression or the onset and recovery of the depressive and manic phases of the illness. Further discussion of the possible involvement of vanadium in the aetiology of manic depressive psychosis is given in Section 1.VII. after the chemistry and biological actions of vanadium have been reviewed.

The effectiveness of lithium has been attributed to its chemical similarity with sodium and potassium. Normally, under steady-state conditions, the red blood cell lithium concentration is approximately one third that of the plasma although considerable interindividual variation is seen (Dorus et al. 1983, Dagher et al. 1984). This concentration gradient is primarily maintained by a lithium-sodium exchange (1:1), whereby sodium entering the cell down its electrochemical gradient exchanges with intracellular lithium. This transport system is phloretin-sensitive, ouabain-insensitive and normally functions as a sodium-sodium exchange (Dorus et al. 1983),

Considerable interindividual variation in the lithium ion ratio (i.e. the ratio of red blood cell lithium concentration to plasma lithium concentration) has been found in family studies within the general population, with strong indications that genetic factors are involved (Dorus et al. 1980). In a study involving the first-degree relatives of 31 bipolar patients and 120 normals, segregation analysis indicated the involvement of an autosomal major gene locus in influencing the lithium ion ratio normally, the expression of which is modified by considerable multifactorial variability. The allele at the major locus was

associated with an elevated lithium ion ratio and an increased risk of psychiatric hospitalisation amongst the relatives of the bipolar patients (Dorus et al. 1983). A study involving 25 pairs of monozygotic twins has shown that lithium ion ratios are significantly greater in twins concordant for affective illness compared with twins discordant for the illness (Verbanck and Mendlewicz 1981). This suggests a membrane transport anomaly as a possible susceptibility factor in subjects genetically disposed to affective illness. However other factors are also important in determining the lithium ion ratio, such as intestinal absorption and renal excretion.

The Na^+/K^+ -ATPase hypothesis based on the previously reviewed data and put forward by El-Mallakh (1983a), explains both mania and depression on the basis of a reduced sodium pump activity. In excitable cells this reduced activity of the sodium pump leads to a depolarisation of the membrane potential towards the action potential threshold and with the reduction in sodium extrusion by the pump leading to increased intracellular sodium levels, sodium-calcium exchange which is partly responsible for the removal of intracellular calcium would be slower, resulting in prolonged transmitter release. This increased neuronal activity results in a period of mania. If the decrease in activity continues so as to decrease the membrane potential beyond the action potential threshold, depression occurs. This would imply that a manic phase occurs every time preceding a depressed phase but may not always be evident due to the rapidity of the change in activity of the pump. However this hypothesis fails to take into account the fact that most of the calcium involved in

transmitter release is removed as a result of active calcium transport (Bowman and Rand 1980) and that sodium-calcium exchange plays only a small part. In addition, this hypothesis implies that in depression the defect is more severe than in mania, which is the opposite of the evidence from genetics. Indeed it would make more sense to postulate that neuro-overactivity produces depression, as for example a person in a depressive stupor recovers rapidly following intravenous administration of sodium amytal (Naylor - personal communication).

As a continuation of the above hypothesis, El-Mallakh (1983b) has explained the therapeutic action of lithium. As lithium enters cells by lithium-sodium exchange or by diffusing in place of sodium through sodium channels normally or during an action potential, and is only extruded significantly by lithium-sodium exchange which is dependent mainly on the extracellular sodium concentration, it is preferentially accumulated in the more active excitable cells where the influx rate of lithium exceeds the efflux rate. This accumulation of intracellular lithium in excitable cells leads to a decrease in sodium entry during subsequent action potentials and an eventual reduction in intracellular sodium, which in turn leads to increased calcium extrusion via sodium-calcium exchange and decreased cellular excitability, returning the membrane potential to normal. This hypothesis goes some of the way towards integrating the catecholamine and ion transport hypotheses although the nature of the endogenous factor responsible for the varied activity of the sodium pump, the primary defect in the sodium pump of bipolar patients, and the phasic nature of the illness are still not

explained.

Perturbations in anion transport, namely erythrocyte phosphate transport, have also been reported in bipolar and unipolar patients (Szentistvanyi et al. 1980). Plasma and erythrocyte inorganic phosphate levels, passive phosphate transport and transfer of inorganic phosphate into the membrane ATP pool but not the cytosol pool were all significantly lower in the depressed patients compared with the controls. No significant difference was seen in ATP levels or in the rate of glucose utilisation. Other membrane abnormalities have also been reported e.g. calmodulin-activated Ca^{2+} -ATPase activity in the presence of lithium ions was greater in manic-depressives whether or not treated with lithium previously, than in controls (Meltzer and Kassir 1983), and in a study using fluorescent membrane probes, Pettegrew et al. (1982) reported alterations in membrane dynamics which indicated abnormalities in the hydrocarbon region of erythrocyte membranes and lymphocyte cell surfaces in manic-depressive patients. Overall it is possible that some fundamental abnormality in cell membranes underlies all the above observations with varied effects being seen from patient to patient depending upon other possible predisposing factors.

Further discussion of the possible involvement of vanadium in the aetiology of manic-depressive psychosis is given in the following sections after a brief illustration of its chemistry and occurrence.

1.V. Chemistry and occurrence of vanadium

Vanadium, atomic number 23 and atomic weight 50.94, is a transition element of Group Vb. Originally discovered by the Swedish scientist Sefstrom in 1831 and named after "Vanadis", the Scandinavian goddess of beauty, love, youth and lustre (Sage 1981, Nechay et al. 1986), vanadium is a trace element found in over 50 different minerals e.g. in combination with uranium and radium as carnotite and roscoelite respectively, and occurs in the earth's crust at a mean concentration of approximately 100 ppm (MARC Report 1976, Sage 1981). Main deposits are in Scandinavia, South Africa and the USA (Sage 1981). It is also found in some crude oils from where it is released in to the atmosphere upon combustion. Its chief commercial uses are in iron and steel alloys where it imparts ductility and shock resistance, and as a catalyst in chemical processes (Cotton and Wilkinson 1972, MARC Report 1976).

Oxidation states range from -1 to +5 but the most commonly occurring are from +2 to +5. V^{2+} and V^{3+} are unstable and are immediately oxidised to V^{4+} which is only stable in acidic pH as the blue vanadyl cation, VO^{2+} . In oxygen or air, V^{4+} is readily oxidised to V^{5+} and this is the oxidation state in which vanadium normally exists under physiological conditions (Ramasarma and Crane 1981, Rubinson 1981).

The solution chemistry of V^{5+} ionic species is complex. It is pH and concentration dependent as can be seen in Figure 1.1., with V^{5+} existing mainly as the monomeric orthovanadate anion, VO_4^{3-} in the most basic solutions and as the monomeric dioxovanadium cation, VO_2^+ , in the most acidic solutions. With

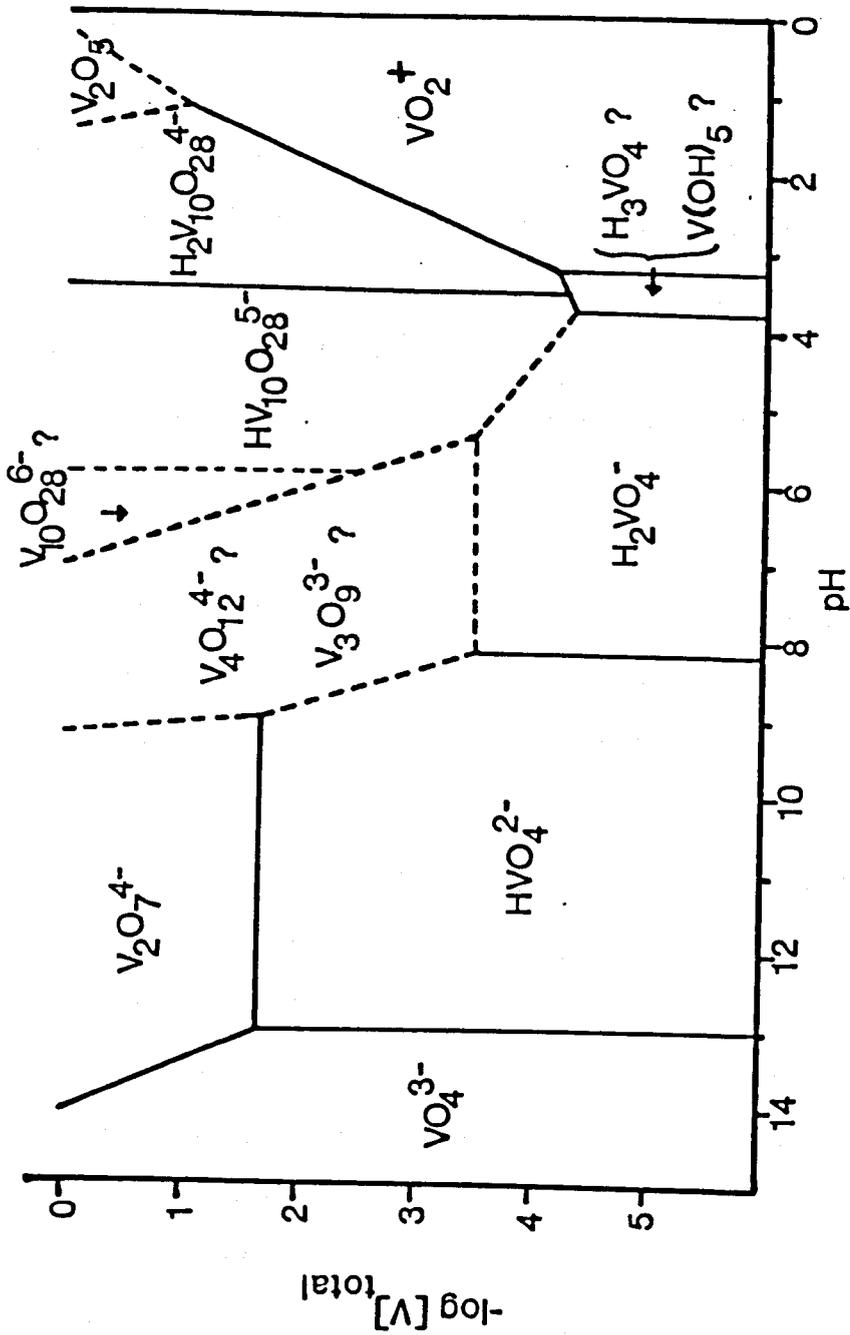


Figure 1.1. Diagram showing the major species of vanadium (+5) which exist depending upon the pH and vanadium concentration

(from Cotton and Wilkinson 1972)

an increase in concentration these species tend to polymerise yielding e.g. tri-, tetra- and decavanadate ions, dependent upon the pH (Cotton and Wilkinson 1972, Rubinson 1981). At the pH and concentration present physiologically and in the absence of any interfering effect from other ions, vanadium would be expected to exist in the +5 oxidation state, largely as the monomeric metavanadate ion, H_2VO_4^- or more simply VO_3^- , unless reduced to the vanadyl ion VO^{2+} intracellularly (Nechay et al. 1986). Such a change results in not only an alteration of the oxidation state of vanadium but also an alteration from a negatively charged species to a positively charged species.

1.VI. Biological effects of vanadium

For the purpose of illustrating the biological distribution of vanadium, the references cited are mainly review articles due to the large amount of literature on this aspect. Regarding the biological aspects of vanadium, this section is largely confined to the vanadate ion as this is the most biologically active form of vanadium (e.g. Schmitz et al. 1982), and is the species of vanadium normally present physiologically unless intracellularly reduced as discussed below. Original references cited here are discussed in greater detail in the appropriate experimental chapters of this thesis.

The essentiality of vanadium is well documented for some organisms e.g. rat and chicken (Nielsen and Sandstead 1974, Erdmann et al. 1984), with deficiency symptoms occurring at dietary levels of less than 10-100 ng vanadium/g food. These

include retardation of growth, bone malformation and impairment of reproductive function. However Nechay et al. (1986) dispute the essentiality of vanadium as being proven, due to inconsistencies of other dietary factors in the various studies. In man however, essentiality is less clear but the toxic effects are well known, mainly due to industrial exposure via the lungs. Most of the ingested vanadium is not absorbed. The primary route of excretion for absorbed vanadium is via the kidney (Nechay et al. 1986). Depression, bronchial spasm, vomiting, diarrhoea, anaemia, inhibition of cholinesterase activity, and death have all been reported (MARC Report 1976).

Tissue levels of vanadium vary from study to study depending on the sensitivity and accuracy of the analytical method used, and the degree of contamination. The total body burden of an adult human has variously been estimated in the range 100 ug to 43 mg, and the mean daily intake at between 10 ug and 4 mg depending on the study (MARC Report 1976, Jandhyala and Hom 1983, Nechay et al. 1986). In man, vanadium tends to accumulate in the kidneys (mainly cortex), liver, spleen and lungs (MARC Report 1976, Ramasarma and Crane 1981). Normal serum levels vary from study to study with values ranging from 0.6 nM to 8 uM being reported (Jandhyala and Hom 1983). More recent measurements using the more sensitive and accurate technique of neutron activation analysis have been in the range 2-20 nM (Erdmann et al. 1984).

Many of the biological effects below have been attributed to the similarity of the chemistry of vanadium and phosphorous e.g. the V-O bond length is only about 1 Angstrom longer than the P-O

bond length (Ramasarma and Crane 1981) and vanadate is able to form a trigonal bipyramidal structure which is analogous to the proposed transition state of phosphate during enzymatic hydrolysis (Lopez et al. 1976, Cantley, Jr. et al. 1978a). However Rubinson (1981) believes that it is unlikely that vanadate and phosphate have similar biological chemistries in anything other than perhaps the simplest anion transport, as profound chemical differences exist in the rate of ligand exchange in their co-ordination complex chemistry.

Following the identification of sodium orthovanadate (Na_3VO_4) as the potent Na^+/K^+ -ATPase inhibitor present as a contaminant in commercially available "Sigma-grade" ATP prepared from equine muscle (Josephson and Cantley, Jr. 1977, Cantley, Jr. et al. 1977, Quist and Hokin 1978), but not present in "Boehringer" ATP or ATP prepared from yeast (Josephson and Cantley, Jr. 1977, Beauge and Glynn 1977, 1978), vanadate has been extensively investigated as a possible endogenous regulator of the sodium pump.

In intact cells, vanadate was found to act from the cytoplasmic side, unlike ouabain or other cardiac glycosides (Cantley, Jr. et al. 1978b, Cantley, Jr. and Aisen 1979). In human erythrocytes, vanadate entered the cells with half the affinity of phosphate ($K_{\text{aff}} \sim 60$ mM for vanadate - the affinity constant analogous to K_d at equilibrium i.e. in this case, the concentration where the uptake is half the maximum), and by the same anion-exchange pathway, as evidenced by the inhibition of vanadate entry by DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) and by vanadate's competitive inhibition of

phosphate entry (Cantley, Jr. et al. 1978b). However in cardiac cells, uptake was postulated to be by a different saturable, non-energy dependent pathway with a K_{aff} of 60-100 μM (Werdan et al. 1980). This was not competitively inhibited by phosphate.

Whereas the apparent K_i values for vanadate in isolated Na^+/K^+ -ATPase systems were very low e.g. 40 nM for dog kidney under optimal conditions of high magnesium concentrations (25 mM) and 100 nM under physiological conditions (Cantley, Jr. et al. 1977), and 1 μM for rabbit heart sarcolemma under physiological conditions (Quist and Hokin 1978), it was higher in intact cells e.g. 40 μM in human erythrocytes under physiological conditions (Cantley, Jr. et al. 1978b). This discrepancy was attributed to the intracellular reduction of the vanadate ion to the less biologically active vanadyl ion and its subsequent binding to intracellular proteins (Cantley, Jr. and Aisen 1979, Macara et al. 1980). More recently the vanadyl ion has also been shown to be a potent inhibitor of highly purified fractions of Na^+/K^+ -ATPase with a K_i of 10 μM , but no inhibitory action was seen using more crude enzyme preparations (North and Post 1984).

Inhibition of Na^+/K^+ -ATPase by vanadate required the presence of potassium ions (extracellularly in intact cells) and magnesium ions (intracellularly in intact cells) which facilitated the binding of vanadate although there was uncertainty about the requirement for potassium ions (Josephson and Cantley, Jr. 1977, Quist and Hokin 1978, Bond and Hudgins 1979, Beauge et al. 1980, Robinson and Mercer 1981). Unlike ouabain, the inhibitory action of vanadate was augmented by increasing the extracellular potassium concentration (Beauge et al. 1980). Decreasing the

extracellular sodium concentration also augmented the inhibition whilst ATP had a protective effect against inhibition (Josephson and Cantley, Jr. 1977). Inhibition of the isolated enzyme was totally reversed by millimolar concentrations of noradrenaline, probably by complexation (Josephson and Cantley, Jr. 1977, Quist and Hokin 1978).

A ouabain-like inhibition of potassium uptake and a concomitant decrease in intracellular potassium concentration has been seen in heart non-muscle cells from neonatal guinea-pigs and chick embryos ($K_{1/2} = 40$ and 100 μM respectively), although the alterations in potassium concentration were not as large as those seen in the presence of ouabain, indicating the possibility of other effects as well as the inhibition of the sodium pump (Werdan et al. 1982). However in heart muscle and non-muscle cells prepared from neonatal rats, and in Girardi human heart cells, vanadate stimulated both the ouabain-sensitive potassium uptake ($K_{\text{aff}} = 22, 140$ and 34 μM respectively) and the sodium influx, resulting in an increased intracellular potassium concentration but no change in the sodium concentration (Werdan et al. 1980, 1982), probably due to the increase in sodium influx. This stimulation was seen despite the inhibition of the isolated enzyme by vanadate. Positive inotropic effects and chronotropic effects were also seen at these vanadium concentrations (Werdan et al. 1980). This stimulation of the ouabain-sensitive potassium flux was mimicked by insulin, and incubation in vanadate, like insulin, stimulated the uptake of 2-deoxy-D-glucose in these cells. This demonstration of a ouabain-like inhibition and an insulin-mimetic stimulation of

potassium uptake in the various cell types may be due to differing reducing abilities, thereby resulting in either predominantly vanadate ions which would inhibit the sodium pump, or predominantly vanadyl ions which produce the insulin-mimetic effect. Other as yet unknown membrane permeability differences may also play a role in these results (Werdan et al. 1982).

The mode of action of vanadate in binding to the sodium pump and thereby inhibiting it is complex and is discussed in more detail in Chapter 3, together with the postulated conformational changes involved in the normally operating sodium pump. Essentially vanadate is considered to bind to ATP binding sites and stabilise the enzyme in the (K)E₂ conformation (Cantley, Jr. et al. 1978a, Karlisch et al. 1979).

When the oxidation of NADH was used to assay for Na⁺/K⁺-ATPase activity, by coupling of the ATP hydrolysis to the oxidation of NADH through pyruvate kinase and lactic dehydrogenase, vanadate appeared to have a stimulatory rather than inhibitory action upon Na⁺/K⁺-ATPase activity in a cat heart cell membrane preparation (Erdmann et al. 1979a). It was later deduced that the decrease in NADH induced by vanadate was not caused by stimulation of the ATPase molecule but by an NADH-vanadate reductase possibly present in the cardiac membranes (Erdmann et al. 1979b). In a later study by Ramasarma et al. (1981), millimolar levels of vanadate (probably decavanadate) stimulated the basal rate of NADH oxidation 10-20 fold in mouse liver plasma membranes and pig erythrocyte membranes, with oxygen as the acceptor, i.e. the oxidation occurred not as a result of NADH-dependent reduction of vanadate but as the result of a vanadate-stimulated NADH oxidase.

present in these membranes. This was inhibited by noradrenaline with a K_i of approximately 2 μM , and also by superoxide dismutase indicating a possible involvement of superoxide anions in the effects of vanadate. Vanadate was also shown to be able to oxidise NADH non-enzymically in this study. Such results show that caution is needed in the interpretation of results produced using such assays.

Initially vanadate's inhibitory action was thought to be specific to the Na^+/K^+ -ATPase following the claim that it was ineffective on sarcoplasmic reticulum Ca^{2+} -ATPase (Josephson and Cantley, Jr. 1977). However it was later shown that the lack of inhibition was due to a protective effect of low calcium concentrations (O'Neal et al. 1979), and in the presence of the ionophore A23187 vanadate was shown to inhibit rabbit muscle sarcoplasmic reticulum Ca^{2+} -ATPase. Several other ATPases have also since been shown to be inhibited by vanadate e.g. Mg^{2+} -ATPase from ascites plasma membranes and the K^+ -ATPase of hog gastric mucosal cell membranes (O'Neal et al. 1979). However mitochondrial ATPase was not inhibited (Josephson and Cantley, Jr. 1977).

Ca^{2+} -ATPase from erythrocyte membranes was inhibited by vanadate but with 3x less sensitivity than the Na^+/K^+ -ATPase - this inhibition also exhibited a requirement for magnesium and potassium ions which appear to increase the apparent affinity of the enzyme for vanadate (Bond and Hudgins 1980, Barrabin et al. 1980). Only calcium concentrations greater than 50 micromolar resulted in a progressive reduction of the vanadate-induced inhibition. Vanadate exerted its inhibitory action

intracellularly and the potentiating effects of potassium and magnesium were also both exerted intracellularly (Rossi et al. 1981). Similar inhibition of Ca^{2+} -ATPase has been observed in several other tissues e.g. squid axons, rat brain synaptosomes, dog heart cells and kidneys - see Jandhyala and Hom 1983 for references). In squid axons, DiPolo et al. (1979) reported an inhibition of uncoupled calcium efflux by vanadate but no inhibition of sodium-calcium exchange suggesting that uncoupled calcium efflux occurs via an ATP-driven pump and that sodium-calcium exchange occurs via a different pathway.

Vanadate at micromolar levels has also been shown to inhibit a variety of other enzymatic reactions involving phosphorylation and dephosphorylation steps and these are reviewed in Chapter 4.

Vanadate was found to stimulate adenylate cyclase in several tissues including human platelets and plasma membranes from rat adipocytes, guinea pig hearts and turkey erythrocytes, (Schwabe et al. 1979, Krawietz et al. 1979, Krawietz et al. 1982, Ajtai et al. 1983). K_m values (the substrate concentration producing half-maximal activation of the enzyme) ranged from 100 μM to 1 mM and it was postulated that this stimulatory action may partly underlie the positive inotropic effects seen with vanadate, as described later. In adipocyte membranes, fluoride was shown to have a greater maximal activating effect than vanadate on adenylate cyclase, and the effects were not additive (Schwabe et al. 1979). Beta-adrenergic agonists had no additive effect on this vanadate-induced activation.

In guinea-pig heart membranes however, effects of vanadate and fluoride were additive and increasing concentrations of vanadate

in the presence of the non-hydrolysable analogue of GTP, Gpp(NH)p, demonstrated a competitive effect (Krawietz et al. 1979). These results suggest that vanadate stimulates adenylate cyclase by interacting with the guanine nucleotide regulatory unit but by a different mechanism to fluoride. This mechanism of action was also supported by the results of Krawietz et al. (1982) using turkey erythrocytes, where like fluoride, vanadate stimulation of adenylate cyclase involves the GTP regulatory unit but by a different mechanism as although the effects of fluoride and vanadate were not additive, the modification of the regulatory unit by two separate agents resulted in different consequences for fluoride- and vanadate-stimulated activity. At optimal fluoride concentrations for activation (10 mM), activation was inhibited by concentrations of vanadate greater than 3 mM. No effect on beta agonist or antagonist binding was seen (Krawietz et al. 1982).

It is known that stimulation of adenylate cyclase in broken cell preparations does not necessarily imply increased cAMP levels in intact cells (see Hackbarth et al. 1980 for references). However increases in cAMP following treatment with 100 uM vanadate have been shown in e.g. cat papillary muscles (Hackbarth et al. 1980) and frog skin epithelia (Cuthbert et al. 1980). No alteration in phosphodiesterase activity was seen. In intact rabbit ventricular myocytes, no accumulation of intracellular cAMP or inhibition of ouabain-sensitive potassium influx was found until concentrations of vanadate greater than 0.5 mM when toxicity was also seen (Aiton and Cramb 1985). These concentrations are higher than the levels at which positive

inotropic effects are seen in the same preparation suggesting that activation of adenylate cyclase or inhibition of the sodium pump may not be involved in these effects. In contrast using the broken cell membrane preparation, vanadate at concentrations less than 100 μM ($K_m = 2 \mu\text{M}$) stimulated adenylate cyclase activity upto 2-3 fold, with higher concentrations being inhibitory ($K_i = 0.5 \text{ mM}$, Aiton and Cramb 1985). Effects of fluoride were not additive. Differences observed between the two preparations may be due to the possible intracellular reduction of vanadate in the whole cell preparation and/or to permeability difficulties.

Whereas in rat adipocyte membranes there was no change in phosphodiesterase activity with vanadate treatment (Schwabe et al. 1979), in intact adipocytes, activation of phosphodiesterase was seen (Souness et al. 1985). From the results it seems likely that this was due at least in part, to the vanadyl ion rather than the vanadate ion.

Vanadate at micromolar levels has also been shown to inhibit membrane phosphotyrosyl-protein phosphatase activity but not the phosphoserine-specific activity, in A-431 cell membranes (Swarup et al. 1982). This may explain the synergistic action of vanadate with mitogens e.g. with epidermal growth factor (EGF) resulting in enhanced DNA synthesis in human fibroblasts (Carpenter 1981), since the initial interaction of EGF with its receptor brings about an increased activity of a membrane protein kinase which is tyrosine specific. Through its inhibitory action, vanadate could possibly increase the level of phosphotyrosine present in the phosphoproteins.

The physiological effects of vanadate have largely been attributed to its ability to inhibit Na^+/K^+ -ATPase and/or stimulate adenylate cyclase. A positive inotropic effect with vanadate was observed in electrically-stimulated cat and guinea-pig papillary muscles (Hackbarth et al. 1978, Borchard et al. 1979), and in ventricular muscle from rat, rabbit, guinea-pig and cat, and in atria of rat and rabbit (Grupp et al. 1979, Schwartz et al. 1980). Half maximal effects were seen at concentrations of vanadate ranging from 20 μM to 500 μM . These effects were reversible and were unaffected by beta agonists or antagonists. At the same vanadate concentrations a marked activation of adenylate cyclase has been described in some tissues (Grupp et al. 1979) but not all (Aiton and Cramb 1985), making the possible involvement of adenylate cyclase in the positive inotropic response unclear. In contrast, a negative inotropic effect was seen in guinea-pig and cat atria at concentrations slightly lower than those producing the positive inotropic response in the tissues above (Grupp et al. 1979, Borchard et al. 1979), and as the isolated enzyme from atria and ventricles in these animals were both inhibited with a K_i of approximately 0.6 μM vanadate despite the differences in basal activity, this effect was not due to differential effects of vanadate upon the ATPase activity unless the tissues had different reducing abilities. No inotropic effects were seen with vanadium in the +3 or +4 oxidation state when tested on cat papillary muscles (Schmitz et al. 1982).

The positive and negative inotropic actions of vanadate correlated with a broadening and shortening of the action

potential respectively in the guinea-pig but in the rat atria, action potentials were also shortened despite the positive inotropy. Although the time courses of the alterations in the action potential duration were the same in both, the time course for the development of the respective negative and positive inotropism differed (Borchard et al. 1980). Borchard et al. (1979, 1980) concluded that the inotropic effects of vanadate are due to alterations in transmembrane potential, but not primarily from the inhibition of the sodium pump. This was also supported by the demonstration of positive inotropy and chronotropy even in preparations where the sodium pump was stimulated by vanadate (Werdan et al. 1980).

When isolated heart preparations are electrically stimulated, at least two different calcium pools are thought to be involved in the contraction (Takeda et al. 1982). One is a superficial pool predominant at high stimulation frequencies, which is related to beat-to-beat control of the force of contraction and is inhibited by verapamil. The other pool is related to post-rest potentiation of contraction and is inhibited by ryanodine. In rat atria, increasing the frequency of stimulation led to minor effects on the force of contraction whereas in guinea-pig atria substantial increases in force were seen with each increase in stimulation frequency (Takeda et al. 1982). This was interpreted as being due to an increase in the magnitude of the superficial pool calcium at higher stimulatory frequencies in the guinea-pig, but not in the rat. The effects of vanadate were unaffected by the frequency of stimulation in the rat where it produced a positive inotropism, but were greater at higher

frequencies of stimulation in the guinea-pig, consistent with the idea that vanadate affects the superficial calcium pool. In contrast vanadate failed to modify the post-rest contraction. It was concluded that the response of the vanadate-sensitive calcium pool must be dependent on species and perhaps the area of the heart (Takeda et al. 1982).

Catalan et al. (1985) have described a vanadate-induced activation of acetylcholinesterase (maximum at 100 uM) in erythrocytes and rat ventricular strips. This may also contribute in some way to the inotropic effects.

The predominant effect of vanadate in vivo is vasoconstriction suggesting that arterial smooth muscle has a greater sensitivity to vanadate than does cardiac muscle. Myocardial contractility was depressed and blood pressure increased in blood-perfused organs from dogs, and in anaesthetised cats and dogs, resulting in a negative inotropism and chronotropism (Schwartz et al. 1980, Borchard et al. 1981). It was concluded that this was due to coronary vasoconstriction at lower vanadate concentrations than those at which the positive inotropic and chronotropic effects were normally observed (Borchard et al. 1981). The mechanism of this is not clear but may be due to an inhibition of the sodium pump although the concentrations needed to inhibit the enzyme in vitro are much lower than those producing the vasoconstriction, possibly due to reduction of the vanadate (Schmitz et al. 1982). However no effect on ouabain-sensitive rubidium influx was seen in isolated vascular preparations at the concentrations where vasoconstriction was produced (Huot et al. 1979). Similar results were obtained using rat vascular smooth

muscle cells grown in vitro and this was not due to a lack of sensitivity of the sodium pump in this tissue to vanadate, as the isolated enzyme was inhibited (Searle et al. 1983). These results make an involvement of the sodium pump in the vasoconstriction produced by vanadate seem unlikely. In rat vas deferens, the constrictive effects of vanadate (greater than 100 μM) were found to be dependent upon external calcium and were blocked by the calcium antagonist nifedipine (Garcia et al. 1981) indicating the possible involvement of Ca^{2+} -ATPase.

Balfour et al. (1978) demonstrated a reversible potent diuresis and natriuresis in rats following intravenous injection of vanadate. There was no significant effect on urinary potassium output. These results were confirmed by Day et al. (1980) with the exception of the potassium excretion which they found to be increased although the urinary potassium concentration was decreased. Serum vanadium concentrations were approximately 40 μM . No changes in GFR were seen, indicating that the increased excretion of sodium was possibly due to a decreased tubular reabsorption, probably proximally as vanadate is accumulated in the renal cortex. This was also supported by the finding of an increased calcium and phosphate excretion and a non-additive effect of furosemide. Peritubular vascular effects may have also played a role due to the vasoconstrictory actions of vanadate. Westenfelder et al. (1981), in a more extensive study, found no change in potassium excretion and also suggested that Na^+/K^+ -ATPase activity in all the renal tubular segments was affected with no change in adenylate cyclase activity.

In vitro, Kumar and Corder (1980), using isolated perfused rat

kidney found that vanadate (0-32 μM) led to a dose-dependent increase in GFR, urine flow, total peripheral resistance and inhibition of sodium reabsorption. A post-capillary vasoconstriction was postulated as both the changes in GFR and vascular resistance occurred simultaneously. From several studies it would appear that inhibition of the Na^+/K^+ -ATPase is largely responsible for the renal tubular effects of vanadate (see Jandhyala and Hom 1983 for references). However in rats fed on high vanadium diets, no effects were seen on basal Na^+/K^+ -ATPase activity despite extremely high renal levels of vanadium (Higashino et al. 1983). Vanadate also had no effect on the stimulation of sodium pump activity which resulted as an adaptation to chronic potassium loading.

Churchill and Churchill (1980) reported that vanadate (greater than 5 μM), like ouabain, reduced basal as well as isoproterenol-stimulated renin secretion in rat renal cortical slices and was potentiated by external calcium. This suggests that an increase in intracellular calcium resulting from a vanadate-induced inhibition of Ca^{2+} -ATPase and/or Na^+/K^+ -ATPase mediates this response. This is consistent with the idea that beta-adrenergic stimulation of renin secretion is mediated by decreases in intracellular calcium caused by stimulation of Ca^{2+} -ATPase or Na^+/K^+ -ATPase.

In contrast however, anaesthetised cats and dogs produced a decrease in both basal and furosemide-induced urine flow when treated with a dose of vanadate comparable to that used in the rat studies. This appeared to be due to a vasoconstriction effect on renal blood vessels leading to a fall in glomerular

capillary pressure and GFR (Larsen et al. 1979). It appears as though in cats and dogs, renal vascular effects predominate over tubular effects in a similar way that the ability of vanadium to enhance myocardial contractility in vivo was diminished by its ability to produce coronary vasoconstriction.

Vanadate has also been found to inhibit saltatory organelle movement in permeabilised cultured human skin fibroblasts, at 10-100 μM , possibly by inhibiting a dynein-ATPase (Forman 1982). In addition, chromosome movement in lysed mitotic PtK₁ cells was inhibited at 10-100 μM (Cande and Wolniak 1978). No inhibition of microtubule polymerisation or depolymerisation was seen and it was thought that this effect was due to a reversible inhibition of dynein-ATPase activity in the spindle. Micromolar concentrations of vanadate also enhanced the stimulatory effect of insulin on DNA synthesis in cultured mouse mammary gland explants, but decreased that of lithium and had only a slight effect by itself (Hori and Oka 1980). It was concluded that insulin stimulation involves an ion-sensitive process and that vanadate and lithium interact at some site which is important in the regulation of this process.

There are many other diverse biological actions of the vanadate ion, too numerous to mention here. These are covered in reviews by Ramasarma and Crane (1981), Jandhyala and Hom (1983), Boyd and Kustin (1984), Erdmann et al. (1984) and Nechay et al. (1986).

1.VII. Vanadium and bipolar manic-depressive psychosis

The previously discussed work of Naylor and his colleagues

(see Section 1.IV.iii) indicated a reduced ability of manic-depressive patients to produce new sodium pump sites in response to increased intracellular sodium levels. This defect was postulated to increase their vulnerability to a modifying plasma factor tentatively suggested as vanadium or more specifically vanadate, and further studies were carried out to test the effects of altering cellular vanadate levels. In erythrocytes it had been shown that most of the vanadate taken up was reduced to the vanadyl ion which is much less biologically active (Cantley, Jr. and Aisen 1979). Ascorbic acid (vitamin C) is known to be effective in counteracting vanadium toxicity, possibly by the reduction of the vanadate ion to the less biologically active vanadyl ion (Adam-Vizi et al. 1981). Following a single dose of ascorbic acid, both manic and depressed patients were significantly better than following a placebo (Naylor and Smith 1981b). Similar improvements were seen in patients on EDTA (chelates vanadium and is poorly absorbed) plus reduced vanadium intake (Naylor and Smith 1981b) and in patients taking methylene blue (reducing agent), supporting the idea of an involvement of vanadium (Naylor et al. 1981, Narsapur and Naylor 1983). In a later study however, the combination of Vitamin C plus EDTA was as effective as the standard antidepressant treatment amitriptyline, but in manic patients was not as effective as the standard antimanic treatment, lithium (Kay et al. 1984). This is in keeping with vanadium being more important in the aetiology of depression rather than mania.

Phenothiazines, but not thioxanthenes or tricyclic

antidepressants were also able to catalyse the reduction of vanadate to vanadyl in vitro and this was postulated as possibly being the basis for part of their therapeutic action (Naylor and Smith 1982). As vanadate is slow to dissociate from the Na^+/K^+ -ATPase molecules, this would account for the slow onset of action of these drugs despite the rapid appearance of other effects such as sedation (Naylor and Smith 1982).

The therapeutic action of lithium can also be explained on the basis of an involvement of vanadium. Significantly higher Na^+/K^+ -ATPase activity/pump site was found in manic-depressive patients taking lithium for over 6 months, when compared with manic-depressives who had never taken lithium (Naylor et al. 1981), suggesting that lithium increases sodium pump activity and not numbers in vivo. This was also supported in vitro as when erythrocyte membranes from manic-depressive patients and normal subjects were prepared and assayed for Na^+/K^+ -ATPase activity in varying concentrations of vanadate, the expected inhibition of activity was seen with a K_i of approximately 0.3 μM . Addition of lithium reduced the inhibitory effect of vanadate with a maximum effect at approximately 6 mM lithium (Naylor et al. 1981). This could explain the above actions of lithium in vivo, if vanadium concentrations were high enough to be inhibitory. However MacDonald et al. (1982) were not able to confirm these actions of lithium. This conflict in the results of many such studies still has not been resolved.

Many of the results seen in studies investigating the amine hypothesis are not irreconcilable with the Na^+/K^+ -ATPase and

vanadate hypothesis. There is considerable overlap between the two areas as already indicated and additionally e.g. neurotransmission is dependent upon a fully functioning ATPase system and catecholamines have been shown to be able to activate rat brain Na^+/K^+ -ATPase, partly by a specific receptor-mediated mechanism and partly by a non-specific mechanism (Wu and Phillis 1980). In addition, incubation with millimolar levels of vanadate induced a loss of cholinergic binding sites in homogenates of rat corpus striatum (Danielsson et al. 1983) but a greater number of muscarinic cholinergic receptors were found in cultured skin fibroblasts of some manic-depressive patients and their relatives, compared with controls (Nadi et al. 1984). Subacute and chronic vanadium exposure has led to decreased levels of noradrenaline and increases in dopamine and 5-HT in rat brains (Witkowska and Brzezinski 1979). In mice, non-toxic doses of vanadate have led to decreases in hypothalamic noradrenaline and dopamine but no alterations in striatal dopamine, the area where it is richest. This suggests that vanadate selectively acts on adrenergic pathways with other pathways being affected secondarily (Sharma et al. 1986). Vanadate is bound by noradrenaline (Quist and Hokin 1978) and vanadate-induced inhibition of both Na^+/K^+ -ATPase and Ca^{2+} -ATPase in vitro can be reversed by catecholamines possibly by either a complexation or an oxidation/reduction reaction (Josephson and Cantley, Jr. 1977, O'Neal et al. 1979). Vanadate is able to oxidise noradrenaline and adrenaline thus producing the vanadyl ion and inactivating the catecholamines (Cantley, Jr. et al. 1978a).

From all the data reviewed here it is clear that the aetiology of bipolar manic-depressive psychosis is likely to have a genetic component but the primary biological perturbation underlying the illness is impossible to define with certainty, due to confounding secondary features and complex interactions. However ion transport and cerebral monoamines are two of the most likely candidates for the areas where a defect or defects may lie.

CHAPTER 2. THE EFFECTS OF CHRONIC VANADATE TREATMENT ON HELA
CELLS; CELL GROWTH, MORPHOLOGY, AND INTRACELLULAR REDUCTION OF
VANADATE

2.I. INTRODUCTION

2.I.i. Tissue culture

Tissue culture is used in many diverse areas of study e.g. cell growth, ion transport, receptors, viral or bacterial infection, immunology, genetics, cancer research, embryology, drug actions and toxicology. The general term "tissue culture" is concerned with the study of cells, tissues and organs maintained in vitro. It encompasses:- a). organ culture - the growth in vitro of tissues or the whole or parts of an organ in conditions which allow the differentiation and preservation of the original architecture and/or function; b). tissue culture - the growth in vitro of tissue fragments in conditions which do not necessarily preserve the original tissue structure; and c). cell culture - the growth in vitro of cells which are no longer organised into tissues (Paul 1970). Such systems allow the study of cellular or tissue processes in a controlled environment, free from the normal whole-body homeostatic mechanisms and endocrine influences.

The first successful culture of organised tissue was carried out by Roux in 1885 who maintained the neural plate of a developing chick embryo in warm saline for several days, and proved that the closure of the neural tube was controlled by the constituent cells and not by structures normally surrounding it in vivo (Parker 1961). Tissue fragments and cells isolated from many parts of the body have since been cultivated, following the development of suitable media which allow the maintenance of the correct pH, osmolarity and concentrations of essential inorganic

salts, vitamins, amino-acids and various growth factors.

A culture initiated from cells, tissues or organs taken directly from an organism is termed a "primary culture". This may survive for days or months either dividing or non-dividing and eventually die, or may divide repeatedly and require sub-culturing or passaging (the removal of cells to a new culture vessel with fresh medium). The culture is then termed a "primary cell line" which may die after several passages or may become an "established cell line", with the apparent potential to be sub-cultured indefinitely. This transition between the primary and established state may occur gradually, or suddenly as a result of "transformation" which can be monitored by the appearance of a few colonies of rapidly-dividing cells which become the predominant cell type. These cells often differ from the primary cell line in several ways e.g. abnormal chromosome number, loss of contact inhibition, shorter population doubling times and sometimes a loss of specialised function. Transformation can be either spontaneous or caused by agents such as viruses or carcinogenic chemicals. In addition, primary cell lines can be established from tumours and in this case often behave as established cell lines from the start. Paradoxically, many cell lines established from tumours frequently show better retention of specialised function than those derived from normal tissue (Paul 1970).

Established cell lines show essentially the same growth kinetics as bacteria. After being seeded from a stationary culture, there is a "lag phase" varying from hours to days before growth begins. Growth then progresses steadily with the

population doubling usually every 12 to 24 hours in the "logarithmic phase". Following the exhaustion of a nutrient or the accumulation of a toxic product, growth stops and the cells enter the "stationary phase". The processes underlying the "lag phase" are not known but may possibly be associated with the removal of intracellular waste metabolites and/or replenishment of essential intracellular metabolites (Paul 1970). Rates of growth depend not only upon inherent properties of the cells but also upon nutrient supply, seeding density and the proliferative fraction of the cell population (Macieira-Coelho 1973),

In this project the preliminary investigations into the cellular actions of vanadate were carried out using the epithelial HeLa cell line. This line was originally derived from human cervical carcinoma cells (Gey et al. 1952) and strains exist which may be grown either in suspension or as a monolayer. For the purposes of this study a monolayer strain was used. The reasons for selecting the HeLa cell line were as follows:- a). it is of human derivation which makes it more comparable with the lymphoblastoid cell lines used later, although functionally different cells; b). its routine use as the major human cell line in this laboratory for several years, during which time the optimum conditions for culture have been established; and c). the thorough characterisation of the cation transport parameters in this cell line which make it ideal for an investigation of the effects of a compound such as vanadate upon ion transport. The results obtained in this part of the study have then been used to aid the interpretation of the results obtained in the second half of the project, when the less-well characterised lymphoblastoid

cell lines derived from manic-depressive patients were used.

2.I.ii. Cytotoxicity of vanadate

In any study of the effects of a compound upon parameters such as ion transport, possible toxic effects must also be evaluated in order that a distinction can be made between effects caused directly by the compound and effects occurring secondarily to toxicity. Toxicity as measured by a decrease in cell numbers relative to controls, has been reported in Madin Darby bovine kidney (MDBK) cells at micromolar levels of vanadate when incubated for 24 hours (Bracken and Sharma 1985, Bracken et al. 1985). This was accompanied by a progressive change in cell morphology from a polygonal form to bipolar, spindle-shaped cells (Bracken et al. 1985). Decreases in the incorporation of thymidine into DNA and leucine into proteins were also reported at toxic levels of vanadate, with leucine incorporation appearing to be more sensitive to the actions of vanadate (Bracken and Sharma 1985). Paradoxically, increased cellular levels of DNA and protein were found. Possible explanations given for this were decreased degradation rates of DNA and protein, decreased rates of cell division and/or decreased precursor transport.

A decreased rate of exogenous and endogenous protein degradation was reported in rat hepatocytes after 30 minutes incubation in 10 mM vanadate (Seglen and Gordon 1981). This was thought to be due to an inhibition of lysosomal cathepsins and protein synthesis was not affected under these conditions. No effects on cell viability were seen until after a 2 hour incubation in 20 mM vanadate. At the same concentrations of

vanadate as those producing an inhibition of protein degradation, changes in the cellular morphology of hepatocytes were also seen which were similar to those caused by cytochalasin, implicating an action of vanadate upon cytoskeletal elements (Seglen and Gordon 1981).

Shape changes in erythrocytes have also been described following incubation in 20 mM vanadate for 2 hours at 25°C. More than 80% of the cells assumed equinocytic forms (Vives-Corrans et al. 1981). The osmotic fragility and deformability of these cells was also reduced. As the lateral mobility of erythrocyte membrane proteins has been shown to be modified by polyphosphates such as 2,3-DPG, ATP and GTP, it was postulated that vanadate may be affecting either the levels or regulatory action of these compounds, resulting in changes in the membrane skeletal organisation and hence the changes in shape and physical properties of the cells.

Inhibition of saltatory organelle movement in permeabilised fibroblasts has also been seen at vanadate levels from 10 μ M to 1 mM (Forman 1982). At similar levels, chromosome movement was inhibited in lysed mitotic PtK₁ cells with no effect on microtubule polymerisation (Cande and Wolniak 1978). Both these effects were postulated as being due to an action of vanadate on a dynein-like molecule.

A mitogenic action of vanadate has been reported in quiescent human fibroblasts with an increase in cell numbers of 61% in the presence of 4 μ M vanadate for 48 hours, compared with an increase of only 9% in the absence of vanadate (Carpenter 1981). Toxicity was seen at vanadate concentrations greater than 40 μ M.

Vanadate also potentiated the mitogenic effect of EGF in these cells as assessed by the increased incorporation of thymidine into DNA (although total DNA levels were not measured), and at concentrations of 2 - 10 μM potentiated the mitogenic action of insulin on mammary gland explants from mice but inhibited that of lithium (Hori and Oka 1980). Vanadate by itself was only slightly mitogenic in the latter tissue. It was postulated that the stimulatory effect of insulin on cell division involves an ion-sensitive process and that vanadate and lithium act at some regulatory site in this process, possibly an ATPase molecule.

It is evident from the above examples that in a variety of cell types, vanadate is capable of affecting many processes involved in cell division and growth, each of which could cause indirect effects upon ion transport in addition to any direct effects which may be present.

2.I.iii. Intracellular reduction of vanadate

Any investigation of the cellular effects of vanadate is complicated by its intracellular reduction to the vanadyl ion. In erythrocytes, uptake of vanadate was found to occur in two distinct phases; a rapid initial uptake lasting 15-30 minutes ($t_{0.5} = 4$ minutes) at an extracellular vanadate concentration of 250 μM , corresponding to chemical equilibration through the anion exchange pathway, and a slower intracellular concentration of vanadium by an energy-dependent uptake ($t_{0.5} = 90-130$ mins). This has been shown to represent the intracellular reduction of V^{5+} to V^{4+} and the subsequent stabilisation of the vanadium in this form by binding to haemoglobin (Cantley, Jr. et al. 1978b,

Cantley, Jr. and Aisen 1979, Macara et al. 1980).

The reduction of vanadate in erythrocytes is thought to be non-enzymatic and due to glutathione (GSH) rather than NADH, as the rate of reduction by NADH is much slower. However a vanadate-stimulated NADH oxidase has been shown to be present in mouse liver plasma membranes and pig erythrocyte membranes (Ramasarma et al. 1981) so it is possible that reduction may be due to a combination of enzymatic and non-enzymatic mechanisms. Intracellular reduction of vanadate has also been described in rat adipocytes with binding of the resultant vanadyl to GSH (Degani et al. 1981). Again it was not clear whether reduction was enzymatic or non-enzymatic.

Such a reduction would explain the resistance of the Na^+/K^+ -ATPase in intact cells to the inhibitory actions of vanadate as already discussed in Chapter 1. Vanadyl is a much less powerful inhibitor of this enzyme unless a highly purified membrane preparation of the enzyme is used (North and Post 1984), although the reasons for this are not known. Unlike vanadate, vanadyl can be detected using electron spin resonance (ESR). Following a ligand binding study and theoretical consideration of cellular vanadium accumulation, Nechay et al. (1986) calculated that in most cell types, less than 1% of intracellular VO^{2+} is free, with the intracellular binding accounting for the vanadium accumulation. Spontaneous oxidation of the vanadyl to vanadate would be prevented by the binding. By assuming that 90% of the plasma VO_3^- was bound to proteins, Nechay et al. (1986) calculated that the free plasma concentration of VO_3^- would be approximately 10^{-9}M , resulting in an intracellular concentration

of approximately $3 \times 10^{-11} \text{M}$ in the absence of active transport (from Nernstian principles - Nechay et al. 1986). Because of the weaker intracellular binding of VO_3^- compared with that of VO^{2+} , the equilibrium favours reduction and subsequent binding of the vanadyl. The free concentrations of both vanadyl and vanadate would thus be below those reported to have any effects upon the Na^+/K^+ -ATPase molecule under optimum conditions and would therefore account for the resistance of intact cells to the inhibitory actions of vanadate seen in membrane preparations. From the above discussion it can be appreciated that it is difficult to establish whether any effects seen in vanadate treated cells are due to vanadate, vanadyl or to a depletion of e.g. NADH or GSH.

As previously mentioned vanadyl ions, unlike vanadate ions, can be detected using ESR. A simplified view of the principles of ESR is given below. A more detailed treatment can be found in several textbooks such as those by Ayscough (1967), Gerson (1970), Banwell (1972), and Straughan and Walker (1976).

Electrons in atoms and molecules move around the nucleus in fixed orbitals and in addition have their own intrinsic spin. Any such spinning or rotating particles act as magnetic dipoles and tend to align themselves in the direction of an applied magnetic field. This occurs in molecules and atoms with either an unpaired electron e.g. NO_2 , or two electrons with parallel spins e.g. O_2 , which are said to be paramagnetic. It does not occur in molecules and atoms which have electrons in closed shells or associated in pairs with opposing spins so that their magnetic dipoles cancel. These are said to be diamagnetic.

There are two possible orientations of the unpaired electrons, either parallel or anti-parallel to the inductive magnetic field. Each orientation represents a discrete energy level. Transitions or resonance between these levels (changes in spin state) may be induced by interaction of the magnetic dipole of the unpaired electrons with an oscillating magnetic field accompanying electromagnetic radiation exerted perpendicularly to an inductive magnetic field. These transitions can be studied using ESR. Resonance conditions are dependent upon both the frequency of the electromagnetic radiation and the strength of the inductive magnetic field. These parameters can be related to one another in a simple equation, hence either one may be kept constant while the other is varied in order to establish the resonance condition. Technically the magnetic field strength is the parameter which is usually varied.

Generally ESR is used for examining the electronic structure of molecules, particularly organic ones. However it can also be used quantitatively. In ESR spectroscopy, microwave radiation of constant frequency (usually 9000 - 10000 MHz corresponding to a wavelength of approximately 3 cm i.e. in the Microwave X-band region), is transmitted via copper tubing to the sample cavity where energy is concentrated on the sample by multiple reflections from the cavity walls. Electromagnets are located on either side of the cavity so that the steady inductive magnetic field and the oscillating field of the electromagnetic radiation are mutually perpendicular. The strength of the steady field is normally in the range of 3000 - 3500 gauss for the resonance condition to be established in most samples at the

electromagnetic radiation frequencies cited previously. A crystal detector measures the intensity of the energy absorption by the sample - this is increased as the magnetic field strength approaches the value needed for resonance when unpaired electrons move to a higher energy level absorbing energy in the process. Energy is emitted if transition of the electrons is to a lower energy level as occurs during relaxation. The magnitude of the radiation absorbed is proportional to the electron population difference between the two energy states. At a particular wavelength and constant temperature, the population difference between the energy states is a constant proportion of the total number of unpaired electrons present. Hence it is possible to relate the magnitude of the ESR signal to the concentration of the sample. Normally the resultant plot is the derivative one as this is technically better i.e. the rate of change of absorption intensity with respect to the change in field strength (y axis) against the field strength (x axis).

The complexity of an ESR spectrum (its hyperfine structure), is determined by interactions between unpaired electrons and the magnetic nucleus and is characteristic for each substance. Vanadium in the +5 oxidation state, for example as the metavanadate ion VO_3^- , is diamagnetic and does not produce an ESR spectrum whereas vanadium in the +4 oxidation state, for example as the vanadyl ion VO^{2+} , is paramagnetic. The spectrum of VO^{2+} is hyperfine with 8 bands of absorption or hyperfine lines each separated by 120 gauss. Different spectra are produced depending upon whether the vanadyl is bound or free unless analysis is carried out at 77°K where the same signal is

produced. An alternative method is to release the protein-bound vanadyl using acid. This also has the added advantage of reversing any hydrolysis which may have occurred, as vanadyl hydroxide which forms at neutral pH is not detectable by ESR. This is due to the fact that vanadium in the +4 oxidation state requires a non-cubic field for observation by ESR, such as that found in square pyramidal vanadyl type complexes (Sakurai et al. 1980). Vanadium in the +3 oxidation state is also paramagnetic but a spectrum is not observable due to an internal electric field effect (Sakurai et al. 1980).

2.II. MATERIALS AND METHODS

2.II.i. Reagents

Reagents used were purchased from the following:-

Aldrich Chemical Company Ltd. (Gillingham, Dorset):- Na_3VO_4
(99% pure).

BDH Chemicals (Poole, Dorset):- Concentrated HCl (Aristar grade) and EDTA (Analar grade).

Coulter Electronics Ltd. (Luton, Beds.): - Isoton.

Edinburgh Cameras (Edinburgh, Scotland):- Ilford FP4, EM and Pan F black and white films.

EMscope Laboratories Ltd. (London):- all electron microscopy supplies as detailed in Section 2.II.v..

Flow Laboratories (Rickmansworth, Herts.): - HeLa cells - originally purchased in 1974 and subcultured and frozen at intervals with stocks maintained in liquid nitrogen.

Fluka (Switzerland):- $\text{VOSO}_4 \cdot 5\text{H}_2\text{O}$ (98% pure).

Gibco-Biocult (Paisley, Scotland):- Basal Medium Eagle (BME) tissue culture medium with Earle's salts, new-born calf serum, L-glutamine (200 mM), kanamycin (10mg/ml), Earle's balanced salt solution (EBSS - Ca and Mg free), trypsin (2.5% w/v), and "Nunclon" tissue culture plates (5 cm and 9 cm diameter).

Sigma (Poole, Dorset):- Ascorbic acid.

Water used for solutions was produced using a Milli-Q water system (Millipore S.A., France). All solutions were dispensed using Gilson adjustable pipettes during experiments.

2.II.ii. Sub-culturing of HeLa cells

All routine tissue culture procedures were carried out using standard aseptic techniques in a "Gelaire Class 100" laminar air flow cabinet (Gelman Instruments). All sterile glassware was autoclaved at 121°C (15 psi) before use. Medium was made from powdered stocks, sterilised by passage through a 0.22 µm Millipore filter and stored at 4°C in sterile bottles. HeLa cells were normally grown in BME with Earle's salts, supplemented with 10% (v/v) new-born calf serum, 2 mM glutamine and 50 µg/ml kanamycin (See Table 2.1.). All solutions were warmed to 37°C before use.

HeLa cells were normally maintained in a 120 glass Roux bottle i.e. a Roux with 120 cm² of flat growth surface. To subculture cells from an existing monolayer, the growth medium was poured off and the cells rinsed with approximately 4.5 mls of trypsin solution (0.25% w/v in EBSS - Ca and Mg free - see Table 2.2.). 5 mls of the trypsin solution were then added to the bottle which was sealed and incubated at 37°C for 5-10 minutes until the cells were detached from the glass surface. The trypsin solution was then neutralised with the addition of 45 mls of complete BME to the bottle, and a single cell suspension was prepared by the repeated syringing or "blasting" of the suspension through a wide-bore (1-2 mm) needle. This procedure does not affect the integrity of the cells. A 1 ml aliquot of the suspension was then added to 19 mls of Isoton and the cell number, cell volume and water content determined using a Coulter Counter Model ZF with Channelyzer (C1000), interfaced to a Sharp MZ-80K personal computer. Intracellular water was computed by assuming that 80%

Table 2.1. Composition of Basal Medium Eagle (BME) tissue culture medium with Earle's salts

<u>Inorganic Salts</u>	<u>mg/l</u>
CaCl ₂ (anhyd.)	200.00
KCl	400.00
MgSO ₄ (anhyd.)	97.67
NaCl	6800.00
NaHCO ₃	2250.00
NaH ₂ PO ₄ ·H ₂ O	140.00
<u>Other Components</u>	
D-Glucose	1000.00
Phenol Red	6.00
Sodium Succinate	100.00
Succinic Acid	75.00
<u>Amino Acids</u>	
L-Arginine (HCl)	21.00
L-Cystine (2HCl)	15.65
L-Histidine HCl (H ₂ O)	15.00
L-Isoleucine	26.00
L-Leucine	26.00
L-Lysine HCl	36.47
L-Methionine	7.50
L-Phenylalanine	16.50
L-Threonine	24.00
L-Tryptophan	4.00
L-Tyrosine	18.00
L-Valine	23.50
<u>Vitamins</u>	
Biotin	1.00
D-Ca Pantothenate	1.00
Choline Bitartrate	1.80
Folic Acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

In addition, the medium was supplemented with 10% (v/v) new-born calf serum, 2mM L-glutamine and 50 ug/ml kanamycin before use.

Table 2.2. Composition of calcium and magnesium-free Earle's
Balanced Salt Solution (EBSS)

<u>Inorganic Salts</u>	<u>mg/l</u>
NaCl	6800.00
KCl	400.00
NaH ₂ PO ₄ ·2H ₂ O	158.00
NaHCO ₃	2200.00
<u>Other Components</u>	
Glucose	1000.00
Phenol Red	10.00

of the cell volume is water (Lamb and McCall 1972). This method of measuring cell volume assumes that the volume is not affected by trypsinisation.

An aliquot containing 5×10^6 cells was taken from the cell suspension and added to 100-125 mls of fresh BME in a clean Roux. This was equilibrated with a 95% air/5% CO₂ mixture, sealed and placed in the 37°C hot room. The remaining cell suspension was discarded or used for plating as described later. Usually after about 5 days growth such a Roux would be confluent and ready for sub-culturing (splitting) as above, with a normal yield of 40-50 $\times 10^6$ cells.

2.II.iii. Plating of HeLa cells

After a cell suspension had been prepared as described in the previous section, an appropriate amount was taken and further diluted with BME to give a density of 0.11×10^6 cells/ml. This was dispensed in 3 ml aliquots into 5 cm diameter sterile plastic "Nunclon" tissue culture plates. The plates were then stacked in plastic boxes, equilibrated with 95% air/5% CO₂ mixture, sealed and incubated at 37°C. Normally, for experiments in this study, the medium was replaced on day 3 (day 0 = the day of plating) with fresh medium \pm vanadate (10^{-9} M- 10^{-4} M), minimising any disruption to the cell monolayer. The plates appeared to be nearly confluent (viewed on an Olympus CK inverted microscope) when used in an experiment 24 hours after the medium change. Vanadate-containing medium was prepared by dissolving Na₃VO₄ in an appropriate volume of BME to yield a 10^{-3} M solution. Lower concentrations were prepared by serial dilutions of this stock

solution and usually stored for 24 hours before use in order to minimise the formation of decavanadate ions.

2.II.iv. Effect of 24 hours growth in vanadate upon HeLa cell numbers

HeLa cells were plated out on 5 cm plates as described in the previous section at 0.33×10^6 cells/plate (day 0). On day 3, the plates were numbered and the medium was replaced with fresh medium \pm Na_3VO_4 (10^{-9}M - 10^{-4}M), each concentration in triplicate. The plates were then incubated for a further 24 hours at the end of which time they were transferred to a bench-top incubator at 37°C . For each plate in turn the medium was aspirated off, the cells washed 4x with Krebs solution (pH 7.4 at 37°C , see Table 2.3.), and 1 ml of trypsin solution (0.25% w/v in EBSS - Ca and Mg free) containing 2 mM EDTA was added. The plates were then replaced on the incubator until the cells had detached whereupon the trypsin was neutralised by addition of 2 mls of Krebs solution. The cell suspension was then blasted through a wide-bore needle producing a single-cell suspension, 1 ml of which was taken and added to 19 mls of Isoton for the determination of cell numbers as previously described. Using the appropriate dilution factors etc., the total cell number/plate, the mean cell volume and the total plate water were determined. The experiment was repeated several times and the results analysed by Student's t-test (unpaired) using the "Minitab" statistical package on the VAX mainframe computer. This experiment was also carried out with incubation times of 3, 6 and 12 hours in vanadate-containing growth medium.

Table 2.3. Composition of Krebs solution

<u>Component</u>	<u>mmoles/l</u>
NaCl	140.0
KCl	5.4
MgSO ₄ ·7H ₂ O	1.2
NaH ₂ PO ₄	0.3
KH ₂ PO ₄	0.3
CaCl ₂	2.8
HCl	12.0
Tris Base	13.7
Glucose	11.1

In addition the Krebs solution was supplemented with 1% (v/v) new-born calf serum. The pH was adjusted to 7.4 at 37°C.

2.II.v. Effect of 24 hours growth in vanadate upon the morphology of HeLa cells

HeLa cells were plated out as described in Section 2.II.iii. and a sterile 1.5 x 1.5 cm glass coverslip was placed in the base of each plate (day 0). The cells were then grown until day 3 when the medium was changed to BME \pm Na₃VO₄ (10^{-9} M- 10^{-4} M) as previously described, and then grown for a further 24 hours. Following this, each coverslip was carefully removed from the plate, washed in Krebs solution, and placed cell-side down on a drop of Krebs solution on a clean labelled glass microscope slide. The edges of the coverslips were sealed with nail varnish to prevent evaporation and the cells were examined using a Leitz Dialux 20 microscope with camera attachment, an objective lens of x40 and x12.5 eyepiece lenses. Photographs were taken using Ilford Pan F black and white film.

Transmission and scanning electron micrographs of HeLa cells are also included in the "Results" section of this chapter to illustrate the normal ultrastructure and morphology of HeLa cells. Specimen preparation is only outlined here but further details can be found in standard electron microscopy textbooks.

Briefly, for transmission electron microscopy, cells were grown on plates as previously described, trypsinised and a single cell suspension prepared. This was fixed in gluteraldehyde, osmicated, dehydrated in a series of alcohols and embedded in Epon (Agar resin). Ultrathin (approx. 80 nm thick) sections were cut using a Reichert OM.U2 ultramicrotome, collected on copper grids, double stained with uranyl acetate and lead citrate, and examined at magnifications ranging from x1900 to

x91000 on a Philips 301 electron microscope at 60 kV. Photographs were taken on Ilford EM film using the camera incorporated in the microscope.

For scanning electron microscopy, cells were grown on glass coverslips as previously described, fixed with gluteraldehyde, osmicated, dehydrated in alcohols and then transferred to acetone. After critical point drying, the samples were coated with a thin layer (200 Å) of gold in an Edwards High Vacuum Coater and viewed in a Jeol JSM 35-CF scanning electron microscope at magnifications ranging from x500 to x1500 at 15 kV. Photographs were taken using a 35 mm camera attachment and Ilford FP4 black and white film.

2.II.vi. Measurement of the intracellular reduction of vanadate to vanadyl in HeLa cells

The measurement of total vanadium present in the cells may have been possible using neutron activation analysis or atomic absorption spectroscopy but the facilities were not available to us, and the cost of radiolabelled vanadium together with its short half-life prohibited radioisotopic uptake experiments. For these reasons it was decided to measure the intracellular V^{4+} using ESR, reduce any V^{5+} present using ascorbic acid and measure the resultant V^{4+} concentration. By a suitable subtraction the amount of vanadium present in the +5 oxidation state could then be determined.

Preliminary studies were carried out using absorption spectroscopy, in order to evaluate the stability of solutions of vanadate and vanadyl solutions under various conditions. This

study was undertaken in order to find a suitable extraction procedure which would not only precipitate the proteins but would also maintain the true intracellular oxidation states of vanadium during extraction. The method used was based on that of Fitzgerald and Chasteen (1974) which involved an extraction using hydrochloric acid in which the vanadyl ion is stable. The stability of vanadate under these conditions was not detailed by Fitzgerald and Chasteen (1974) but was investigated here. In addition it was proposed to quantify the reduction of vanadate by ascorbic acid under these conditions. Wherever possible, new disposable plastic utensils were used in order to minimise contamination.

Vanadyl-containing solutions were prepared by dissolving vanadyl sulphate (VOSO_4) in 1M HCl to form a 10^{-2}M solution which was then serially diluted as required. Stability and concentration of these solutions was monitored by absorption spectroscopy using a Cecil CE 393 Digital Grating Spectrophotometer (Series 2) and a molar extinction coefficient of 17.6 at 750 nm (Chasteen et al. 1973). Absorbance was monitored over a 24 hour period with the samples being stored at 4°C between readings. Vanadate-containing solutions were also prepared by dissolving sodium orthovanadate (Na_3VO_4) in 1M HCl to form a 10^{-2}M solution and serially diluting this stock as necessary. The absorbance of these solutions was also measured at 750 nm where V^{5+} ions show no absorbance; hence any absorbance would be most likely indicative of reduction of the V^{5+} ions to V^{4+} ions under the acidic conditions employed here. A solution of 10^{-2}M Na_3VO_4 in 1M HCl containing 5 mM ascorbic

acid was also prepared and the absorbance monitored at 750 nm in order to check to what extent the ascorbic acid reduced the vanadate to vanadyl ions.

A suspension of HeLa cells was prepared from a Roux as described previously and diluted to 0.07×10^6 cells/ml with fresh BME. This suspension was dispensed in 18 ml aliquots onto 9 cm diameter sterile plastic "Nunclon" tissue culture plates which were then incubated at 37°C (day 0). This seeding density of 1.3×10^6 cells/9 cm plate was found to yield plates with the same degree of confluency as the 5 cm plates seeded at 0.33×10^6 cells/plate used in the earlier experiments, but a smaller extraction volume could be used on the 9 cm plates thus minimising dilution of intracellular contents.

On day 3, the medium was replaced with fresh medium $\pm \text{Na}_3\text{VO}_4$ (10^{-9}M - 10^{-4}M) with 9 plates for each condition. Twenty-four hours later, the medium was rapidly aspirated from 3 plates per condition, the plates washed 4x with ice-cold Krebs solution (pH 7.4 at 4°C), placed on ice and immediately 750 ul of 1M HCl (4°C) was added to each plate. The plates were swirled at intervals to ensure constant coverage. After 30 minutes, the contents of each extracted plate were removed using pasteur pipettes and placed in disposable plastic test-tubes. These were centrifuged in a Fisons MSE Coolspin for 15 minutes at 3,000 rpm, 4°C, the supernatants pooled within each treatment condition in order to obtain a suitable volume for analysis and the resultant samples were stored on ice until analysed using ESR, 2-3 hours later. Blanks were prepared by washing plates which had contained 10^{-4}M vanadate solution but no cells and extracting as above.

For each treatment condition, 3 plates of cells were also extracted as above but using 1M HCl containing 5 mM ascorbic acid as the extractant. The remaining 3 plates per treatment condition were used to ascertain cell numbers as described previously with the exception that 2 mls of the trypsin solution was used per plate and this was neutralised with 6 mls of Krebs. 200 ul of the resultant single cell suspension was added to 19.8 mls of Isoton for the determination of cell numbers.

Optimal conditions for ESR analysis were established using the standardised VOSO_4 solutions prepared previously, both in 1M HCl and in 1M HCl containing 5mM ascorbic acid to check for any possible interference. Samples were run in triplicate (by Dr. J. Walton of the University Chemistry Department) on a Bruker ER 200D system - the operating conditions were as given in the appropriate figure legends in the following "Results" section. The signal from a ruby mounted in the sample chamber was used as an internal standard, and the results were normalised both with respect to this signal and to the gain used for each experiment.

Samples were also run which were from blank plates containing medium plus 10^{-4} Na_3VO_4 which had been incubated for 24 hours as if cells were present and the medium then acidified - this was to check that the extracellular vanadate was not being reduced during the incubation period. Also plates of cells not previously treated with vanadate were extracted with 1M HCl containing 10^{-4} M Na_3VO_4 in order to see if any non-enzymatic reduction of the vanadate occurred during the extraction process. A similar procedure was carried out with 1M HCl containing 10^{-4} M VOSO_4 . By comparison of the signal from this sample with that

from a 10^{-4} M VOSO_4 solution containing no cell precipitate, any oxidation of the vanadyl ions occurring due to e.g. cellular metabolites could be determined, together with any interference of the ESR signal by soluble cellular products and/or adsorbance of vanadyl to the protein precipitate.

As the actual ESR analysis was carried out courtesy of another department and maximum use had to be made of the day available to us, it was not possible to establish optimum conditions for analysis before preparing and running the biological samples. One previous run had been carried out using a standard solution in order to check that detection of the vanadyl ion was possible with the reported settings and the equipment available.

2.III. RESULTS

2.III.i. Effects of 24 hours growth in vanadate upon HeLa cell numbers

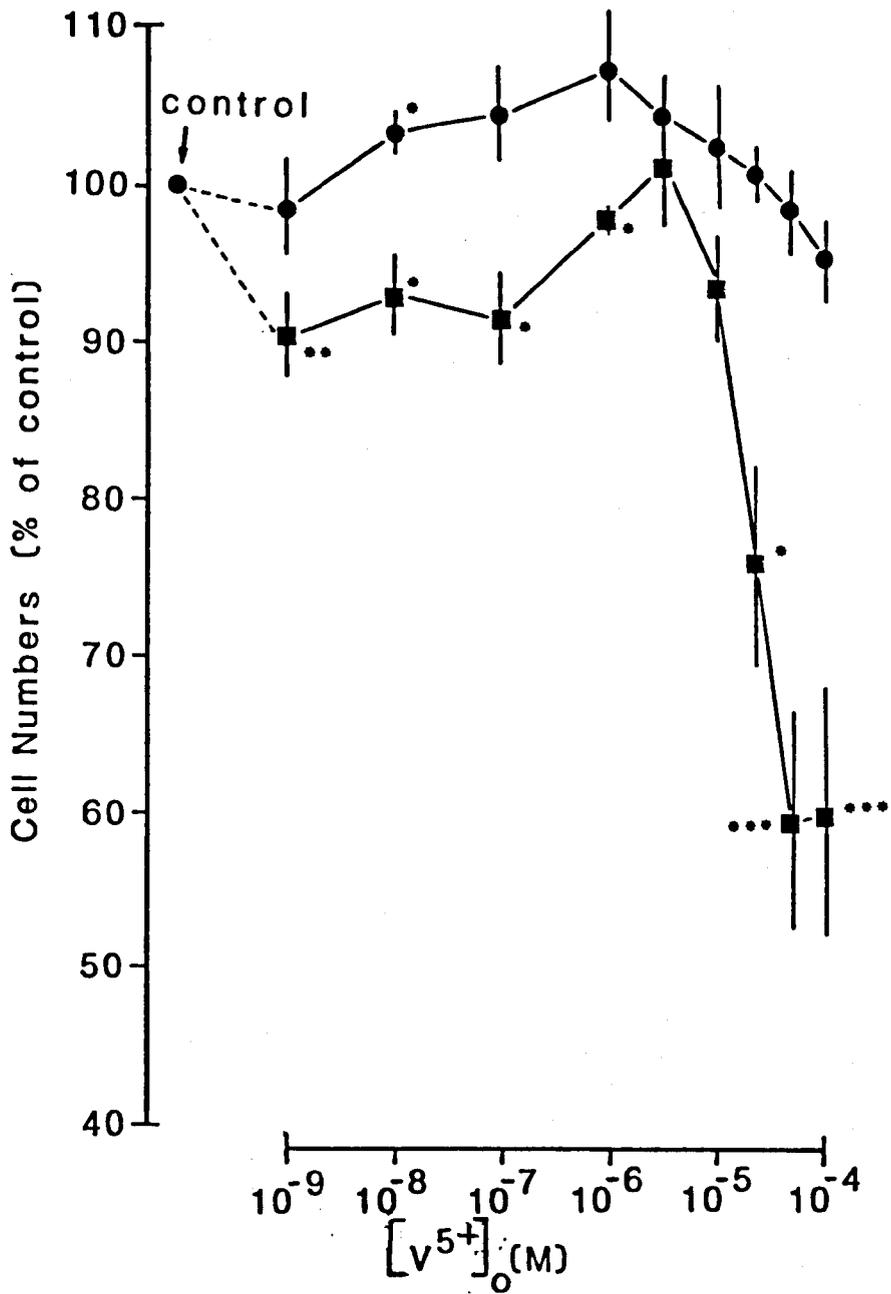
Incubation in vanadate-containing medium (10^{-9}M - 10^{-4}M) produced no significant effects upon HeLa cell numbers with incubation periods of 3 or 6 hours (results not shown). However after 12 hours growth in vanadate, a very slight but significant increase (3.4%) in cell numbers was seen at a vanadate concentration of 10^{-8}M , together with further slight but non-significant increases at 10^{-7}M and 10^{-6}M (Figure 2.1. and Table 2.4.). At vanadate concentrations greater than 10^{-6}M , cell numbers tended to decrease down to and marginally below control values, although non-significantly.

After 24 hours growth in vanadate, HeLa cell numbers were decreased significantly (less than 10% decrease) at vanadate concentrations from 10^{-9}M to 10^{-6}M (Figure 2.1. and Table 2.5.) in a dose-independent fashion, increasing back to 1.3% above control values at $3.2 \times 10^{-6}\text{M}$ vanadate. Dose-dependent decreases in cell numbers were seen at vanadate concentrations above $3.2 \times 10^{-6}\text{M}$ with cell numbers decreasing to less than 60% of control values at $4.6 \times 10^{-5}\text{M}$ vanadate. No further decrease in cell numbers was seen at 10^{-4}M vanadate.

2.III.ii. Effects of 24 hours growth in vanadate upon the morphology of HeLa cells

Transmission and scanning electron micrographs of HeLa cells are shown in Figures 2.2. and 2.3. respectively, illustrating the

- p < 0.05
- p < 0.01
- p < 0.005



●-● 12 hour incubation ■-■ 24 hour incubation

Figure 2.1. Effects on HeLa cell numbers of incubation in vanadate-containing medium for 12 or 24 hours

Each point represents the mean \pm S.E.M. of 3 experiments (24 hours) or 4 experiments (12 hours), each condition within each experiment being performed in triplicate.

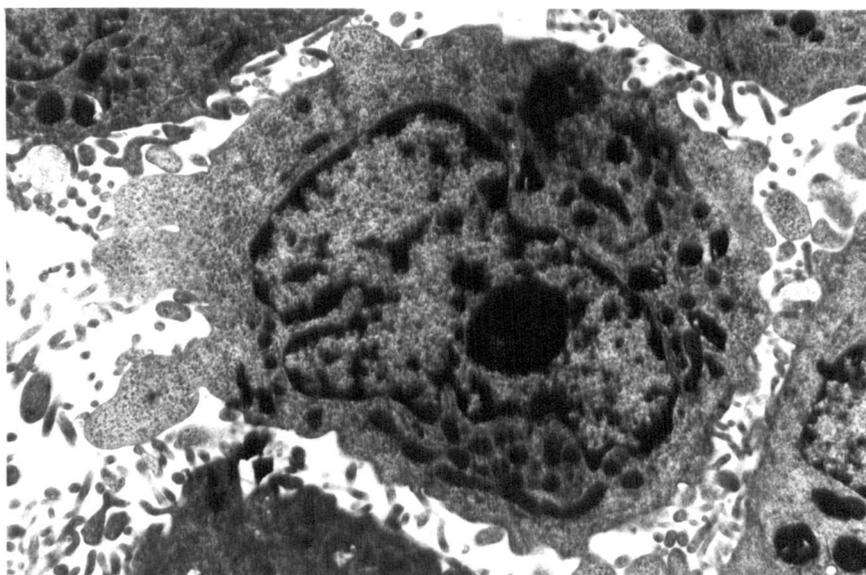


Figure 2.2. Transmission electron micrograph of a HeLa cell

2um

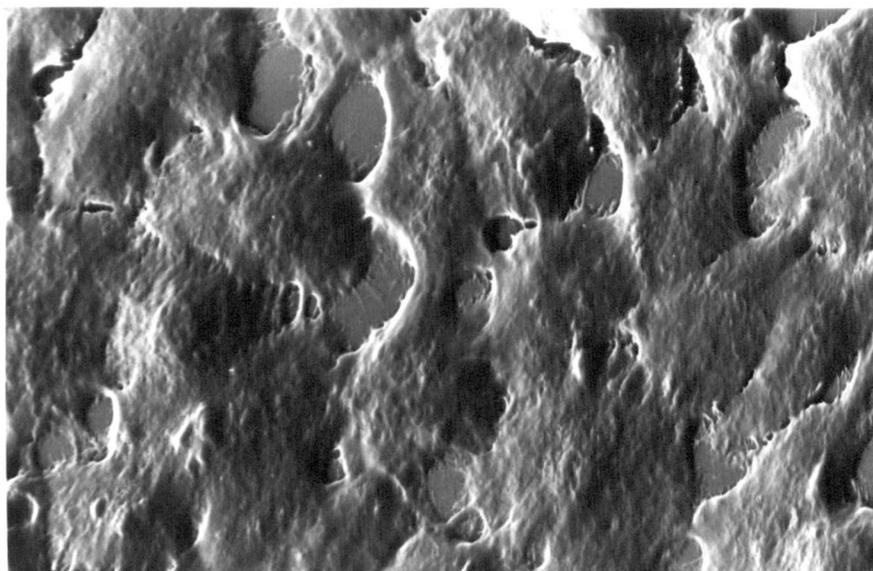


Figure 2.3. Scanning electron micrograph of a HeLa cell
monolayer

10 um

Table 2.4. Effect of 12 hours growth in vanadate on HeLa cell numbers

The values below represent the mean \pm S.E.M. of 4 experiments with each condition being triplicated within each experiment. Significance testing is by two-way analysis of variance.

$[V^{5+}]_0$ (M)	Mean cell number \pm S.E.M. (% of control)	Level of Significance
0	100.0 \pm 0	-
10^{-9}	98.7 \pm 1.7	N.S.
10^{-8}	103.4 \pm 1.2	$p < 0.05$
10^{-7}	104.7 \pm 3.0	N.S.
10^{-6}	107.6 \pm 3.7	N.S.
3.2×10^{-6}	104.3 \pm 2.6	N.S.
10^{-5}	102.6 \pm 3.9	N.S.
2.1×10^{-5}	100.8 \pm 1.4	N.S.
4.6×10^{-5}	98.7 \pm 2.3	N.S.
10^{-4}	95.6 \pm 2.3	N.S.

Table 2.5. Effect of 24 hours growth in vanadate on HeLa cell numbers

The values below represent the mean \pm S.E.M. of 3 experiments, with each condition being triplicated within each experiment. Significance testing is by two-way analysis of variance.

$[V^{5+}]_0$ (M)	Mean cell number \pm S.E.M. (% of control)	Level of Significance
0	100.0 \pm 0	-
10 ⁻⁹	90.6 \pm 2.4	p < 0.01
10 ⁻⁸	92.8 \pm 2.3	p < 0.05
10 ⁻⁷	91.4 \pm 3.0	p < 0.05
10 ⁻⁶	97.9 \pm 0.6	p < 0.05
3.2 x 10 ⁻⁶	101.3 \pm 3.4	N.S.
10 ⁻⁵	93.5 \pm 3.3	N.S.
2.1 x 10 ⁻⁵	76.1 \pm 6.4	p < 0.05
4.6 x 10 ⁻⁵	59.4 \pm 7.1	p < 0.005
10 ⁻⁴	60.2 \pm 7.9	p < 0.005

general appearance of the cells. The transmission electron micrograph shown in Figure 2.2. illustrates the irregular shape of the cells showing numerous cytoplasmic protrusions. The cell shown has a large lobulated nucleus with a prominent nucleolus and densely staining chromatin around the nuclear periphery. Often HeLa cells are multinucleate (not shown). Numerous mitochondria are present with few cristae. Arrays of endoplasmic reticulum can also be seen in the cytoplasm together with densely staining multilamellar bodies. Upon examination at higher magnification these appeared to be membrane-bound and contained a circular array of membrane-like strands bordering a central amorphous zone, also densely staining. The function of these structures is not known unless possibly lysosomal-related.

The epithelial sheet-like appearance of the HeLa cells can be seen in the scanning electron micrograph, showing the numerous intercellular connections. In the more confluent areas the divisions between individual cells are not entirely clear. The electron micrographs were taken to illustrate the general appearance of the cells. The effect of vanadate on cell structure was only examined at the light microscope level.

Under the light microscope the sheet-like appearance is less apparent (Figure 2.4.), possibly due to the fact that the cells are unfixed and, despite precautions, may be undergoing some shrinkage or drying whilst under examination. The control cells appear polygonal in shape with a granular cytoplasm and prominent nuclei. The majority of the cells have multiple nucleoli. After treatment with vanadate (10^{-4} M) for 24 hours, the cells appear to be much less confluent and more rounded (Figure 2.5.),

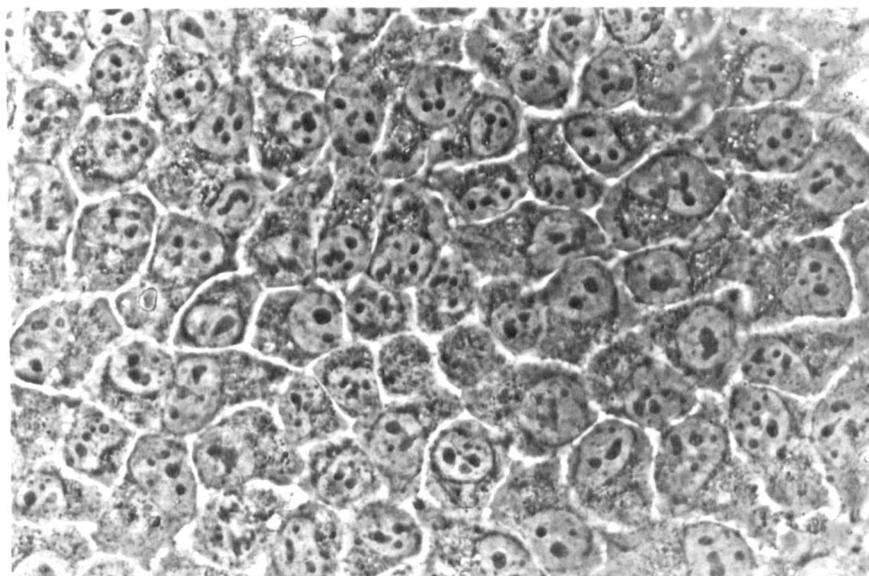


Figure 2.4. Light micrograph of a HeLa cell monolayer

20um

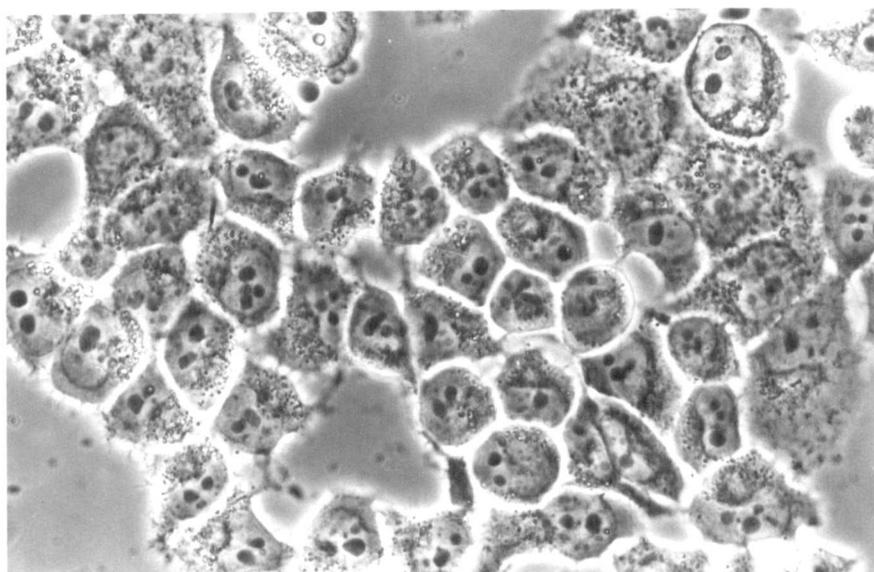


Figure 2.5. Light micrograph of a HeLa cell monolayer after a
24 hour incubation in medium containing 10^{-4} M vanadate

20um

with some spindle-shaped cells also present (not shown in this picture). Refractility is also more evident around the cell peripheries. Such changes in cell appearance were not evident until vanadate concentrations greater than $10^{-5}M$.

2.III.iii. Intracellular reduction of vanadium (+5) to vanadium (+4) in HeLa cells

Standard solutions of $VOSO_4$ in 1M HCl appeared to be stable to oxidation over at least a 24 hour period as evidenced by no change in their absorption at 750 nm. Similarly no reduction of solutions of Na_3VO_4 in 1M HCl was seen over a similar time period, as indicated by a lack of absorption at 750 nm. The addition of 5mM ascorbic acid to the vanadate solutions resulted in a reduction of the vanadium (+5) to vanadium (+4) as monitored by the appearance of a blue vanadyl solution and absorption at 750 nm. Reduction appeared to be incomplete with absorption measurements being approximately 90% of those expected if the total vanadate present had been reduced to vanadyl. Further addition of ascorbic acid produced no additional increase in absorption indicating that the reduction of vanadate was probably complete.

The above discrepancy between the predicted and actual absorption values could not be accounted for by a quenching effect of ascorbic acid as no effect on absorption was seen when ascorbic acid was added to standard vanadyl solutions. Similarly the possible presence of vanadate was shown to have no effect on the absorption of vanadyl solutions. Vanadyl sulphate absorbs water readily and normally has varying amounts of water

associated with it per molecule depending upon the manufacturer. However possible inaccuracies in the concentration of the vanadyl solutions due to variations in water content were also not responsible for the discrepancy as the absorption values for vanadyl sulphate solutions in this study agreed well with the reported molar extinction coefficient (Chasteen et al. 1973) and the water content was checked by drying down a sample. It was also unlikely that the concentrations of the vanadate solutions were inaccurate unless the manufacturer's purity specifications were incorrect, as consistent results were obtained with solutions prepared at various times. It is possible however that the presence of different ligands in the two standard vanadyl solutions prepared either by dissolution of vanadyl sulphate or by reduction of sodium orthovanadate may be responsible for the differences, as different molar extinction coefficients have been reported for VOSO_4 and VOClO_4 solutions for example (Chasteen et al. 1973).

The ESR signal for a 10^{-4}M solution of VOSO_4 in 1M HCl is shown in Figure 2.6.. The characteristic hyperfine structure of vanadyl solutions is shown, with 8 bands at intervals of approximately 120 gauss. It was decided to use the 3rd band for quantitation as it was the largest. However when samples were run on the day of the experiment a few months later, considerable interference with the signal was found as shown in Figure 2.7., a biological sample, necessitating the use of the 2nd band for quantitation. Such interference was seen with either standard or biological samples and was traced to the quartz capillary sample cell used in the analysis, probably due to an imperfection

100 Gauss

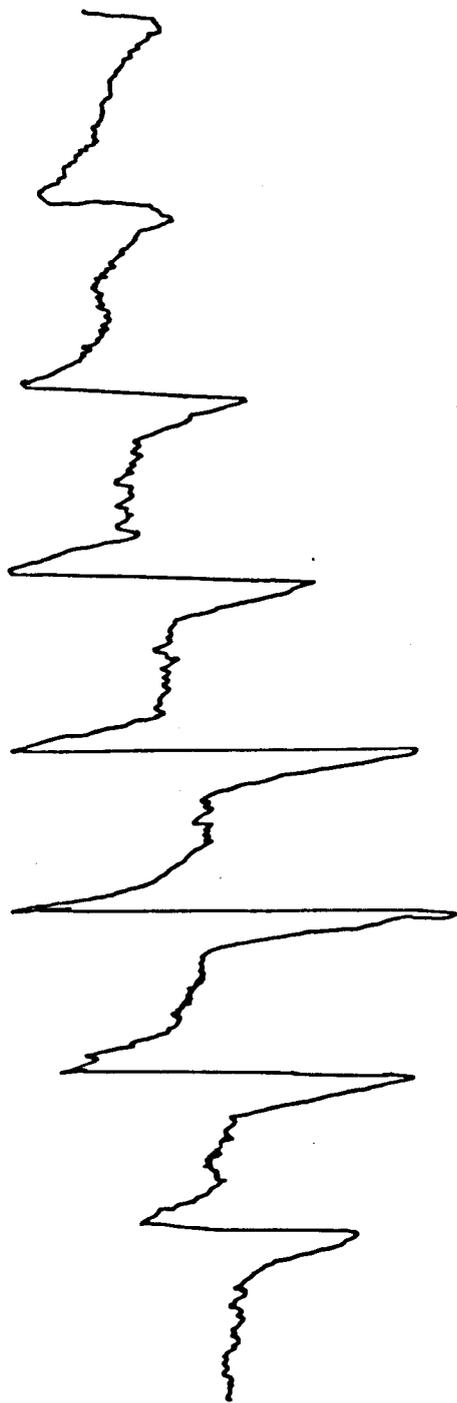


Figure 2.6. ESR spectrum of 10^{-4} M VOSO_4 in 1M HCl

Equipment - Bruker ER 200D, microwave frequency - 9.32 GHz, power - 8 dB, gain - 2.5×10^5 , modulation - 20 Gpp, time constant - 500 msecs, scan time - 1000 secs, field centre - 3330 G, scan - 1000 G, temperature - ambient, cell - quartz capillary. Internal standard height = 7.9 cm.

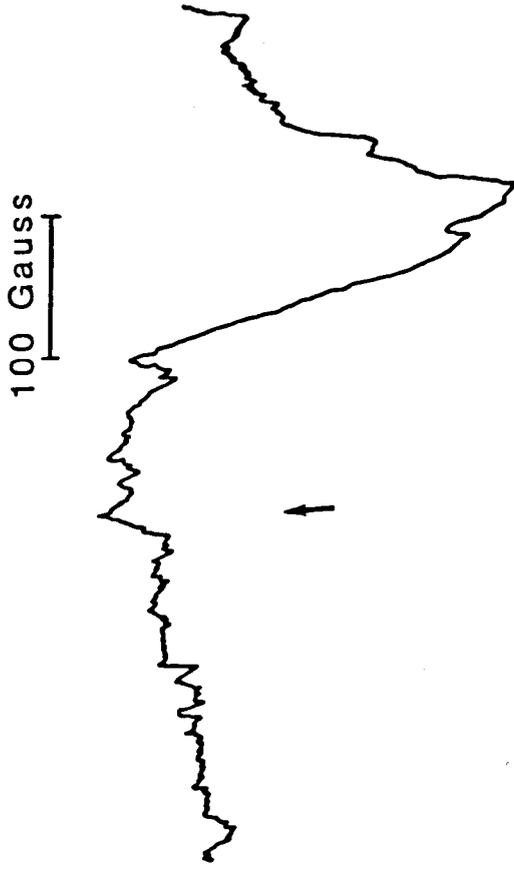


Figure 2.7. ESR spectrum of HeLa cells treated with $10^{-4}M$

Na_3VO_4 for 24 hours and extracted in 1M HCl

Equipment and conditions as described for Figure 2.6..
↑ indicates the 2nd hyperfine band which was used for quantitation. Internal standard height = 7.8 cm.

in the structure. This sample cell was a replacement for the one used in the initial sample run which apparently had been broken in the intervening time period.

The calibration graph for the ESR signal for various concentrations of VO_2^{+} is shown in Figure 2.8. with the lowest detectable vanadyl standard being approximately 5 μM , in agreement with Fitzgerald and Chasteen (1974). Although it was possible to run only a few standards due to the time limitations, the ESR signal over the concentration range used had previously been shown to be linear (Fitzgerald and Chasteen 1974). A regression line through the origin was plotted using the GLIM statistical package on the VAX mainframe computer. Ascorbic acid did not interfere with the signal. The "typical" signal for a biological sample is shown in Figure 2.7., with the hyperfine band used for quantitation indicated, although the signal was magnified further before being measured. No signal was detected from the blanks and the calculated intracellular vanadyl concentrations resulting from incubation of HeLa cells in vanadate-containing medium are given in Table 2.6.. The values reported are per litre of cell water which assumes the vanadyl is free, however some or all of it may be bound as discussed later.

Because of the necessary dilutions involved during extraction, only the cells incubated in the two highest concentrations of vanadate had detectable amounts of vanadyl present. There appears to be no difference between the vanadyl concentrations from cells extracted in 1M HCl and those extracted with 1M HCl containing 5mM ascorbic acid, indicating that all the vanadium present in the cells was in the +4 oxidation state. However it

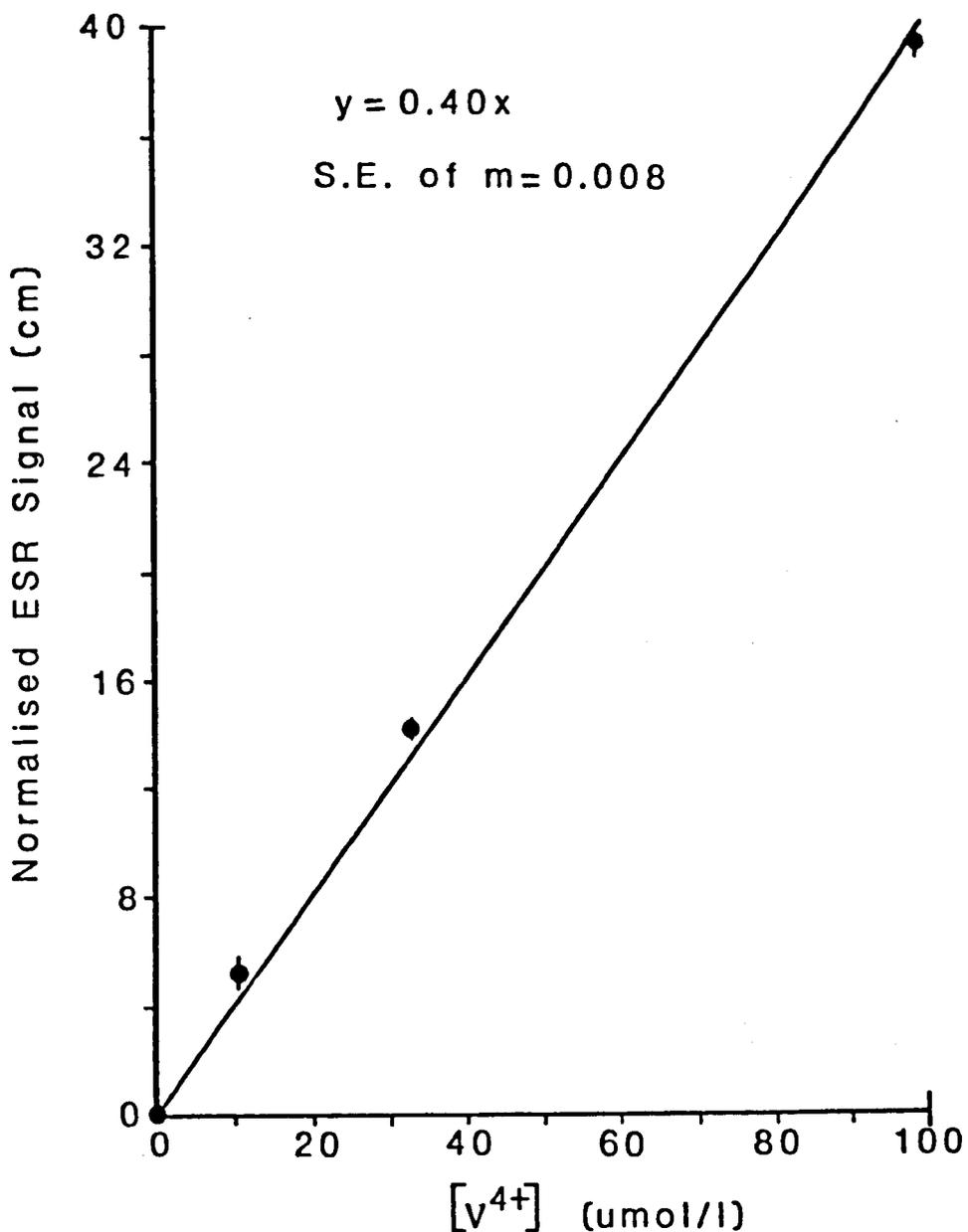


Figure 2.8. Calibration graph of various concentrations of VOSO₄ in 1M HCl against the resultant ESR signal

The 2nd hyperfine band was used for quantitation. Signals were normalised for a gain of 1.6×10^6 and an internal standard height of 10 cm. Equipment and operating conditions as detailed in Figure 2.6. Points are the mean \pm S.E.M. of 2 readings.

Table 2.6. Measurement by ESR of the intracellular concentration of vanadium (+4) in HeLa cells, following 24 hours incubation in vanadium (+5)

The values below represent the mean \pm S.E.M. of the results from 1 experiment, each condition within the experiment being performed in triplicate.

$[V^{5+}]_0$ (M)	Extractant	Mean $[V^{4+}]_i \pm$ S.E.M. (umoles/lcw)
10^{-4}	1M HCl	598 ± 20
10^{-4}	1M HCl/5mM Ascorbic Acid	608 ± 14.7
4.6×10^{-5}	1M HCl	256 ± 0
4.6×10^{-5}	1M HCl/5mM Ascorbic Acid	258 ± 6.6

must be emphasised that this technique would not be sensitive enough to measure any very small concentrations (less than micromolar) of intracellular vanadate which may be present. As previously discussed in Chapter 1, concentrations of vanadate less than 10^{-6} M would presumably be capable of causing some inhibition of e.g. Na^+/K^+ -ATPase, and would not have been detected in this experiment.

In addition the results show that the vanadium is accumulated within the cells at a concentration some 5 to 6 times that of the external medium. At the concentrations of vanadate used here, the vanadium accumulated appears to be proportional to the extracellular concentration i.e. a 2.2x increase in extracellular vanadate concentration resulted in a 2.3x increase in intracellular vanadium concentration.

When the acidified samples from the vanadate-containing medium which had been incubated for 24 hours at 37 °C without cells were analysed, there was too much interference from medium components to detect any vanadyl that may have been present. Hence it was impossible to establish whether or not any of the vanadate was reduced extracellularly before entering the cells. No difference was seen between the signal from a standard solution of VOSO_4 in 1M HCl and the signal resulting from cells extracted with the same solution indicating that no oxidation of vanadyl due to cellular metabolites occurred during the extraction, and/or that vanadyl was not adsorbed to the protein precipitate. However vanadyl was detected in cells extracted with 10^{-4} M Na_3VO_4 in 1M HCl despite the fact that the samples were kept on ice, with approximately 15% of the vanadate present in the 2.25 mls of

sample being reduced after 6 hours (i.e. 34 nmoles with precipitated proteins from 39×10^6 cells). This indicates the presence within the cells of acid-stable substances capable of reducing vanadate non-enzymatically, as protein precipitation and low temperatures would have been expected to have abolished any enzymatic activity present in the intact cells.

2.IV. DISCUSSION

The apparent toxicity of vanadate in HeLa cells after 24 hours incubation appears to be similar to that previously described in MDBK cells (Bracken et al. 1985), both in time of onset and the lowest effective dose. Slight differences were apparent however e.g. a 5x increase in vanadate concentration from $2 \times 10^{-5}M$ to $10^{-4}M$ was paralleled by an increase in cytotoxicity of 1.6x in HeLa cells and 3.2x in MDBK cells. A plateau in response was seen in both MDBK and HeLa cells where further increases in extracellular vanadate produced no further increases in cytotoxicity. This occurred at vanadate concentrations of $2 \times 10^{-4}M$ and $4.6 \times 10^{-5}M$ in MDBK and HeLa cells respectively, with cell toxicity of 60-70% and 40% respectively, in spite of the fact that the vanadium content of both the HeLa and MDBK cells increased further at higher extracellular vanadate levels. Such a plateau was also seen with respect to the vanadate-induced inhibition of acid phosphatase in MDBK cells. It would appear that there is a critical range of intracellular vanadium concentrations below which cellular effects are not apparent, and above which no further change is seen. This critical level is obviously not reached until 12-24 hours in HeLa cells.

Whether the decrease in HeLa cell numbers with vanadate treatment is due to cell death as a result of e.g. alterations in protein synthesis or degradation as has been shown to occur in MDBK cells at similar concentrations (Bracken and Sharma 1985) and in rat hepatocytes at higher concentrations (Seglen and Gordon 1981), or is due to effects on cell division as shown in

lysed mitotic PtK₁ cells (Cande and Wolniak 1978) can not be concluded without further investigation. The slight non-significant stimulation of growth at lower vanadate levels, especially after 12 hours incubation, may be related to the mitogenic actions of vanadate as demonstrated in human fibroblasts (Carpenter 1981). Toxicity was also seen in these cells at similar vanadate concentrations as those producing cytotoxicity in HeLa cells.

The changes in morphology of the HeLa cells occurring concurrently with the cytotoxicity are also similar to those described in MDBK cells (Bracken et al. 1985), although the change in appearance of some cells to a bipolar spindle-type cell may be due to the loss of cell-cell contact, promoting outgrowth of cells to re-establish contact. This can often be seen in less confluent cultures of HeLa cells. The possibility of an involvement of vanadate with cytoskeletal elements or polyphosphates in causing the shape change as has been suggested by Seglen and Gordon (1981) and Vives-Corróns et al. (1981) can not be ruled out however.

The interpretation of the effects of chronic vanadate treatment is complicated by the fact that in HeLa cells, as in many other cell types, vanadate is reduced intracellularly to the vanadyl ion. The time course of reduction in HeLa cells has not been determined. In erythrocytes for example, 250 uM vanadate has been shown to equilibrate across the membrane within 15-30 minutes via the anion-exchange pathway (Cantley, Jr. et al. 1978b). This is followed by a slower intracellular accumulation which is thought to be due to the subsequent intracellular

reduction of the vanadate to vanadyl (Cantley, Jr. et al. 1978b, Cantley, Jr. and Aisen 1979). The vanadyl is then stabilised by binding to proteins, in this case haemoglobin. The rate-limiting step in the accumulation is thought to be the intracellular reduction. In an erythrocyte suspension incubated in 1.14 mM vanadate, more than 90% of the vanadate had been reduced within 23 hours, with an intracellular vanadium concentration of 7.2 mM compared with 0.29 mM extracellularly (Macara et al. 1980). This is approximately 5x greater than the 5-6x accumulation shown in HeLa cells in this study and initial extracellular vanadium concentrations were approximately 10-20x greater than the concentrations used for the HeLa cells. At the much lower vanadium concentrations normally present in man, the ratio of whole blood to serum total vanadium concentration is about 2:1 (Naylor - personal communication). Assuming a haematocrit of approximately 50%, these erythrocytes in vivo have a total vanadium concentration of approximately 3x that of the serum. It would appear that the concentration of vanadium within cells is not necessarily linearly related to the extracellular concentrations, with a proportionately greater accumulation occurring in erythrocytes at least, at higher extracellular concentrations. The converse situation may have been expected in view of the fact that the intracellular reduction of vanadate is thought to be the rate-limiting step in the accumulation and the reducing capability may be exceeded at higher vanadate levels. The accumulation of vanadium in MDBK cells however has been shown to be linearly proportional to the extracellular concentration of vanadate over the range 20-1000 μ M

(Bracken et al. 1985). This was also seen to be the case in HeLa cells at the two vanadate concentrations used in the ESR study. Obviously the uptake of vanadate is complex, probably largely due to the intracellular reduction and subsequent binding of vanadium, and may vary between cell types.

At a concentration of 10^{-4} M vanadate for 24 hours, the vanadium concentration of MDBK cells was approximately 2.5 nmoles/ 10^6 cells, with a cytotoxicity of 50% (Bracken et al. 1985). In HeLa cells the vanadium concentration was approximately 600 umoles/lcw (equivalent to 1.3 nmoles/ 10^6 cells) with a cytotoxicity of 40%. Although the vanadium content is comparable in the two cell types, the volumes of the MDBK cells are not given and hence the intracellular concentrations of vanadium may be different between the two cell types, possibly accounting for any slight differences in cytotoxicity.

In rat adipocytes reduction was faster, appearing to have stopped after 3-4 hours (Degani et al. 1981), although it was not ascertained whether all the vanadate had been reduced. Reduction of vanadate has been reported to occur enzymatically in cardiac membranes (Erdmann et al. 1979b) but it appears as though in erythrocytes at least, reduced glutathione (GSH) may be responsible for the non-enzymatic reduction of vanadate. Glucose starvation which decreases GSH levels led to a decrease in the rate of vanadate uptake whereas glycolytic inhibitors had no effect (Macara et al. 1980). Paradoxically, an increase in cellular GSH levels with increasing vanadate treatment was demonstrated in MDBK cells (Bracken and Sharma 1985) although the GSH/vanadium molar ratio decreased from 1.7 to 0.52 as toxicity

increased at vanadate concentrations from 50 μM to 500 μM . This increase in GSH levels was thought to be a compensatory response to the initial depletion of GSH thought to occur as a result of its role in the reduction of vanadate. From the results with HeLa cells in this study it would appear that the intracellular reduction of vanadate is due, at least in part, to non-enzymatic processes as it occurs even after protein precipitation of the cells with acid. This may be due to GSH as described above since GSH is acid-stable.

Assuming that in HeLa cells the reduction is totally non-enzymatic and proceeds at the same rate as the reduction of vanadate by acid-extracted cells i.e. 34 nmoles/6 hours (cell precipitate from 39×10^6 cells), the 600 μmoles vanadyl/lcw (1.3 nmoles/ 10^6 cells) in the HeLa cells treated with 10^{-4}M vanadate, would have been produced by reduction in approximately 9 hours. This is probably a minimum estimate, as in intact cells, the initial rate of uptake may limit the reduction in the first instance. Additionally the reducing agent may be present at much lower levels in the vanadate-treated cells as a result of the vanadate treatment, than in the extracted control cells in which the reduction rate of vanadate was measured, and also may be compartmentalised in the intact cell being released upon acidification. Furthermore, enzymatic reduction may also occur in the intact cell and predictions involving the rate of reduction if this were the case can not be made.

Assuming that reduction is slower than the 9 hours above, this may account for the delay before toxic effects are seen if in fact they are due not vanadate but to either vanadyl or

indirectly as a result of the depletion of e.g GSH etc. In addition if the effects are due to vanadyl they may be direct or indirect as a result of binding to proteins as no distinction has been made in this study between bound and free vanadyl. Differences in cytotoxicity between the various cell types may be as a result of different reducing abilities and hence possibly different intracellular vanadium accumulation, or may be due to differing sensitivities to the reduced vanadyl, and/or indirect effects accompanying the reduction.

Further experiments which would be constructive if the facilities and time were available would include the measurement of the uptake of radiolabelled vanadium at various time points and the characterisation of the uptake pathway (for example using DIDS), measurement of GSH, NADH, protein synthesis and degradation etc. The results here could also be improved by further repetition, monitoring of possible loss of cellular vanadium (using radiolabelled vanadium) during the washing procedure pre-extraction, better ESR analysis using different sample cells and frozen samples (to minimise cellular changes occurring during extraction), and with the inclusion of more vanadyl standards. Ideally these standards would all be prepared using the acidic supernatant of acid-extracted cells in order to check for any interference of soluble cell substances which may not have precipitated out with the protein, or any adsorbance of the vanadyl onto the proteins. Such effects are unlikely however, based on the results with the 10^{-4} M VOSO_4 standard which gave the same signal irrespective of whether or not extracted cells were present.

CHAPTER 3. THE EFFECTS OF CHRONIC VANADATE TREATMENT ON THE
VOLUME, ION CONTENTS, SODIUM PUMP NUMBERS, AND K⁺ INFLUX PATHWAYS
OF HELA CELLS

3.I. INTRODUCTION

The following section serves as a basic introduction to the normal distribution of ions in cells. This is followed by a more detailed description of the transport processes involved in establishing and maintaining such a distribution, and an examination of the effects of vanadate upon these transport processes. Much of the information in the first two sections has been taken from review articles or textbooks by Baker (1966), De Weer (1985), Dudel (1983), Glynn (1968), Glynn and Karlish (1975), Jorgensen (1982), Robinson (1975), and Skou (1985). Original research articles, where used, are cited.

3.I.i. Ion distribution

The unequal distribution of ions across cell membranes i.e. generally high intracellular K^+ , high extracellular Na^+ , low extracellular K^+ , and low intracellular Na^+ , results from a combination of selective membrane permeability, the sodium pump (Na^+/K^+ -ATPase), and the presence of large impermeant anions such as proteins, within the cells. The actual ion concentrations vary with the type of cell. Normally the intracellular $[K^+]$ is high (approx. 150 mM) compared with the extracellular (approx. 5 mM). As the membrane is freely permeable to K^+ , diffusion out of the cell occurs down the concentration gradient. This outward movement of K^+ ions, which is not accompanied by an equivalent movement of anions, leads to a charge separation across the membrane giving rise to a potential difference with the intracellular environment negatively charged relative to the

extracellular. This results in an inwardly directed electrical gradient for K^+ and eventually an equilibrium is established where the tendency for K^+ to diffuse down its concentration gradient out of the cell is balanced by the tendency of K^+ to diffuse down its electrical gradient into the cell. This is the K^+ equilibrium potential (E_K) and is normally -90 mV (intracellular K negative) in most animal cell types.

In contrast, the intracellular $[Na^+]$ is normally low (approx. 15 mM) compared with extracellular (approx. 150 mM). Consequently both the electrical and chemical gradients are inwardly directed for Na^+ and the equilibrium potential for Na^+ (E_{Na}) is normally $+60$ mV (intracellular positive). The overall membrane potential (E_M) i.e. when both Na^+ and K^+ are present, is not an average of E_K and E_{Na} but is normally -80 mV (intracellular negative). This is due to the relative impermeability of the membrane to Na^+ (approx. $100x$ less permeable to Na^+ than to K^+). As a result of the selective permeability of the membrane, the major determinant of the resting potential is therefore K^+ .

A distribution of this type where neither Na^+ or K^+ is at equilibrium would soon result in the running down of the ion gradients but for the action of the sodium pump (Na^+/K^+ -ATPase molecule). In most cell types, sodium pumps within the membrane maintain the ion gradients and hence the resting potential, by extruding $3 Na^+$ ions from the cell and pumping $2 K^+$ ions into the cell for each cycle of the pump (i.e. per molecule of ATP hydrolysed). Normally the selective permeability of the membrane plays a major role in establishing the negative membrane

potential, with the electrogenic nature of the pump only producing a slight hyperpolarisation (less than 10 mV) of the membrane compared with if the pump were not electrogenic.

The membrane is also permeable to Cl^- ions which are usually distributed in a reciprocal fashion to the K^+ ions. E_{Cl} is normally the same as E_{M} since Cl^- is usually distributed passively across the membrane. The distribution of Cl^- ions adjusts in response to changes in membrane potential in such a way that E_{Cl} remains equal to E_{M} .

The maintenance of this pump-leak steady-state situation in cells, and hence the unequal distribution of ions, is important in many aspects of cellular function, e.g. in cell volume regulation, regulation of intracellular $[\text{Ca}^{2+}]$ via Na/Ca exchange, cotransport of sugars or amino acids, enzyme function, and electrical activity. The diuretic-sensitive cotransport of Na^+ , K^+ , and Cl^- ions, which is present in many cells, is also possibly involved in volume regulation. The activity of the sodium pump and cotransport systems can be ascertained by measuring the K^+ influx of cells radioisotopically. By using specific pharmacological inhibitors, the total K^+ influx can be partitioned into the 3 main K^+ influx pathways:- i). the ouabain-sensitive or sodium pump flux, ii). the diuretic-sensitive or ($\text{Na}^+ + \text{K}^+ + \text{Cl}^-$) cotransport flux, and iii). the passive leak influx (Figure 3.1.). The properties of these are discussed in turn in the following sections.

3.I.ii. Major K^+ influx pathways

a). Ouabain-sensitive (sodium pump) K^+ influx:- The sodium

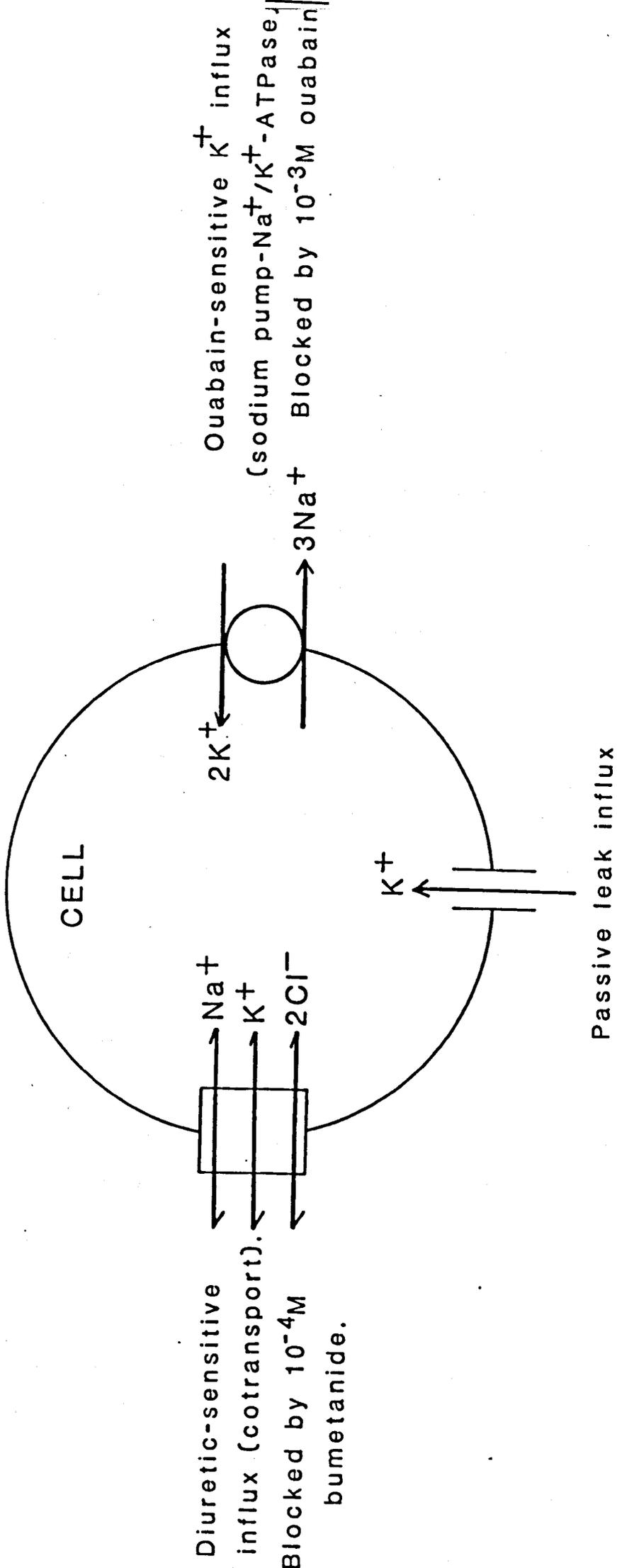


Figure 3.1. Major K^+ influx pathways

pump protein is a membrane-bound enzyme which utilises ATP to transport ions across the membrane. In HeLa cells for example, the ouabain-sensitive K^+ influx normally accounts for approximately 40% of the total K^+ influx. The transport protein consists of α and β sub-units with molecular weights of about 100,000 and 50,000 respectively. Although there is controversy about the polymerisation of these sub-units during purification, the majority of views support a dimeric (i.e. $\alpha_2 \beta_2$) complex as being the functional sodium pump, transporting 3 Na^+ ions outwards and 2 K^+ ions inwards for each molecule of ATP hydrolysed (e.g. Glynn 1968, De Weer 1985).

Various models have been proposed regarding the conformational changes involved in the hydrolysis of ATP and the translocation of Na^+ and K^+ ions. The most widely accepted models are based on the Albers-Post scheme proposed in the late 1960's which is schematically represented in Figure 3.2. and outlined below (from De Weer 1985).

The pump protein is thought to exist in 2 conformations, E_1 (high affinity for Na^+) and E_2 (high affinity for K^+), which have ion-binding sites facing the cytoplasm and extracellular medium respectively. Both E_1 and E_2 exist in either unphosphorylated or phosphorylated forms with E_1P possessing a high-energy bond (\sim) which is able to rephosphorylate ADP to ATP. ATP binds to E_1 with high affinity (K_d less than 1 μM) and, in the presence of Na^+ and Mg^{2+} which also bind to the enzyme, phosphorylates E_1 to $E_1\sim P$. With the ensuing conformational transition from $E_1\sim P$ to E_2-P (favoured by the difference in energy), the Na^+ ions are released to the extracellular medium and K^+ ions bind to the

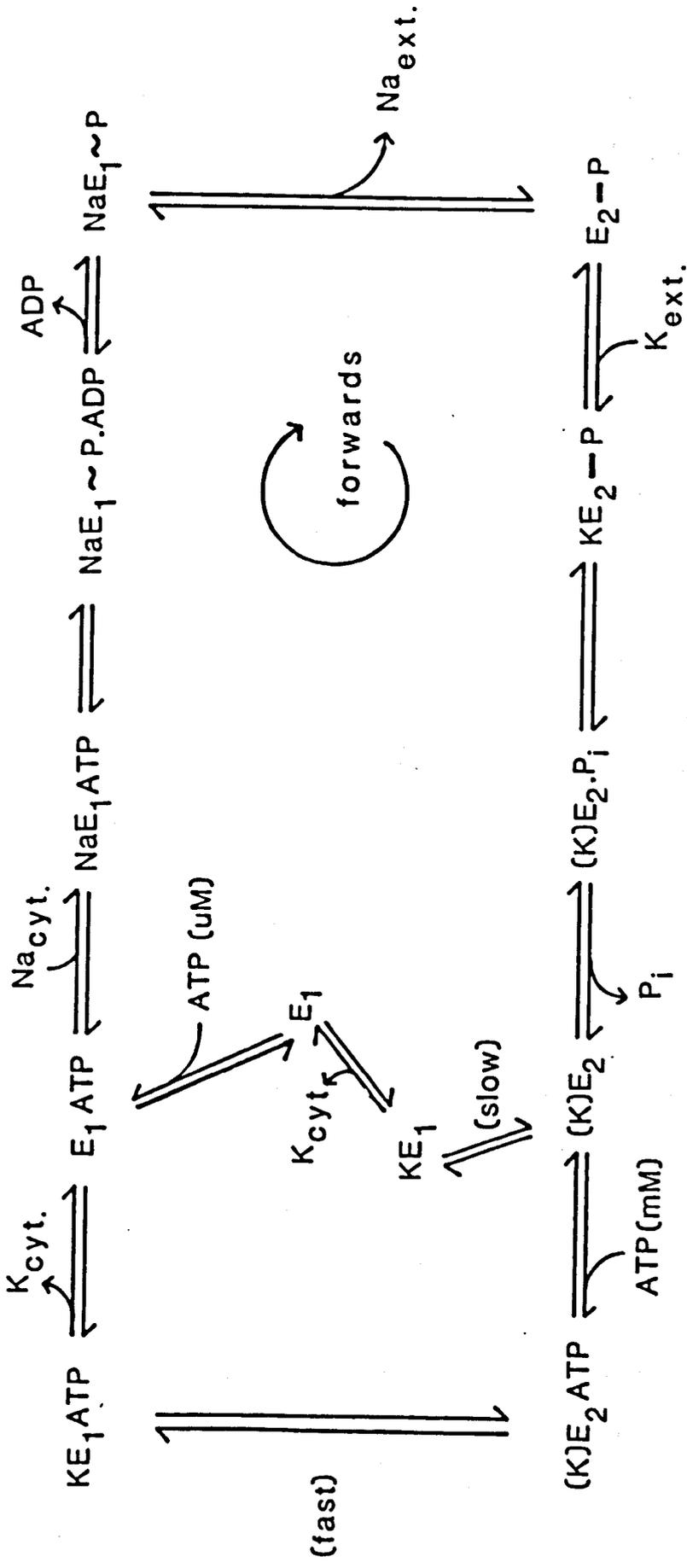


Figure 3.2. Schematic diagram of the current Albers-Post model for the intermediate steps of the sodium pump cycle (adapted from De Weer 1985)

E_1 and E_2 are conformations of the enzyme with ion binding sites facing the cytoplasm and the extracellular medium respectively. A non-covalent bond is indicated by a dot and a covalent bond by a dash. A wavy line indicates a high-energy bond. (K) refers to an occluded state in which the bound ion is unable to exchange with the aqueous phase. The multiple ion stoichiometry of the pump, the various transport modes, and the role of magnesium are discussed in the text.

enzyme which still has Mg^{2+} associated with it. The binding of extracellular K^+ ions, which, unlike Na^+ , can be replaced by Li^+ , Rb^+ or Cs^+ to varying extents, enhances the rate of dephosphorylation of E_2-P , with the K^+ ion becoming "occluded" or trapped in the enzyme (probably the translocation step). The occluded $(K)E_2$ form reverts very slowly to the E_1 form, but alternatively $(K)E_2$ can bind ATP with low affinity ($K_d = 0.3$ mM) and the rate of conversion of $(K)E_2-ATP$ to the E_1 form is much faster. The pathway taken depends upon the concentration of available ATP. Thus the Na^+/K^+ -ATPase kinetics are biphasic, with both high and low K_m values for ATP. Opinions are divided as to whether there are 2 separate ATP binding sites with different affinities on the α -subunit, or whether there is one site which has an affinity determined by the conformation of the α -subunit (e.g. Cantley et al. 1983, Askari and Huang 1985). In further modifications to this model it has been proposed that Na^+ exists in an occluded form $(Na)E_1-P$ during its translocation (Skou 1985).

Besides the "forward" pumping described above, the pump can also run in reverse if the intracellular and extracellular ion concentrations are reversed and the ADP and P_i concentrations greatly exceed the intracellular ATP concentration. Provisions are also made in this model for "uncoupled" Na^+ efflux in the absence of extracellular K^+ or Na^+ (Na^+ -ATPase activity), $Na^+:Na^+$ exchange, and $K^+:K^+$ exchange which have been shown to occur in some cells. A discussion of these modes is outwith the scope of this chapter however.

The number of sodium pump molecules per cell varies depending

on the cell type e.g. 100-200 per human erythrocyte (less than $1/u^2$) and 4.1×10^6 /cell in rabbit kidney thick ascending limb cells (approx. $4400/u^2$; De Weer 1985). A recycling process occurs with pumps continually being removed and inserted into the membrane e.g. in HeLa cells both these processes occur at a rate of approximately 9%/hour. It is thought that cells can "upregulate" their pump numbers in order to match the leak flux but whether this is due to a change in the rate of internalisation or of insertion, is not clear (Algerably et al. 1985). This is discussed further in Chapter 6.

Intracellular Na^+ and extracellular K^+ stimulate the pump activity with sigmoidal kinetics (De Weer 1985). The K_m for Na^+ is 15-25 mM depending on the cell type, and the pump is usually only about 50-75% saturated with respect to extracellular K^+ with a K_m of approximately 1.5 mM. As the extracellular Na^+ is decreased, the K_m for extracellular K^+ decreases until in Na^+ -free medium, the apparent affinity of the pump for extracellular K^+ is 5-10x that in normal medium (De Weer 1985). This is possibly due to a simple competition of Na^+ and K^+ at the same site.

Cardiac glycosides such as digitalis and ouabain are known to bind extracellularly to the α -subunit of the sodium pump in the E_2 -P conformation, and inhibit the translocation of ions (Allen et al. 1985, Forbush III 1983). This is antagonised by extracellular K^+ which normally accelerates the hydrolysis of E_2 -P. Ouabain binds practically irreversibly and can be used in the measurement of sodium pump numbers and activity as described later in this chapter.

b). Diuretic-sensitive K^+ influx:- In many diverse cell types e.g. MDCK cells, HeLa cells, avian erythrocytes and flounder intestinal cells, a large proportion of the ouabain-insensitive K^+ influx is mediated by an electroneutral cotransport with Na^+ and Cl^- ions, in a 1:1:2 stoichiometry. This is inhibited by loop diuretics such as furosemide and bumetanide. The properties of this transport system have recently been reviewed by Saier, Jr. and Boyden (1984) and are summarised below.

The operation of this transport system is dependent on the simultaneous binding of Na^+ , K^+ and Cl^- ions to the transport protein. Na^+ can be replaced by Li^+ , Cl^- by Br^- , and K^+ by Rb^+ and to a lesser extent, NH_4^+ and Cs^+ . Strong co-operativity exists between the 3 ion binding sites with the apparent affinity constants of each ion being influenced by the binding of the other 2 ions. The system is readily bi-directional and it is thought that the direction of net transport is determined by the overall sum of the transmembrane chemical gradients for the 3 ions (Haas et al. 1982). Normally the system functions as a passive exchanger with no net transport. Due to its electroneutrality it does not affect the membrane potential and in turn is not affected by the membrane potential.

ATP functions to activate the system but as the relationship is not stoichiometric, the activation is thought to be due to a protein-kinase mediated reaction. In some cell types, such as turkey and frog erythrocytes, this is thought to be via a cAMP-dependent system as, for example, it is stimulated by beta-adrenergic agonists or cholera toxin, and is potentiated by IBMX.

Such agents have also been shown to result in the phosphorylation of a membrane protein of molecular weight about 240,000 which has been postulated to be the transport protein. In MDCK cells however, no involvement of cAMP has been found and it is thought that a cAMP-independent protein kinase is involved.

In secretory epithelium, the diuretic-sensitive cotransport is important in regulating chloride absorption or secretion. In other cell types however, the role of the diuretic-sensitive cotransport is uncertain. This arises largely from the differences between cell types and the difficulties in determining, for example, whether transport of ions is via a cotransport molecule or two coupled exchangers such as the Na^+/H^+ or K^+/H^+ , and $\text{Cl}^-/\text{HCO}_3^-$ coupled exchangers present in Amphiuma red blood cells (Cala 1980). Such coupling produces an overall Na^+ or K^+ and Cl^- transport but can be distinguished from the diuretic-sensitive cotransport on the basis of sensitivity to inhibitors such as amiloride and DIDS or SITS.

One of the postulated roles of the diuretic-sensitive cotransport is in volume regulation. When duck erythrocytes, for example, are placed in hypertonic solutions, the cells shrink but return to control volumes with continued incubation. This regulatory volume increase (RVI) has been attributed to the diuretic-sensitive transport which increases producing a net influx when the cells are placed in the hypertonic medium, thus increasing ion concentrations intracellularly and restoring the volume osmotically. However such an RVI is only seen if the extracellular K^+ is raised above physiological levels (Kregenow - from Saier Jr. and Boyden 1984).

In cells which exhibit no RVI e.g. HeLa cells or MDCK cells, an increase in diuretic-sensitive flux is seen in response to hyperosmotic medium, but this occurs in both the efflux and influx and therefore produces no net flux (Tivey 1986). On the basis of the increase in ion concentration in shrunken cells, it would be predicted that a net efflux of ions would be produced, resulting in further shrinkage. The reason why this does not occur is not clear but it is proposed that the ion contents measured may not all be osmotically available.

Alternatively the diuretic-sensitive flux may not be predominantly volume regulatory but be concerned with potassium homeostasis (Duhm and Gobel 1984). This hypothesis is also consistent with the observation in Ehrlich ascites tumour cells of an electroneutral Na^+ and Cl^- cotransport which is diuretic-sensitive, and is stimulated by cell shrinkage only in cells which have previously been depleted of KCl (Hoffmann et al. 1983). The net influx of Na^+ and Cl^- causes a RVI. The increased Na^+ levels in turn stimulate the sodium pump resulting in the restoration of intracellular K^+ levels. The exact stimulus for the increased influx and efflux is not known in these cells.

In many cells, including HeLa cells, an initial increase in cell volume caused by exposure to hypo-osmotic medium is reversed by a loss of KCl together with osmotically-obliged water - a regulatory volume decrease (RVD; Tivey 1986). This is mediated by a passive increase in membrane K^+ permeability but whether this is diuretic-sensitive or due to other mechanisms such as calcium-activated K^+ channels is not clear and possibly

depends upon the cell type.

c). **Passive leak K^+ influx:-** This comprises the diffusion of K^+ ions down their electrical gradient into the cell, which together with the influx through the sodium pump equals the K^+ efflux down its concentration gradient, and an exchange diffusion of K^+ ions across the membrane. Overall these mechanisms result in no overall net loss or gain of K^+ by the cell unless e.g. the membrane potential is altered.

3.I.iii. Vanadate and ion transport

There have been no reported studies of the effects of vanadate on the diuretic-sensitive cotransport system. In contrast, there have been many studies into the properties of vanadate as an inhibitor of Na^+/K^+ -ATPase, following its discovery as a contaminant in commercial ATP supplies (Josephson and Cantley, Jr. 1977, Cantley, Jr. et al. 1977, Quist and Hokin 1978). It should be noted that it has been proposed that vanadium was present "in the bottle" as the vanadyl ion and was slowly oxidised when dissolved at physiological pH (Grantham and Glynn 1979). Thus some of the early results reported using the ATP as the source of vanadate may be slightly in error as regards the concentration of vanadate present. The studies concerning the effects of vanadate on Na^+/K^+ -ATPase in isolated enzyme systems, its cellular uptake and subsequent reduction to vanadyl, and the physiological effects of vanadate have been reviewed in Chapter 1. It is proposed here to review in greater detail the mode of action of vanadate in inhibiting the Na^+/K^+ -ATPase molecule, and the effects of vanadate on ion fluxes in the intact

cell.

Inhibition of Na^+/K^+ -ATPase by vanadate requires the presence of potassium ions (extracellularly in intact cells) and magnesium ions (intracellularly in intact cells) which facilitate the binding of vanadate, although there is uncertainty about the requirement for potassium ions (Josephson and Cantley, Jr. 1977, Quist and Hokin 1978, Bond and Hudgins 1979, Beauge et al. 1980, Robinson and Mercer 1981). Unlike ouabain, the inhibitory action of vanadate is mediated intracellularly and is augmented by increasing the extracellular potassium concentration (Beauge et al. 1980). The sites occupied by K^+ and Mg^{2+} in augmenting the inhibitory actions of vanadate are thought to be different to those normally involved in their activation of the enzyme (Bond and Hudgins 1979). Increasing the extracellular sodium concentration antagonises the inhibition, possibly by displacing K^+ from its inhibitory site, and ATP has a protective effect against inhibition (Josephson and Cantley, Jr. 1977, Bond and Hudgins 1979). Inhibition of the isolated enzyme is totally reversed by millimolar concentrations of noradrenaline, probably by complexation (Josephson and Cantley, Jr. 1977, Quist and Hokin 1978).

Assuming the model of 2 distinct sites with differing affinities for ATP binding on the sodium pump is correct, vanadate is considered to bind with low affinity ($K_{\text{aff}} = 0.5 \mu\text{M}$) to a site which is probably the high affinity ATP site associated with Na^+ -stimulated ATPase activity, and with high affinity ($K_{\text{aff}} = 4 \text{ nM}$, i.e. several fold higher affinity than that of phosphate) to a site which is identical to that for low affinity ATP binding

(Cantley, Jr. et al. 1978a). This is supported by ouabain binding data, which shows that simultaneous vanadate treatment with ouabain facilitates ouabain binding in a similar manner to phosphate, but at micromolar concentrations of vanadate compared with millimolar concentrations of phosphate (Hansen 1979). However it has been reported that this facilitation is only seen in the absence of K^+ (Myers et al. 1979). The inhibition of Na^+/K^+ -ATPase by vanadate is thought to be due to a stabilisation of the enzyme in the $(K)E_2$ conformation following hydrolysis of the ATP (Cantley, Jr. et al. 1978a, Karlisch et al. 1979).

Few studies have been carried out which examine the effects of vanadate on ion fluxes in intact cells and those which have been done have utilised short incubation times. In rat vascular smooth muscle cells cultured in vitro, no effect on the ouabain-sensitive K^+ influx was seen after 2 hours incubation in $10^{-5}M$ vanadate, despite the inhibition of the Na^+/K^+ -ATPase enzyme in disrupted cells with a K_i of $10^{-6}M - 10^{-7}M$ Searle et al. 1983). Inhibition of the ouabain-sensitive K^+ influx has been reported in heart non-muscle cells from neonatal guinea-pigs and chick embryos ($K_i = 40 \mu M$ and $100 \mu M$ respectively) although the decrease in intracellular $[K^+]$ was not as large as that found with a similar ouabain-induced inhibition of flux (Werdan et al. 1982). However in heart muscle and non-muscle cells prepared from neo-natal rats and in Girardi human heart cells, vanadate stimulated both the ouabain-sensitive K^+ influx ($K_{aff} = 22, 140$ and $34 \mu M$ respectively) and the Na^+ influx. Thus an increase in intracellular $[K^+]$ but no change in $[Na^+]$ was seen (Werdan et al. 1980, 1982). It was postulated that the inhibitory action was

due to vanadate and that the stimulation was due to an insulin-mimetic effect of vanadyl formed by reduction of the vanadate. The differences due to cell type were postulated as therefore being due to differing reducing abilities. However vanadyl has also been reported to have an inhibitory action on Na^+/K^+ -ATPase in very pure enzyme preparations, with no stimulation being reported (North and Post 1984).

In mouse diaphragm muscle, vanadyl, like insulin, produces a hyperpolarisation of 3 - 6 mV within minutes at concentrations of 10^{-6} - 10^{-5} M (Zemkova et al. 1982). At 10^{-4} and 10^{-3} M vanadyl, the hyperpolarisation was preceded by a transient depolarisation. Vanadate had a similar effect but to a lesser extent, and only after a delay attributed to its reduction to vanadyl. Increases in intracellular $[\text{K}^+]$ of approximately 15 mM were seen before the hyperpolarisation. The hyperpolarisation and increase in $[\text{K}^+]$ was blocked by ouabain but was unaffected by the removal of extracellular K^+ . It was proposed that the hyperpolarisation was due either to a stimulatory effect of vanadyl on the pump (the lack of effect of removal of extracellular K^+ does not support this), or that the binding of vanadyl to intracellular proteins caused the release of bound K^+ which resulted in an increase in K^+ concentration when measured using ion-selective electrodes, and a consequent hyperpolarisation. In the latter case the action of ouabain was postulated to be on the vanadyl uptake. The effect of ouabain on the vanadate-induced hyperpolarisation was not examined. Further research is necessary to distinguish between or negate these possible explanations.

In NG108-15 mouse neuroblastoma-glioma hybrid cells, 20-30 mV hyperpolarisations were seen with vanadate treatment with a half-maximally effective dose of 35 μM for Na_3VO_4 and a $t_{0.5}$ of 30 seconds (Lichtstein et al. 1982). Similar results were also seen in various other cell types e.g. BHK cells and rat hepatoma cells. Concomitantly a decrease in membrane resistance was seen indicating an increased conductivity of one or more ions. Ouabain had no effect on the hyperpolarisation, and extracellular Na^+ removal or high extracellular K^+ also had no effect making it unlikely that it was due either to a stimulation of the sodium pump or an increase in K^+ conductivity. The possibility that it was due to an increased Cl^- uptake was not investigated.

In erythrocytes, an increase in K^+ permeability was shown to be due to a vanadate-induced inhibition of Ca^{2+} -ATPase, producing an elevated intracellular $[\text{Ca}^{2+}]$ and a stimulation of Ca^{2+} -dependent K^+ efflux (Gardos effect; Fuhrmann et al. 1984). EDTA abolished the effect. It is conceivable that a similar mechanism may account for the hyperpolarisation in the cell lines above. The increase in K^+ permeability in erythrocytes was also seen with vanadyl in the presence of EDTA and the ionophore A23187, but it was thought that this was due to vanadyl displacing Ca^{2+} from the EDTA. The Ca^{2+} then enters the cell via the ionophore and produces a Ca^{2+} induced K^+ permeability.

In Chapter 1 of this thesis, the possible involvement of a perturbation in ion transport in the aetiology of manic depressive psychosis has been reviewed, together with the evidence for a role of vanadium in the illness. This chapter is aimed at investigating the chronic effects of vanadium

(vanadate/vanadyl) on cation transport in the relatively well-characterised HeLa cell line. The results can then be used to aid the interpretation of results obtained using the less well characterised virally-transformed lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects.

3.II. MATERIALS AND METHODS

3.II.i. Reagents

Reagents used were purchased from the following:-

Amersham International (Amersham, Bucks.):- ^3H -ouabain (42 Ci/mmol), and ^{86}Rb (200-240 mCi/mmol initially).

BDH Chemicals (Poole, Dorset):- EDTA and all the salts (Analar grade) used in the Krebs solution, and "Cocktail T" scintillant.

Boots Chemists Ltd. (Nottingham):- Sorbitol.

Gibco-Biocult (Paisley, Scotland):- all tissue culture supplies as detailed in Section 2.II.i.

Sigma (Poole, Dorset):- Ouabain.

Bumetanide was a kind gift of Leo Laboratories, UK. Water used for solutions was produced using a Milli-Q water system (Millipore S.A., France). All solutions were dispensed using Gilson adjustable pipettes during experiments.

3.II.ii. Effects of 24 hour vanadate treatment on the ion contents of HeLa cells

Intracellular sodium and potassium concentrations can be determined using radioisotopes. By placing the cells in a solution containing either ^{42}K or ^{22}Na , and measuring the radioactive content of the cells after the isotope has been allowed to equilibrate across the cell membrane, the ion contents can be calculated. In practice this is expensive and a flame photometric method is often used (e.g. Aiton and Simmons 1983),

as described below.

Plates of HeLa cells were prepared as described in Section 2.II.iii. (0.33×10^6 cells/ 5 cm plate - day 0), and the medium replaced with fresh medium \pm Na_3VO_4 (10^{-9} - 10^{-4} M) on day 3, with 6 plates for each condition. Twenty-four hours later, half the plates in each treatment group were washed 4x in ice-cold isosmotic sorbitol (Table 3.1.) to remove extracellular sodium and potassium. Deionised water (5 mls) was added to each plate to lyse the cells and the plates left to extract for 2 hours at 20°C . The sodium and potassium concentrations of the extracts were then determined sequentially using an EEL flame photometer (Mark II) and appropriate standards ($100 \mu\text{M Na}^+$ and K^+), diluting the samples if necessary. Suitable plate blanks were run, consisting of cell-free plates containing medium which were washed and extracted in the same way as the plates above.

The remaining plates for each treatment condition were used to determine cell numbers, volumes and plate water as previously described in Section 2.iv.. This use of plates run in parallel was necessitated by the fact that the lysis of cells during extraction of the ions precluded the use of the same plates for the measurement of both ions and numbers. The ion contents were calculated using the equation in Appendix 1 and expressed in mmol/lcw or $\text{nmol}/10^6$ cells.

Experiments were repeated on several occasions with each condition in triplicate, and the results analysed by Student's t-test (unpaired) using the "Minitab" statistical package on the VAX mainframe computer.

Table 3.1. Composition of isosmotic sorbitol solution

<u>Component</u>	<u>mmoles/l</u>
Sorbitol	274.0
HCl	12.0
Tris Base	13.7

The pH of the solution was adjusted to 7.4 at 4°C.

3.II.iii. Effects of 24 hour vanadate treatment on sodium pump site numbers of HeLa cells, as measured by ^3H -ouabain binding

The method used here is based on those of Baker and Willis (1970), and Boardman et al. (1972). There are two components of ouabain binding; i). a "specific" component which is sensitive to extracellular potassium concentration and saturates at low concentrations of ouabain; and ii). a "non-specific" component which increases linearly with ouabain concentration (upto at least 1mM) and is insensitive to extracellular potassium. The saturable component is thought to represent the specific ouabain binding to the sodium pump since binding correlates with the inhibition of the potassium flux through the pump.

In practice, pump site numbers in HeLa cells are measured using ^3H -ouabain as a tracer in a Krebs solution containing $2 \times 10^{-7}\text{M}$ cold (i.e. non-radioactive) ouabain. Total binding (i.e. specific and non-specific) is measured using a potassium-free Krebs solution (K-free Krebs) whilst the non-specific binding is measured using a Krebs solution containing 15mM K^+ (15K-Krebs). By appropriate subtraction, the specific ouabain binding can then be estimated. The ouabain concentration of $2 \times 10^{-7}\text{M}$ is usually chosen for use with HeLa cells as being an excess concentration where the specific and non-specific binding can best be distinguished (Boardman et al. 1972). At this ouabain concentration the non-specific binding is generally less than 10% of the total binding. With lower concentrations of ouabain, much longer incubation periods are required for saturation of the specific binding, and at higher concentrations the non-specific binding comprises a higher proportion of the

total binding, thus making the specific binding more difficult to evaluate accurately. At 37°C and with an incubation time of 20 minutes, specific binding saturates, and dissociation and pump internalisation are insignificant (Lamb and Ogden 1977, Algerably et al. 1985).

K-free Krebs was prepared as per normal Krebs (Table 2.3.) but omitting the KCl, replacing the KH_2PO_4 with NaH_2PO_4 , and using dialysed new-born calf serum. 15K-Krebs was prepared by adding the appropriate amount of 1M KCl to K-free Krebs. Ouabain solutions were prepared by serial dilution of a 10^{-3}M aqueous stock solution which was stored at 4°C in the dark. The specific activity of the stock ^3H -ouabain was 42 Ci/mmol.

Plates of HeLa cells were prepared as described in Section 2.II.iii. with a seeding density of 0.33×10^6 cells/3 mls of BME on 5 cm tissue culture plates (day 0). On day 3 the plates were numbered and the medium was replaced with fresh medium $\pm \text{Na}_3\text{VO}_4$ (10^{-9}M - 10^{-4}M), with 6 plates for each treatment condition. Twenty-four hours later, half the plates from each treatment condition were transferred to a bench-top incubator at 37°C. At 30 second intervals, each plate in turn was washed 4x in K-free Krebs (37°C), the remaining solution aspirated off, the plate replaced on the incubator and approximately 4 mls of radioactive ouabain standard solution was added ($2 \times 10^{-7}\text{M}$ ouabain in K-free Krebs, containing 0.4 uCi of ^3H -ouabain/ml) by syringe. All additions and removals of solution were carried out at the same point on the plate in order to minimise any disruption to the cell monolayer. The plates were then incubated for 20 minutes at 37°C and at the appropriate 30 second intervals each plate was

removed from the incubator and washed 4x (less than 20 secs in total) in ice-cold Krebs (this washing procedure has previously been shown to remove all the extracellular space marker ^{14}C -inulin - Aiton et al. 1981). To each plate, 1 ml of trypsin solution (0.25% w/v in EBSS containing 2mM EDTA) was added. The plates were then returned to the incubator until the cells detached whereupon the trypsin was neutralised by the addition of 2 mls of Krebs to each plate.

For each plate in turn, a single cell suspension was prepared by blasting and 1 ml was taken and added to 19 mls of Isoton for the determination of cell numbers as previously described in Section 2.II.iv.. 1 ml of each cell suspension was added to scintillation vials containing 10 mls of scintillation fluid. The scintillation vials were then capped, shaken and placed in a Packard Tri-carb Liquid Scintillation Spectrometer 3255. After allowing 1-2 hours for chemiluminescence to subside, the samples were counted for 10 minutes or 10,000 counts thus keeping the error below 1%. No quench correction was made for the varying protein content of the samples as the influence was negligible with the small quantities and range involved (Aiton - personal communication).

The same procedure was carried out on the remainder of the plates using a similar radioactive ouabain solution but made up in 15K-Krebs, and using 15K-Krebs for the washes. In both cases blanks were run to account for any residual radioactivity left after the washing. These consisted of cell-free plates to which 4 mls of radioactive standard solution was added and the plates treated as described above. For standard counts (specific

activity), 3 x 100 ul aliquots were taken from each standard solution and added to scintillation vials containing 10 mls of scintillation fluid and 0.9 mls of 0.25% (w/v) trypsin solution/Krebs mixture (1:2, v/v), mimicking the conditions present in the other vials. Machine blanks were also run in order to measure the machine background counts (normally less than 20 cpm). These consisted of 3 vials each containing 10 mls of scintillation fluid and 1 ml of the above trypsin/Krebs mixture.

Total and non-specific binding, and consequently the specific binding, was calculated according to the equation in Appendix 2. The results were expressed as molecules of ouabain bound/cell. Assuming that one molecule of ouabain binds per pump site (Baker and Willis 1972), this represents the number of sodium pump sites per cell.

Experiments were repeated on several occasions, with each treatment condition in triplicate. Results were analysed by Student's t-test (unpaired) using the "Minitab" statistical package on the VAX mainframe computer.

In the light of the results (discussed later) which show a vanadate-induced decrease in the specific ouabain binding, a time course of ouabain binding (30 secs - 16 mins) was carried out on cells previously incubated for 24 hours in the presence and absence of vanadate. This was carried out in order to check that the vanadate pretreatment was not altering the binding kinetics of ouabain and producing an apparent decrease in the number of binding sites. The method used was essentially the same as that above, only with the incubation times in ouabain

solution being varied and a vanadate concentration of 10^{-4} M being used for the 24 hour preincubation.

Another possibility, although unlikely, was that vanadate, despite inhibiting the sodium pump from the intracellular side unlike ouabain, may later have prevented access of ouabain to some sites. This may have produced a similar time course of binding as in the untreated cells but with apparently less pump sites. However this had previously been shown not to be the case (Aiton - personal communication) and was not investigated further in this study.

3.II.iv. Effects of 24 hour vanadate treatment on potassium (^{86}Rb) influx in HeLa cells

As already described in the "Introduction" to this chapter, there are three major pathways by which potassium enters cells; i). a passive leak influx; ii). a diuretic-sensitive cotransport; and iii). a ouabain-sensitive pathway via the sodium pump. The magnitude of these individual pathways can be measured using the radioactive tracer ^{86}Rb (this is handled in the same way as ^{42}K when used in tracer amounts but has a half-life of 18.7 days compared with 12.4 hours - Aiton et al. 1982), as described by Aiton et al. (1981). The term " K^+ influx" will be taken to include ^{86}Rb influx for the purposes of this thesis. By measuring the influx of ^{86}Rb when the cells are incubated in Krebs, the total potassium influx can be determined. By measuring the influx in Krebs containing 10^{-3} M ouabain and subtracting the measured flux from the total flux, the ouabain-sensitive component can be determined, and similarly for the

diuretic-sensitive component using either 10^{-4} M furosemide or bumetanide. The passive leak influx can then be determined by subtractions or by measuring the flux in the presence of both ouabain and furosemide/bumetanide. The flux measurements must be made over a short time period during the linear portion of the uptake curve i.e. whilst back-diffusion of the tracer is still negligible. In HeLa cells a period of less than 10 minutes is usually used (Aiton et al. 1981).

It has previously been shown however, using HeLa and MDCK cells, that there is a substantial stimulation of the cotransport pathway by ouabain (Aiton and Simmons 1983). This may be secondary to the inhibition of the flux through the sodium pump and results in erroneous estimates of the ouabain-sensitive flux if measured as above. In addition, high concentrations of furosemide have been shown to inhibit the ouabain-sensitive flux (Wiley and Cooper 1974). Although these interactive effects predominate at longer time periods than are normally used in flux measurements, it has been recommended that the total potassium influx is measured as described, but that the ouabain-sensitive and diuretic-sensitive pathways be measured using nitrate Krebs (NO_3^- -Krebs; Aiton and Simmons 1983). This is a Krebs solution in which the chloride has been replaced by nitrate which is not transported by the cotransport pathway and hence inhibits it. When the flux is measured using this solution and the appropriate subtraction made, the chloride-dependent pathway (normally equivalent to the diuretic-sensitive pathway in HeLa cells (Aiton and Simmons 1983) can be measured, and the passive leak pathway can be measured in NO_3^- -Krebs containing ouabain. By

subtraction, the ouabain-sensitive pathway can be determined.

Plates of HeLa cells were prepared as described in Section 2.II.iii (0.33 x 10⁶ cells/5 cm plate - day 0), and the medium replaced with fresh medium ± Na₃VO₄ (10⁻⁹-10⁻⁴M) on day 3, with 9 plates for each condition. Twenty-four hours later at appropriate staggered intervals, the plates for one treatment condition were transferred to a bench-top incubator at 37°C. At 30 second intervals, the first 3 plates in turn were washed 4x in Krebs (37°C), aspirated and replaced on the incubator where 4 mls of radioactive standard solution (Krebs containing 0.5 uCi of ⁸⁶Rb/ml) was added with a syringe. The second three plates and remaining three plates in the treatment group were treated similarly except that they were washed with NO₃⁻-Krebs (see Table 3.2. for composition) and the radioactive standard solutions were, respectively, NO₃⁻-Krebs containing 0.5 uCi of ⁸⁶Rb/ml, and the same but also containing 10⁻³M ouabain. The K⁺ concentration of both the NO₃⁻- and normal Krebs solutions were checked by flame photometry before use (5.7 mM normally).

After 5 minutes incubation, the plates were removed from the incubator at the appropriate time intervals and washed 4x (less than 20 secs in total) with ice-cold Krebs (this procedure had previously been shown to remove all of the extracellular marker ¹⁴C-inulin and less than 1% of intracellular ⁸⁶Rb was lost - Aiton et al. 1981). The plates were then returned to the incubator where 1 ml of trypsin solution (0.25% w/v in EBSS containing 2mM EDTA) was added to each plate. When the cells were detached, the trypsin was neutralised by addition of 2 mls of Krebs and a single cell suspension was prepared by blasting.

Table 3.2. Composition of NO₃⁻-Krebs solution

<u>Component</u>	<u>mmoles/l</u>
NaNO ₃	140.0
KNO ₃	5.4
MgSO ₄ ·7H ₂ O	1.2
NaH ₂ PO ₄	0.3
KH ₂ PO ₄	0.3
Ca(NO ₃) ₂	2.8
HNO ₃	12.0
Tris Base	13.7
Glucose	11.1

In addition the Krebs solution was supplemented with 1% (v/v) new-born calf serum. The pH was adjusted to 7.4 at 37°C.

For each plate in turn, 1 ml of the suspension was added to 19 mls of Isoton and used for the determination of cell numbers as described in Section 2.II.iv.. 1 ml of each cell suspension was also taken and added to 10 mls of water in a scintillation vial and counted in a Packard Tricarb Liquid Scintillation Spectrometer 3255 by the Cerenkov method. This is a method of counting high-energy beta-emitters whereby particles of energy greater than 300 KeV travelling through a medium such as water, at a velocity greater than that of light in the same medium, emit a photon of light. This can be detected using a liquid scintillation counter.

The above procedure was repeated for all the treatment conditions. Plate blanks were run as previously described in Section 3.II.iii. and 3 x 100 ul aliquots of each standard were taken (for specific activity measurement) and added to 10 mls of water and 0.9 mls of 0.25% (w/v) trypsin/Krebs mixture (1:2 v/v) in scintillation vials, to ensure the same colour quenching. These were counted as above. Machine blanks were also counted by taking 3 x 1 mls of the above Krebs/trypsin mixture and adding them to 10mls of water in scintillation vials.

Results were calculated according to the equation in Appendix 3 and expressed as $\text{mmol K}^+/\text{lcw}/\text{min.}$ or as $\text{nmol K}^+/\text{10}^6 \text{ cells}/\text{min.}$ for each flux pathway. Experiments were repeated on several occasions, with each condition in triplicate. Results were analysed by Student's t-test (unpaired) using the "Minitab" statistical package on the VAX mainframe computer.

3.III. RESULTS

3.III.1. Effect of 24 hours incubation in vanadate-containing medium on the volume and intracellular $[Na^+]$ and $[K^+]$ of HeLa cells

The effects of vanadate on the mean cell volume are shown in Table 3.3. and Figure 3.3.. Slight but highly significant increases in mean cell volume are produced at $[V^{5+}]_o$ of $10^{-5}M$ - $10^{-4}M$. The increase is dose-dependent from $[V^{5+}]_o$ of $10^{-5}M$ to $4.6 \times 10^{-5}M$ but tends to decrease again towards control values at $10^{-4}M V^{5+}$, although still significantly elevated.

Changes in ion contents are not seen until higher extracellular concentrations of vanadate than those producing the earliest changes in volume. Highly significant increases in $[Na^+]_i$ are seen at $[V^{5+}]_o$ of $4.6 \times 10^{-5}M$ and $10^{-4}M$ with a maximum increase of 133% at $10^{-4}M V^{5+}$ if the results are expressed as mmol/lcw, or an increase of 142% if the results are expressed as nmol/ 10^6 cells (Table 3.4. and Figure 3.4.). The slight differences in the magnitude of this change are due to the slight change in cell volume with vanadate treatment.

No significant changes in intracellular K^+ levels were seen if expressed as mmol/lcw and only slightly significant increases at $[V^{5+}]_o$ of $4.6 \times 10^{-5}M$ and $10^{-4}M$, if expressed as nmol/ 10^6 cells (Table 3.5. and Figure 3.4.). This apparent virtual lack of significant effect of vanadate on $[K^+]_i$ however, is due to the variability of the results between experiments, as can be seen in Figure 3.5. where the results from 8 individual experiments are shown. In contrast to the intracellular sodium levels which

Table 3.3. Effect of 24 hours growth in vanadate on the mean cell volume of HeLa cells

The values below represent the mean \pm S.E.M. of 8 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test.

$[V^{5+}]_0$ (M)	Mean cell volume \pm S.F.M. (μ^3)	Level of Significance
0	2850 \pm 29	-
3.2×10^{-6}	2862 \pm 22	N.S.
10^{-5}	2927 \pm 33	p 0.05
2.1×10^{-5}	3034 \pm 35	p < 0.001
4.6×10^{-5}	3076 \pm 30	p < 0.001
10^{-4}	2983 \pm 46	p < 0.05

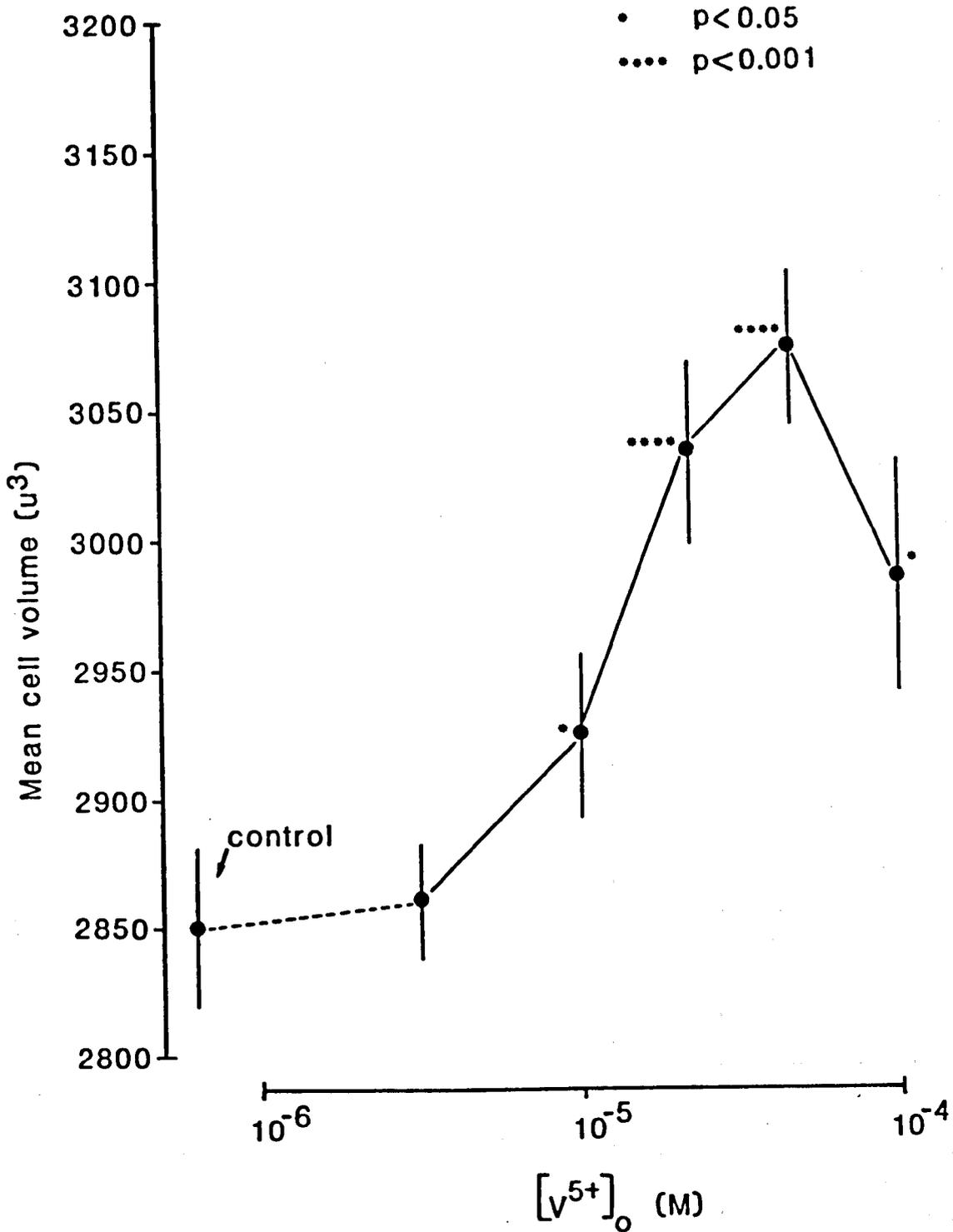


Figure 3.3. Effect of a 24 hour incubation in vanadate-containing medium on the mean cell volume of HeLa cells

Each point represents the mean \pm S.E.M. of 8 experiments, with each condition being triplicated within each experiment.

Table 3.4. Effect of 24 hours growth in vanadate on the intracellular $[Na^+]_i$ of HeLa cells

The values below represent the mean \pm S.E.M. of 8 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test and refers to both columns of results unless otherwise indicated.

$[V^{5+}]_o$ (M)	Mean $[Na^+]_i \pm$ S.E.M. (nmol/ 10^6 cells)	Mean $[Na^+]_i \pm$ S.E.M. (mmol/lcw)	Level of Significance
0	38.9 ± 1.5	17.0 ± 0.7	-
3.2×10^{-6}	41.8 ± 3.9	18.2 ± 1.7	N.S.
10^{-5}	39.1 ± 2.7	16.9 ± 1.4	N.S.
2.1×10^{-5}	45.0 ± 3.8	18.6 ± 1.7	N.S.
4.6×10^{-5}	64.2 ± 3.5	26.0 ± 1.4	$p < 0.001$
10^{-4}	94.1 ± 9.7	39.6 ± 4.4	$p < 0.001$

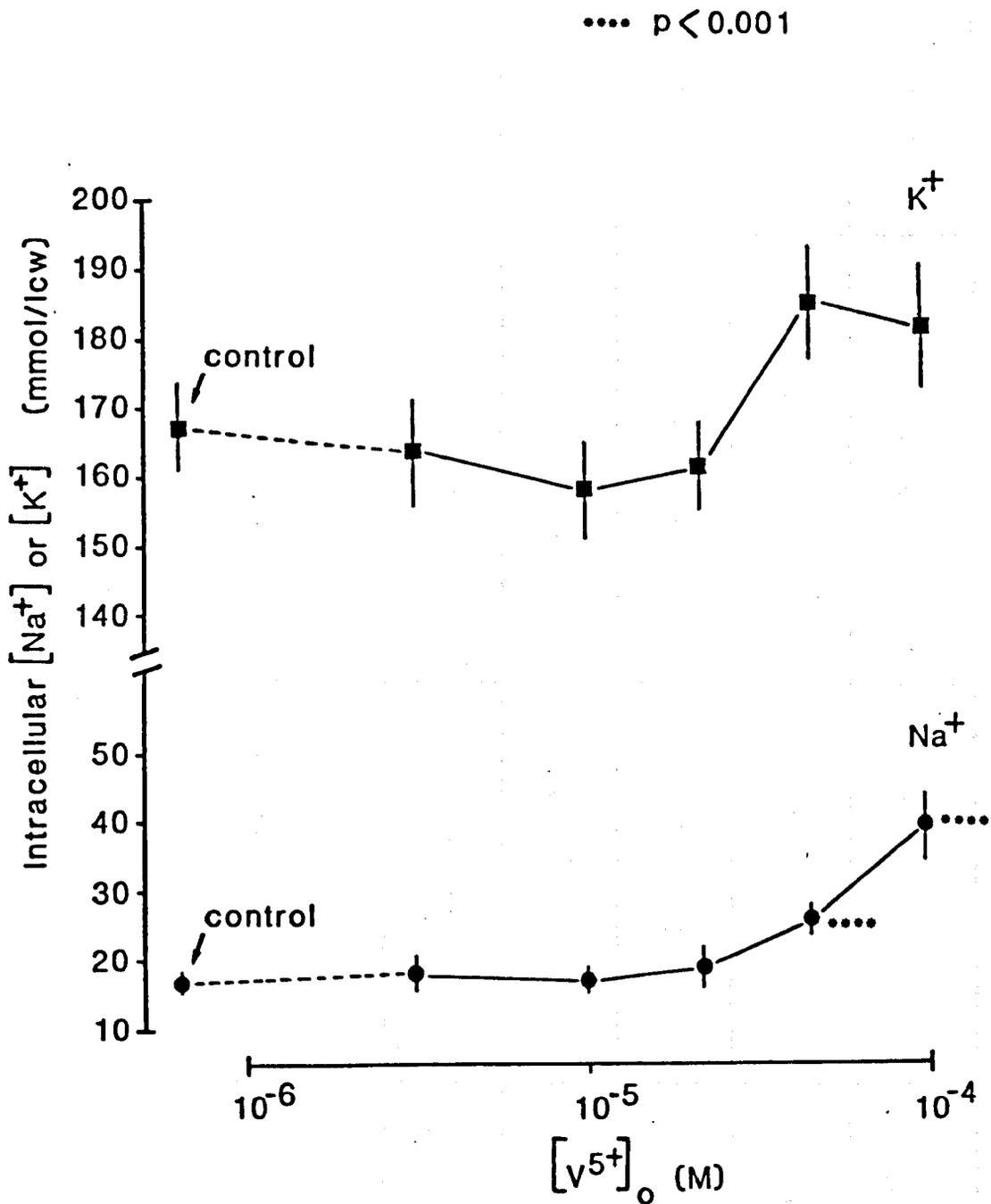


Figure 3.4. Effect of a 24 hour incubation in vanadate-containing medium on the intracellular $[Na^+]$ and $[K^+]$ of HeLa cells

Each point represents the mean \pm S.E.M. of 8 experiments, with each condition being triplicated within each experiment.

Table 3.5. Effect of 24 hours growth in vanadate on the intracellular $[K^+]_i$ of HeLa cells

The values below represent the mean \pm S.E.M. of 8 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test and refers to both columns of results unless otherwise indicated.

$[V^{5+}]_0$ (M)	Mean $[K^+]_i \pm$ S.E.M. (nmol/ 10^6 cells)	Mean $[K^+]_i \pm$ S.E.M. (nmol/lcw)	Level of Significance
0	381.6 ± 15.2	167.3 ± 6.9	-
3.2×10^{-6}	375.3 ± 14.4	163.5 ± 5.6	N.S.
10^{-5}	367.9 ± 13.9	158.1 ± 5.6	N.S.
2.1×10^{-5}	391.5 ± 13.5	161.0 ± 5.4	N.S.
4.6×10^{-5}	456.6 ± 20.4	184.9 ± 8.1	(nmol) $p < 0.01$ (nmol) N.S.
10^{-4}	432.4 ± 21.4	180.9 ± 8.9	(nmol) $p < 0.05$ (nmol) N.S.

- $p < 0.05$
- $p < 0.005$
- $p < 0.001$

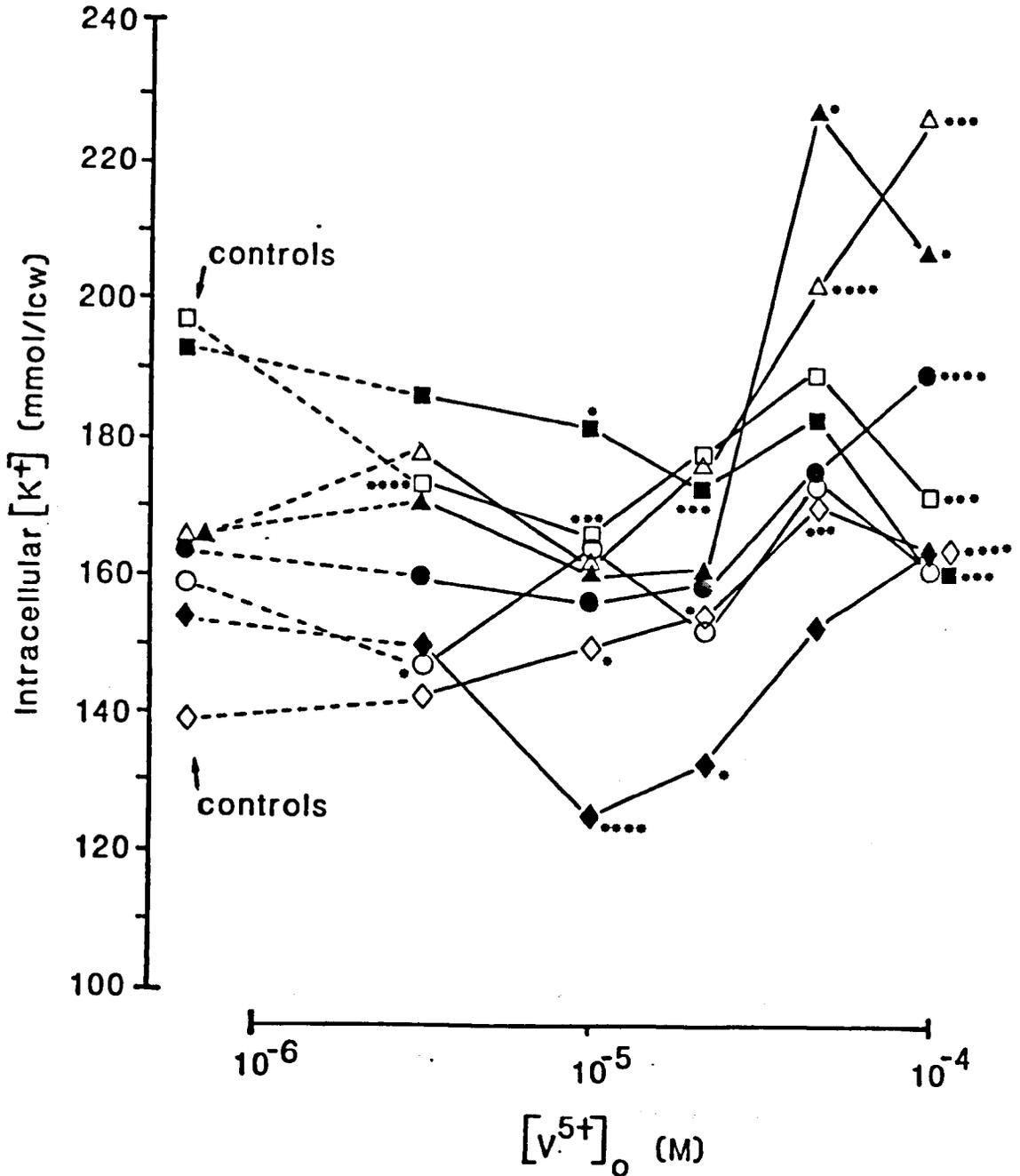


Figure 3.5. Effect of a 24 hour incubation in vanadate-containing medium on the intracellular $[K^+]$ of HeLa cells

Each set of symbols represents the results from one experiment with each point being the mean \pm S.E.M. of triplicate data. The error bars are omitted for clarity but in most cases are less than 3% of the mean value.

were reproducible from experiment to experiment, potassium levels were significantly increased, decreased or remained unaltered at $[V^{5+}]_o$ greater than $10^{-6}M$, depending upon the experiment. The majority of the experiments do, however, tend to show a significant increase in $[K^+]$ at $4.6 \times 10^{-5}M V^{5+}$, decreasing slightly towards control values at $10^{-4}M$. In some cases these changes occurred following a slight decrease in K^+ levels at lower $[V^{5+}]_o$, and hence the resultant level was not significantly different from the control levels. When the results from all the experiments are averaged, vanadate appears to have little effect on intracellular $[K^+]$ (Table 3.5. and Figure 3.4.), due to this variability. No significant changes in either the volume or ion contents of the cells were seen at extracellular concentrations of vanadate from $10^{-9}M$ to $10^{-6}M$ (results not shown).

3.III.ii. Effect of 24 hours incubation in vanadate-containing medium upon the sodium pump numbers (ouabain binding) of HeLa cells

A progressive and significant decrease in the specific ouabain binding (sodium pump number) of HeLa cells was seen at $[V^{5+}]_o$ greater than $10^{-5}M$ although there is not a linear relationship between the dose and response (Table 3.6. and Figure 3.6.). Overall a 20% decrease in pump sites/cell is seen at $10^{-4}M V^{5+}$. The results are not expressed per unit of cell surface area since surface area would have to be calculated from the volume assuming the cells are spherical. However it has been shown that numerous filopodia protrude from the cell (Lamb and McCall 1972).

Table 3.6. Effect of 24 hours growth in vanadate on the specific ouabain binding of HeLa cells

The values below represent the mean \pm S.E.M. of 5 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test.

$[V^{5+}]_0$ (M)	Mean specific ouabain binding \pm S.E.M. ($\times 10^3$ molecules/cell)	Level of Significance
0	766 \pm 31	-
3.2×10^{-6}	737 \pm 26	N.S.
10^{-5}	698 \pm 19	N.S.
2.1×10^{-5}	666 \pm 19	$p < 0.05$
4.6×10^{-5}	651 \pm 30	$p < 0.05$
10^{-4}	611 \pm 23	$p < 0.005$

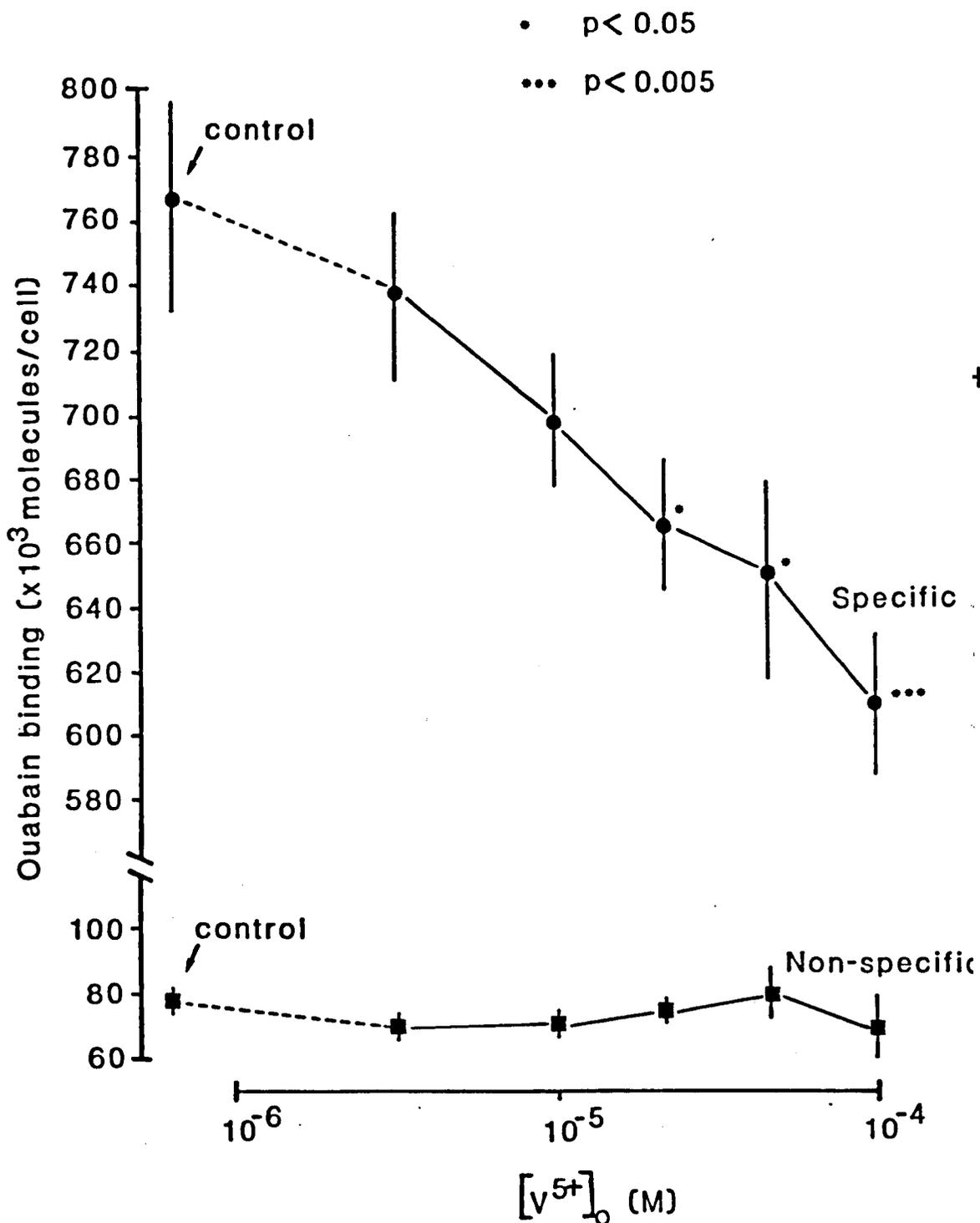


Figure 3.6. Effect of a 24 hour incubation in vanadate-containing medium on the specific and non-specific ouabain binding of HeLa cells

Each point represents the mean \pm S.E.M. of 5 experiments with each condition being triplicated within each experiment.

In addition as vanadate produces changes in cell volume, the relationship between volume and surface area of these cells may not remain the same if the morphology of the cells alters which appears to be the case (see Chapter 2). The apparent decrease in sodium pump numbers appears to be a real effect as the time course of ouabain binding was the same in both control and vanadate-treated cells (Figure 3.7.) indicating that the apparent decrease was not due to a change in binding kinetics. No significant change in non-specific ouabain binding was seen with vanadate treatment (Table 3.7. and Figure 3.6.). The slight increase in binding at $4.6 \times 10^{-5} \text{M V}^{5+}$ followed by a slight decrease at 10^{-4}M V^{5+} (although not significantly different from the control), parallels the changes in cell volume, and hence possibly surface area, seen at these vanadate concentrations.

No significant changes in specific or non-specific ouabain binding were seen at extracellular vanadate levels from 10^{-9}M to 10^{-6}M (results not shown).

3.III.iii. Effect of 24 hours incubation in vanadate-containing medium on the K^+ influx pathways of HeLa cells

At $[\text{V}^{5+}]_0$ greater than 10^{-6}M , the total K^+ influx of HeLa cells was significantly reduced (Table 3.8. and Figure 3.8.). The magnitude of the decrease was dose-dependent although not in a linear fashion, and ranged from a 13% decrease at $3.2 \times 10^{-6} \text{M V}^{5+}$ to a 30% (if expressed as mmol/lcw/min) or 18% (if expressed as $\text{nmol}/10^6 \text{ cells/min}$) decrease at 10^{-4}M V^{5+} . This decrease was due to decreases, similar in magnitude to those above, in the ouabain-sensitive, chloride-dependent, and passive leak K^+ influx

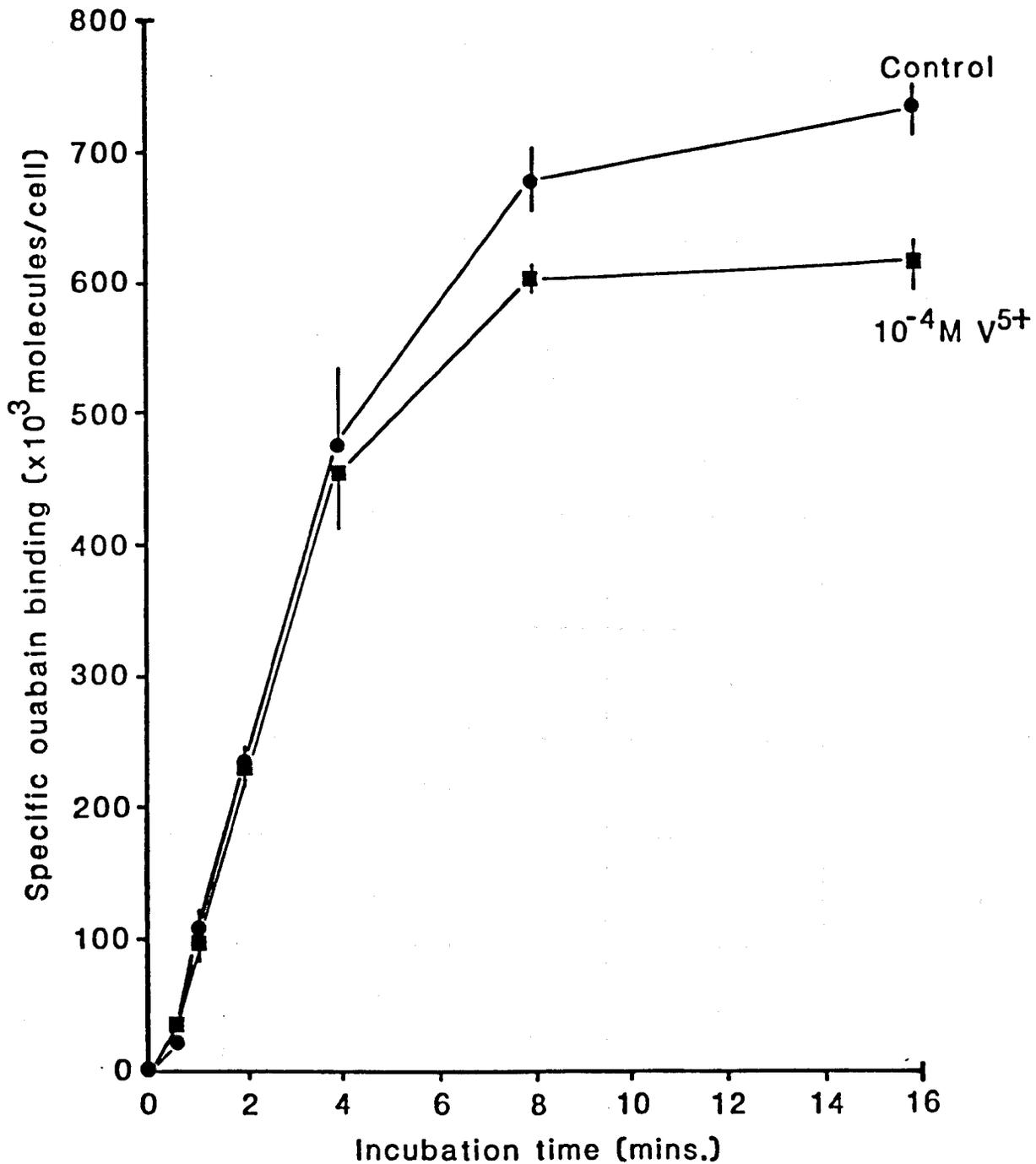


Figure 3.7. Effect of a 24 hour incubation in vanadate-containing medium on the time course of the specific ouabain binding of HeLa cells

The experiment was performed 4 times and the graph illustrates the results from one representative experiment. Each point represents the mean \pm S.E.M. of triplicate data within the experiment.

Table 3.7. Effect of 24 hours growth in vanadate on the non-specific ouabain binding of HeLa cells

The values below represent the mean \pm S.E.M. of 5 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test.

$[V^{5+}]_0$ (M)	Mean non-specific ouabain binding \pm S.E.M. ($\times 10^3$ molecules/cell)	Level of Significance
0	78 ± 5	-
3.2×10^{-6}	70 ± 5	N.S.
10^{-5}	71 ± 5	N.S.
2.1×10^{-5}	75 ± 4	N.S.
4.6×10^{-5}	80 ± 8	N.S.
10^{-4}	70 ± 9	N.S.

Table 3.8. Effect of 24 hours growth in vanadate on the total K^+ influx of HeLa cells

The values below represent the mean \pm S.E.M. of 6 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test and refers to both columns of results unless otherwise indicated.

$[V^{5+}]_0$ (M)	Mean K^+ influx \pm S.E.M. (nmol/ 10^6 cells/min)	Mean K^+ influx \pm S.E.M. (mmol/lcw/min)	Level of Significance
0	9.80 \pm 0.47	4.89 \pm 0.22	-
3.2×10^{-7}	9.06 \pm 0.19	4.51 \pm 0.12	N.S.
10^{-6}	9.46 \pm 0.41	4.67 \pm 0.16	N.S.
3.2×10^{-6}	8.57 \pm 0.28	4.25 \pm 0.16	$p < 0.05$
10^{-5}	8.24 \pm 0.19	4.02 \pm 0.15	(nmol) $p < 0.01$ (mmol) $p < 0.005$
2.1×10^{-5}	8.02 \pm 0.30	3.59 \pm 0.15	(nmol) $p < 0.005$ (mmol) $p < 0.001$
4.6×10^{-5}	8.55 \pm 0.62	3.52 \pm 0.24	(nmol) N.S. (mmol) $p < 0.005$
10^{-4}	8.03 \pm 0.55	3.40 \pm 0.17	(nmol) $p < 0.05$ (mmol) $p < 0.001$

Table 3.8. Effect of 24 hours growth in vanadate on the total K^+ influx of HeLa cells

The values below represent the mean \pm S.E.M. of 6 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test and refers to both columns of results unless otherwise indicated.

$[V^{5+}]_0$ (M)	Mean K^+ influx \pm S.E.M. (nmol/ 10^6 cells/min)	Mean K^+ influx \pm S.E.M. (mmol/lcw/min)	Level of Significance
0	9.80 \pm 0.47	4.89 \pm 0.22	-
3.2×10^{-7}	9.06 \pm 0.19	4.51 \pm 0.12	N.S.
10^{-6}	9.46 \pm 0.41	4.67 \pm 0.16	N.S.
3.2×10^{-6}	8.57 \pm 0.28	4.25 \pm 0.16	$p < 0.05$
10^{-5}	8.24 \pm 0.19	4.02 \pm 0.15	(nmol) $p < 0.01$ (mmol) $p < 0.005$
2.1×10^{-5}	8.02 \pm 0.30	3.59 \pm 0.15	(nmol) $p < 0.005$ (mmol) $p < 0.001$
4.6×10^{-5}	8.55 \pm 0.62	3.52 \pm 0.24	(nmol) N.S. (mmol) $p < 0.005$
10^{-4}	8.03 \pm 0.55	3.40 \pm 0.17	(nmol) $p < 0.05$ (mmol) $p < 0.001$

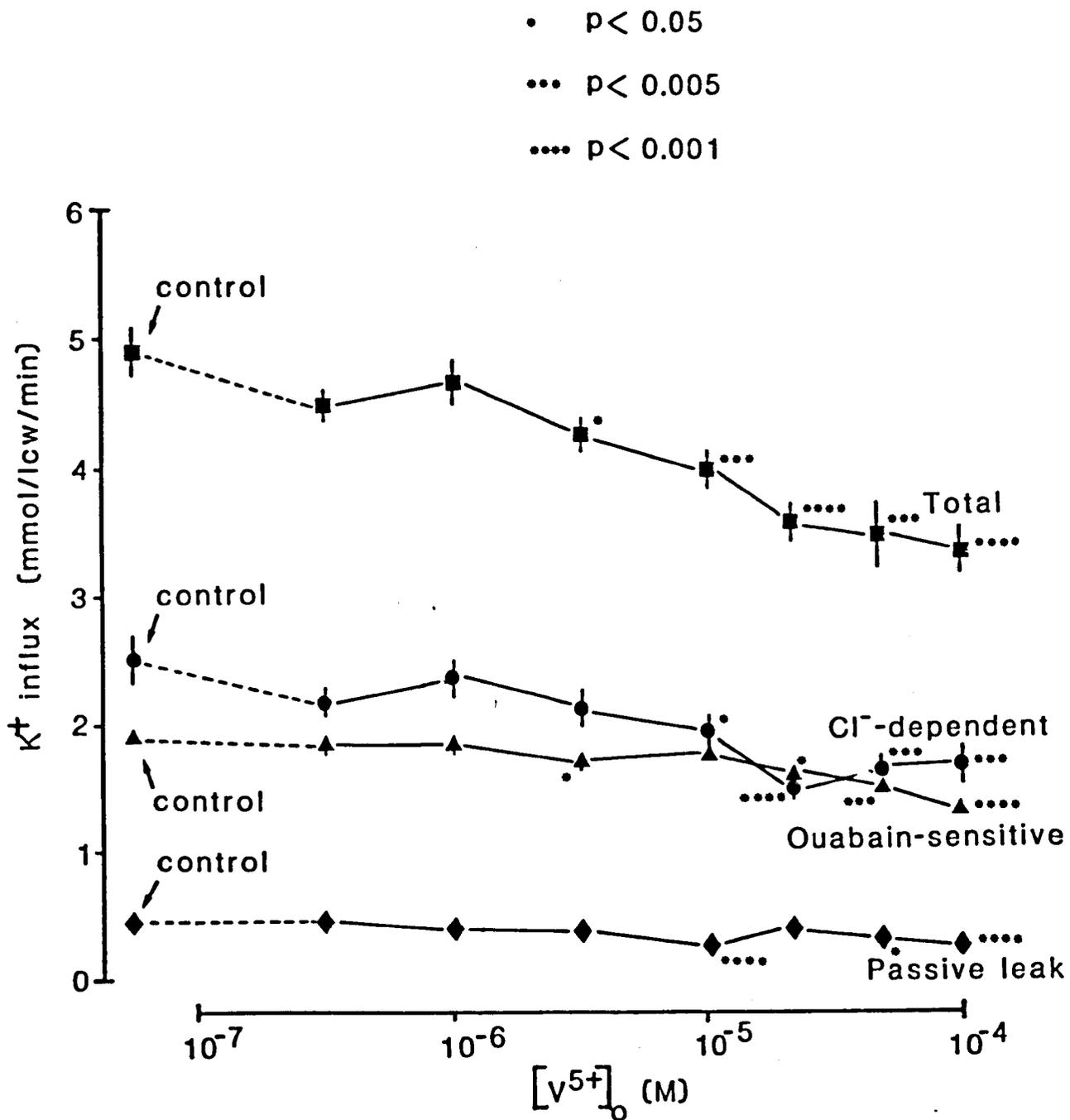


Figure 3.8. Effect of a 24 hour incubation in vanadate-containing medium on the total, chloride-dependent, ouabain-sensitive, and passive leak K⁺ influx pathways of HeLa cells

Each point represents the mean ± S.E.M. of 6 experiments with each condition being triplicated within each experiment.

pathways. Because the decrease was proportionately similar in all of these pathways, their individual contribution to the total flux remained the same at approximately 40%, 50% and 10% respectively (Tables 3.9., 3.10., 3.11., and Figure 3.8.). This was not the case at every level of vanadate treatment however e.g. the chloride-dependent pathway was inhibited to a greater extent than the other pathways at $2.1 \times 10^{-5} \text{M V}^{5+}$.

The ouabain-sensitive K^+ influx was significantly decreased at $3.2 \times 10^{-6} \text{M V}^{5+}$ and at $2.1 \times 10^{-5} \text{M} - 10^{-4} \text{M V}^{5+}$ when expressed as mmol/lcw/min , but only at 3.2×10^{-6} and 10^{-4}M V^{5+} when expressed as $\text{nmol}/10^6 \text{ cells/min}$ (Table 3.9. and Figure 3.8.). The chloride-dependent K^+ influx was significantly decreased at $[\text{V}^{5+}]_o$ greater than $3.2 \times 10^{-6} \text{M}$, irrespective of whether expressed as per lcw or per 10^6 cells (Table 3.10. and Figure 3.8.). The most significant decrease in the chloride-dependent flux was seen at $2.1 \times 10^{-5} \text{M V}^{5+}$ as already mentioned. Significant decreases in the passive leak K^+ influx were seen at 10^{-5}M , $4.6 \times 10^{-5} \text{M}$ and 10^{-4}M V^{5+} (Table 3.11. and Figure 3.8.) with the largest decrease at 10^{-5}M . Similar results were seen if the cotransport flux was measured using bumetanide rather than NO_3^- -Krebs. DIDS had no effect on the K^+ influx. These results indicate that the decrease was in the diuretic-sensitive cotransport flux and not in any other chloride-dependent potassium influx which may have been present in these cells.

No significant changes in any of the K^+ influx pathways were seen at extracellular vanadate concentrations from 10^{-9}M to 10^{-7}M (results not shown).

The mean K^+ influx per pump site is ideally determined by

Table 3.9. Effect of 24 hours growth in vanadate on the ouabain-sensitive K^+ influx of HeLa cells

The values below represent the mean \pm S.E.M. of 6 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test and refers to both columns of results unless otherwise indicated.

$[V^{5+}]_0$ (M)	Mean ouabain-sensitive K^+ influx \pm S.E.M. (nmol/ 10^6 cells/min)	Mean ouabain-sensitive K^+ influx \pm S.E.M. (nmol/1cw/min)	Level of Significance
0	3.78 ± 0.15	1.92 ± 0.08	-
3.2×10^{-7}	3.68 ± 0.15	1.87 ± 0.07	N.S.
10^{-6}	3.72 ± 0.21	1.86 ± 0.08	N.S.
3.2×10^{-6}	3.34 ± 0.12	1.70 ± 0.06	$p < 0.05$
10^{-5}	3.90 ± 0.18	1.78 ± 0.10	N.S.
2.1×10^{-5}	3.63 ± 0.13	1.66 ± 0.07	(nmol) N.S. (mmol) $p < 0.05$
4.6×10^{-5}	3.63 ± 0.16	1.54 ± 0.06	(nmol) N.S. (mmol) $p < 0.005$
10^{-4}	3.28 ± 0.20	1.37 ± 0.07	(nmol) $p < 0.05$ (mmol) $p < 0.001$

Table 3.10. Effect of 24 hours growth in vanadate on the Cl^- -dependent K^+ influx of HeLa cells

The values below represent the mean \pm S.E.M. of 6 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test and refers to both columns of results unless otherwise indicated.

$[\text{V}^{5+}]_0$ (M)	Mean Cl^- -dependent K^+ influx \pm S.E.M. (nmol/ 10^6 cells/min)	Mean Cl^- -dependent K^+ influx \pm S.E.M. (nmol/1cw/min)	Level of Significance
0	5.02 ± 0.33	2.52 ± 0.18	-
3.2×10^{-7}	4.45 ± 0.12	2.22 ± 0.11	N.S.
10^{-6}	4.82 ± 0.30	2.40 ± 0.17	N.S.
3.2×10^{-6}	4.35 ± 0.25	2.15 ± 0.15	N.S.
10^{-5}	3.74 ± 0.25	1.93 ± 0.14	(nmol) $p < 0.01$ (mmol) $p < 0.05$
2.1×10^{-5}	3.49 ± 0.19	1.54 ± 0.10	(nmol) $p < 0.005$ (mmol) $p < 0.001$
4.6×10^{-5}	4.05 ± 0.42	1.63 ± 0.18	(nmol) $p < 0.05$ (mmol) $p < 0.005$
10^{-4}	3.99 ± 0.39	1.71 ± 0.14	(nmol) $p < 0.05$ (mmol) $p < 0.005$

Table 3.11. Effect of 24 hours growth in vanadate on the passive leak K^+ influx of HeLa cells

The values below represent the mean \pm S.E.M. of 6 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test and refers to both columns of results unless otherwise indicated.

$[V^{5+}]_0$ (M)	Mean passive leak K^+ influx \pm S.E.M. (nmol/ 10^6 cells/min)	Mean passive leak K^+ influx \pm S.E.M. (nmol/lcw/min)	Level of Significance
0	1.0 ± 0.08	0.45 ± 0.03	-
3.2×10^{-7}	0.92 ± 0.07	0.43 ± 0.02	N.S.
10^{-6}	0.92 ± 0.09	0.42 ± 0.03	N.S.
3.2×10^{-6}	0.88 ± 0.10	0.40 ± 0.04	N.S.
10^{-5}	0.68 ± 0.05	0.31 ± 0.02	(nmol) $p < 0.005$ (mmol) $p < 0.001$
2.1×10^{-5}	0.90 ± 0.07	0.39 ± 0.03	N.S.
4.6×10^{-5}	0.87 ± 0.08	0.35 ± 0.03	(nmol) N.S. (mmol) $p < 0.05$
10^{-4}	0.77 ± 0.06	0.32 ± 0.02	(nmol) $p < 0.05$ (mmol) $p < 0.001$

measuring the ouabain-sensitive influx and dividing it by the sodium pump numbers determined during the same experiment. This was impossible in this study due to the magnitude of such an experiment. Hence the mean K^+ influx through the sodium pump was determined by dividing the individual ouabain-sensitive flux results by the overall mean sodium pump number for each vanadate treatment (Table 3.12.). Similar results were obtained if the mean flux results were divided by the individual ouabain-binding results. As the ouabain binding and flux experiments had been carried out alternately during the period of this part of the study, the effect of any possible external variable in influencing preferentially one parameter rather than the other during, for example, the first half of this period, was kept to a minimum.

There appears to be small but significant increases in the flux per pump site at extracellular vanadate concentrations of $10^{-5}M$ to $4.6 \times 10^{-5}M$ with a non-significant increase at $10^{-4}M$ v^{5+} . Such changes are due to the greater decreases seen in sodium pump numbers compared with those seen in the ouabain-sensitive flux per cell.

Table 3.12. Effect of 24 hours growth in vanadate on the K^+ influx per sodium pump molecule in

HeLa cells

The values below were obtained by dividing the ouabain-sensitive K^+ influx results from 6 experiments (with triplicates for each treatment group) by the mean ouabain-binding values and are the mean \pm S.E.M. Significance testing relative to the control group is by Student's t-test.

$[V^{5+}]_0$ (M)	Mean K^+ influx per sodium pump molecule \pm S.E.M. (K^+ ions/pump site/min)	Level of Significance
0	2972 \pm 121	-
3.2×10^{-6}	2730 \pm 100	N.S.
10^{-5}	3362 \pm 152	$p < 0.05$
2.1×10^{-5}	3281 \pm 119	$p < 0.05$
4.6×10^{-5}	3360 \pm 150	$p < 0.05$
10^{-4}	3232 \pm 200	N.S.

3.IV. DISCUSSION

The majority of the results reported here show only slight, although significant, changes in the parameters studied as a result of a 24 hour incubation in vanadate-containing medium. This is not unexpected in view of the fact that it has been shown in the previous chapter that most if not all of the intracellular vanadate is reduced to vanadyl within this time period, and vanadyl is thought to be less biologically active generally than vanadate. It is actually unlikely that vanadate is present in any significant quantities in the HeLa cells, as if the reducing capability of the cells was exceeded and vanadate accumulated even at nanomolar levels, a similar degree of inhibition of the Na^+/K^+ -ATPase would be expected as that occurring in isolated enzyme preparations (Josephson and Cantley, Jr. 1977). Such an inhibition is not apparent in this study.

The biological actions of vanadyl are even less well established than those of vanadate owing to its instability under physiological conditions, unless bound within the cell. It is also difficult to differentiate quantitatively between vanadate and vanadyl at the low concentrations at which specific biological effects may occur. Indeed many cellular actions previously attributed to vanadate may actually be caused by vanadyl. Vanadyl inhibits Na^+/K^+ -ATPase, although only in very pure enzyme preparations (North and Post 1984). In addition, in rat adipocytes a stimulation of glucose oxidation by vanadyl has been reported (Shechter and Karlisch 1980). The intracellular reduction of vanadate to vanadyl together with the potential

biological effects of vanadyl obviously makes the interpretation of the results in this study more difficult.

The slight but significant changes in volume are first seen at a lower concentration of vanadate ($10^{-5}M$) than that which produces a significant change in intracellular ion concentration. The reason for this discrepancy is not clear but can not be accounted for on the basis of the variability of the intracellular $[K^+]$ between experiments. When the results from individual experiments are examined, no changes in $[K^+]$ at $10^{-5}M$ V^{5+} are seen. Similarly the uptake of vanadium by the cells (see Chapter 2) would not have produced an increase in osmotic pressure which would cause a significant volume change. The variability of the intracellular $[K^+]$ values from experiment to experiment is also difficult to explain when the remainder of the parameters seem to show relatively consistent changes. Results expressed either in terms of cell number or per litre of cell water may differ in their significance relative to the control group. This is due to the differences in cell volume at the higher vanadate concentrations.

The changes in intracellular ions in this study can be further analysed when examined in conjunction with the results obtained for ouabain binding and K^+ influx. Before this is done however, some necessary assumptions which have had to be made and some disadvantages of the techniques employed are discussed.

When using monolayer cultures of cells in radioisotopic binding or uptake experiments, the assumption is made that the bathing medium has equal access to both sides of the monolayer. This assumption appears to be justified when using HeLa cells

since the ouabain binding of intact cells is not significantly different to that of a membrane preparation (Baker and Willis 1972).

The difficulties involved in measuring the different flux pathways have been mentioned briefly in Section 3.II.iv.. Inaccuracies can arise either from a stimulation of the cotransport flux by ouabain as a result of sodium pump inhibition (Aiton and Simmons 1983), or alternatively from the non-specific inhibitory actions of the loop-diuretics, particularly furosemide, on the sodium pump (Wiley and Cooper 1974) and the DIDS-sensitive anion transport pathway (Geck et al. 1981). These effects are seen over longer time periods than are commonly used in flux experiments and can be largely circumvented by using the higher affinity loop-diuretic, bumetanide, at lower concentrations where non-specific effects are less evident. Alternatively, ouabain together with nitrate medium (where the chloride has been replaced by nitrate) can be used. Nitrate is an anion which behaves in a similar fashion to Cl^- with the notable exception that it is not transported by the diuretic-sensitive cotransport pathway which it inhibits. Like Cl^- , nitrate is distributed passively in accordance with the membrane potential although equilibration may not occur as rapidly, resulting in transient fluctuations in membrane potential. The use of Cl^- to measure the diuretic-sensitive flux would not be appropriate in cells which possess other significant Cl^- -dependent fluxes but this is not the case in HeLa cells (Aiton and Simmons 1983).

Since the experiments have been performed in this study,

concern has been expressed in several laboratories about the possible production of free radicals in nitrate-containing incubation medium (Aiton - personal communication). In this study the decrease seen in the Cl⁻-dependent K⁺ influx was also shown to occur in the bumetanide-sensitive K⁺ influx and DIDS was without effect. These results illustrate the equivalence of the Cl⁻-dependent flux and the diuretic-sensitive flux, and the absence of any other Cl⁻-dependent K⁺ influx pathways. The use of bumetanide and DIDS has confirmed the results obtained with nitrate-containing medium in this study and ruled out the possible ambiguity. Retrospectively, the use of bumetanide may be a better option and potentially less likely to produce problems.

The lack of a significant effect of vanadate on the non-specific ouabain binding would be in accord with the small (though significant) changes in volume and hence surface area of the cells. The decrease in sodium pump numbers however, as measured by specific ouabain binding, together with the subsequent slight but significant decrease in the ouabain-sensitive K⁺ influx, may account for the increased intracellular [Na⁺]. Although the possibility that vanadate may have altered the affinity of the pumps for ouabain binding can not be ruled out without further experimentation, at the concentration of ouabain used here the time course of binding appears to be very similar in vanadate-treated and control cells, indicating a real decrease in pump number.

The interpretation of the results is complicated by the toxic effects of vanadate/vanadyl described in Chapter 2, which

together with other possible indirect actions, may mask any direct actions of vanadate/vanadyl. The results obtained may be a combination of all these possibilities. For example, although a decrease in the ouabain-sensitive K^+ influx was described, the actual flux per pump was increased as the concomitant decrease in sodium pump numbers was significantly greater. This stimulation of flux per pump (approx. 10% increase - from 50 K^+ ions transported per pump per second to 56 per pump per second), was probably caused by the increase in intracellular $[Na^+]$ resulting from the decrease in total pump flux. However on the basis of the known kinetics of activation of the sodium pump by the elevated intracellular Na^+ , an increase in flux per pump of approximately 50% would have been expected (De Weer 1985). This discrepancy between the predicted results and actual findings indicates an inhibition of the ouabain-sensitive flux by vanadate/vanadyl, which is partially masked by the stimulation of the flux by the raised intracellular $[Na^+]$. However the stimulation of flux per pump site was also seen at vanadate concentrations which produced no change in internal sodium levels. An alternative explanation is a possible stimulatory action of vanadate/vanadyl on the ouabain-sensitive K^+ influx, as demonstrated by Werdan et al. (1980, 1982) using heart muscle and non-muscle cells prepared from neo-natal rats, and Girardi human heart cells. If this were to occur in HeLa cells at the lower extracellular vanadate concentrations ($10^{-5}M$), with inhibitory actions predominating at the higher concentrations when a stimulation due to raised sodium levels may also occur, this could account for the changes seen.

The increased intracellular sodium levels would in turn have been expected to produce only a slight, if any, up-regulation of pump numbers at the concentrations of sodium seen in this study. For example, with an ethacrynate-induced increase in intracellular sodium concentration from 13 mM to 60 mM, the specific ouabain binding of HeLa cells was only increased by 39% (Boardman et al. 1974). Whether a slight upregulation has occurred in this study but is masked by the toxic or specific action of vanadate/vanadyl in decreasing the sodium pump numbers is not clear. Alternatively, possible up-regulation may not occur if the toxicity seen in this study occurs as a result of a generalised inhibition of protein synthesis. Such an inhibition may also account for the decrease seen in pump sites.

The minimal effect of vanadate incubation on the ouabain-sensitive K^+ influx per cell which was similar to that described in rat vascular smooth muscle cells cultured in vitro (Searle et al. 1983), was not due to low intracellular vanadium concentrations (see Chapter 2). It is likely that it was due to the reduction of the vanadate to vanadyl as already discussed in Chapter 2. However there is also the possibility that the Na^+/K^+ -ATPase enzyme in HeLa cells is resistant to vanadate. In an attempt to establish that this was not the case, HeLa cell membranes were prepared and assayed for ATPase activity. However the ouabain-sensitive Na^+/K^+ -ATPase activity was only a very small fraction of the total ATPase activity present and could not be studied in such a crude preparation. Although it is relatively easy to purify up this enzyme in tissue homogenates owing to the large amount of material present, it is much more

difficult in cultured cells and this line of research was not pursued further. Insensitivity to vanadate must therefore remain a possibility, although this was not the case with the isolated Na^+/K^+ -ATPase from rat vascular smooth muscle cells for example, despite the lack of effect of vanadate on ouabain-sensitive ion transport in the intact cell (Searle et al. 1983).

The diuretic-sensitive K^+ influx was also decreased in the vanadate-treated cells although with greater significance statistically than the decreases seen in the ouabain-sensitive flux. Without measurements of the diuretic-sensitive K^+ efflux it is impossible to determine whether the decrease in cotransport flux is a bidirectional decrease, or a unidirectional decrease leading to a lowering of the intracellular $[\text{K}^+]$. If the former is the case, the decrease in the diuretic-sensitive flux seen in the slightly swollen vanadate-treated HeLa cells may be the opposite situation to the bidirectional increase in flux seen in HeLa cells when shrunken (Tivey 1986), although the function of it is not known. Such alterations in diuretic-sensitive flux may be volume regulatory although this is normally associated with an increased efflux of KCl in swollen cells (Tivey 1986).

If however the decrease in diuretic-sensitive flux is unidirectional then it may be occurring in order to maintain K^+ homeostasis as proposed by Duhm and Gobel (1984). This would imply that it is occurring to counteract an increased intracellular $[\text{K}^+]$ and therefore is not likely to be the explanation in this study. Alternatively the changes in diuretic-sensitive flux may be non-specific effects seen as the result of the toxicity.

The very significant decrease in the passive leak K^+ influx occurring at an extracellular vanadate concentration of $10^{-5}M$ may be indicative of a reduced membrane permeability to K^+ or of a perturbation of the membrane potential mediated by, for example, an increased membrane permeability to Na^+ ions. However this can not be reconciled with the hyperpolarisation seen in various cultured cell types with vanadate treatment (Lichtstein et al. 1982). The experiments in this thesis however utilised much longer incubation times. The accumulation of vanadate within the cells as vanadyl is not likely to have a direct effect on the membrane potential itself at the concentrations present in this study. It seems strange that the effects on the passive leak K^+ influx occur predominantly at one single concentration of vanadate, returning towards control values before decreasing slightly again. It is possible that this change in the passive leak K^+ influx may be related to the toxicity as it is seen at a vanadate concentration where effects on cell growth are first produced, although non-significantly. Alternatively the decrease in flux may possibly reflect some change in the cell occurring as a consequence of the toxicity, or may be involved in the causation of the toxicity.

In summary therefore, it is impossible to divide the results shown here into those arising from direct or indirect actions of vanadate/vanadyl. In addition, the reduction of vanadate to vanadyl and the toxic effects seen are all confounding factors. In view of the toxicity (see Chapter 2) which appears to be the predominant effect, it is likely that many of the changes seen in this study are secondary to toxicity. Additionally some of the

effects may be secondary to ATP depletion for example, which would directly affect the sodium pump and indirectly the diuretic-sensitive cotransport pathway (Saier, Jr. and Boyden 1984). This possibility is explored and discussed in greater detail in the following chapter.

A possible interaction between cell number and transmembrane flux can not be excluded since the magnitude of the flux pathways varies slightly with the day of culture normally. Whether this is due to the actual differences in cell number per plate or is due to the phase of growth (i.e. whether logarithmic or stationary) on the different days of culture has not been established. In either case such a variation is only slight and would not be sufficient to account for the changes in flux seen here with vanadate treatment, despite the varying cell numbers with treatment level.

The overall conclusion based on the results presented in Chapters 2 and 3 is that the changes seen after vanadate incubation are probably largely due to vanadyl and may occur as a result of secondary and/or toxic effects, rather than any specific inhibitory actions on the transport pathways. A combination of these actions is possible however. The cause of the toxicity has been speculated upon already in Chapter 2.

CHAPTER 4. MEASUREMENT OF INTRACELLULAR NUCLEOTIDES IN HELA
CELLS BEFORE AND AFTER CHRONIC VANADATE TREATMENT, USING HIGH
PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

4.I. INTRODUCTION

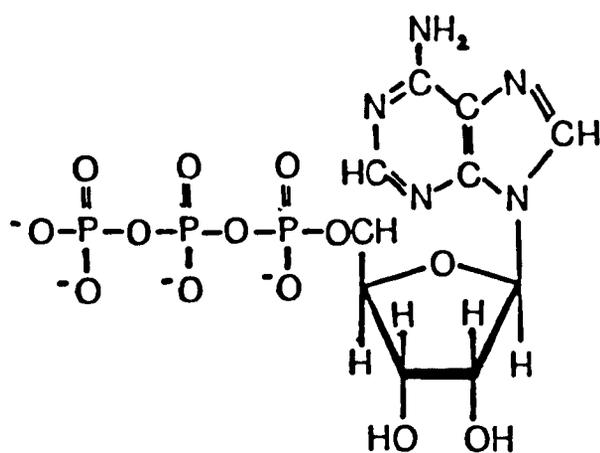
4.I.i. Biological importance of nucleotides

Nucleotides, the phosphate esters of nucleosides (a purine or pyrimidine base linked to the pentose sugars D-ribose or 2-deoxy-D-ribose), are important in many biochemical processes. For example, they act as metabolic regulators, are precursors of the nucleic acids DNA and RNA, and ATP is the universally important energy-rich compound (Stryer 1981). Structural examples are given in Figure 4.1..

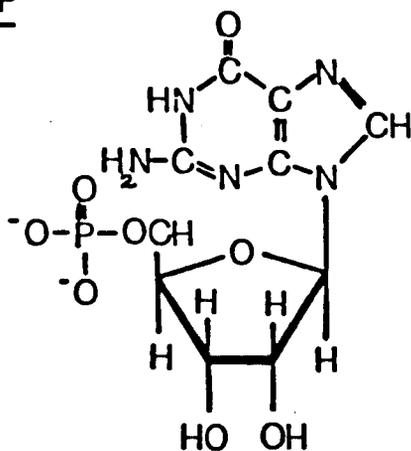
The role of metabolism can be broadly defined as the production of ATP, NADPH (to provide reducing power for the biosynthesis of cell components from more oxidised precursors), and macromolecular precursors. Metabolic regulation is largely achieved by controlling the amounts of key enzymes, controlling their catalytic activity (e.g. either through allosteric inhibition or activation of the enzyme by a product, or through covalent modifications of the enzyme, such as phosphorylation) and by the compartmentalisation of the synthetic and degradative pathways (Stryer 1981). The cellular energy status is also important in metabolic regulation, although the concentration of ATP alone has been found to be inadequate as a reflection of the cellular energy balance (Sauer 1978, Matsumoto et al. 1979). A more generally accepted index of energy status is the adenylate energy charge, proposed by Atkinson and Walton in 1967 where:

$$\text{Energy charge} = \frac{[\text{ATP}] + 0.5 [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

ATP



GMP



CDP

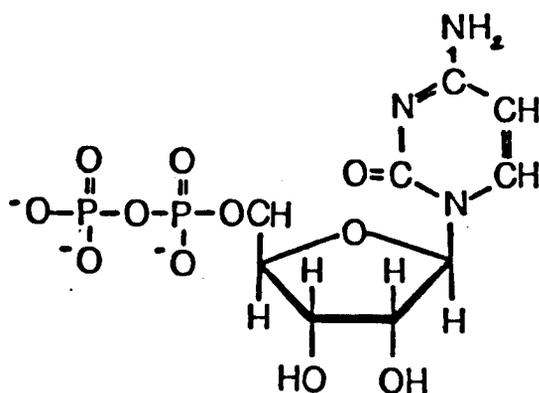


Figure 4.1. Structural examples of nucleotides

Under control conditions this is normally in the range of 0.8 to 0.95 for most cells. As the conservation of ATP is a major feature of metabolic regulation, a logical consequence of an increase in energy charge would be the inhibition of ATP-generating pathways and/or the stimulation of ATP-utilising pathways and this is indeed what occurs e.g. the affinity of phosphofructokinase for fructose-6-phosphate is decreased by ATP and enhanced by AMP or ADP depending upon the species (Atkinson and Walton 1967). This response to a change in energy charge is also increased as a result of feedback inhibition by citrate (Shen et al. 1968).

Significant physiological correlation has been found in cultured fibroblasts, between the energy charge and cellular viability under starvation conditions (Calderwood et al. 1985). No correlation was found however, between the energy charge and cellular viability following exposure to heat. Matsumoto et al. (1979) found a correlation between energy charge and the dephosphorylation of AMP in cultured lymphoblastoid cell lines, following the depletion of ATP as a result of the inhibition of glycolysis and cellular respiration. This dephosphorylation did not correlate with an elevated AMP concentration and appeared to occur as a homeostatic mechanism, restoring the normal energy charge. It occurred only when the adenylate energy charge decreased to less than 0.6. Further examples of the involvement of the energy charge in metabolic regulation have been discussed by Atkinson (1968).

4.I.ii. Nucleotides and ion transport

The dependence of the sodium pump (Na^+/K^+ -ATPase) upon ATP has been well documented (for reviews see e.g. Baker 1966, Glynn 1968, Glynn and Karlsh 1975, De Weer 1985), albeit that different isozymes exist which have differing affinities for ATP (Sweadner 1985). Although it was originally thought that approximately 50% of the body's total ATP production was used by the Na^+/K^+ -ATPase enzyme in maintaining cellular ion gradients, this concept was based on measurements made on cut or homogenised tissues. More recent measurements made using intact tissues, organs or limbs have yielded lower estimates which are generally nearer 10% than 50% (De Weer 1985).

Few studies have been carried out investigating the kinetics of the activation of sodium transport by ATP in intact cells, due to the experimental difficulties involved. Using dialysed squid axons, Beauge and DiPolo (1981) showed that the activation of Na^+ efflux by ATP appeared to follow Michaelis-Menten kinetics with a K_m for ATP of 0.2 mM under physiological conditions. Similar results were found using canine cardiac sarcolemmal vesicles (Philipson and Nishimoto 1983) with a K_m value for ATP of 0.21 mM. A competitive inhibition of ATP-dependent Na^+ transport by ADP was also seen. It should be noted however, that in isolated Na^+/K^+ -ATPase preparations, biphasic Lineweaver-Burke plots have been found indicating the existence of both high affinity sites and low affinity sites for ATP, e.g. K_m values for ATP were approximately 1 μM and 0.5 mM respectively in rat brain preparations (Robinson 1976) and ADP or CTP were both found to competitively inhibit the ATP-dependent activation of Na^+/K^+ -

ATPase. Whether the high affinity and low affinity sites are two distinct sites or are different states of the same catalytic site is still the subject of much debate (e.g. Cantley, Jr. et al. 1983, Askari and Huang 1985).

From the evidence presented recently in a review by Saier, Jr. and Boyden (1984), it appears that the diuretic-sensitive cotransport pathway also shows an ATP-dependency. However rather than a stoichiometric relationship between ATP hydrolysis and ion transport, it appears that ATP functions to activate the system, possibly by a protein kinase-catalysed phosphorylation mechanism which in some cell types is cAMP dependent. This has been discussed in the previous chapter. Further experimental evidence is needed to validate this hypothesis however.

4.I.iii. Vanadate and phosphoryl transfer enzymes

Vanadate has been shown to have inhibitory actions on Ca^{2+} - and Na^+/K^+ -ATPases both in membrane preparations and in whole cells from many tissues (for reviews see Ramasarma and Crane 1981, Jandhyala and Hom 1983, Erdmann et al. 1984), whilst in previous chapters an inhibitory action on both the Na^+/K^+ -ATPase and diuretic-sensitive cotransport activities of HeLa cells has been demonstrated, although these may be non-specific effects as cytotoxicity also occurred at these vanadate concentrations. Inhibition by vanadate of some phosphoryl transfer enzymes involved in glucose metabolism has also been reported. Examples include rat liver microsomal glucose-6-phosphatase which was totally inhibited at endogenous vanadate levels (Singh et al. 1981), yeast phosphoglucomutase and phosphoglycerate mutase which

were totally inhibited by micromolar concentrations of vanadate, and rabbit muscle hexokinase and phosphoglycerate kinase which were only partially inhibited (Climent et al. 1981). In red blood cells vanadate caused a rapid breakdown of 2,3-bisphosphoglycerate which in turn increased cellular pyruvate and the cellular glycolytic flux (Ninfali et al. 1983).

The ability of vanadate to inhibit enzymes which catalyse phosphoryl transfer has been attributed to its adoption of a stable trigonal bipyramidal or distorted octahedral configuration when hydrated or chelated with oxygen, nitrogen or sulphur ligands. This structure is thought to resemble the proposed transition state of phosphate during the reaction (Lopez et al. 1976). However Rubinson (1981) believes that this is not the case as profound chemical differences exist between phosphate and vanadate with regard to the rate of ligand exchange in their coordination complex chemistry.

In addition, vanadyl, which is formed by the reduction of vanadate, possesses many insulin-mimetic effects. As previously described in Chapter 3, both vanadyl and insulin produce an increase in intracellular potassium levels and a subsequent slight hyperpolarisation within minutes in mouse diaphragm muscle at 10^{-6} to 10^{-3} M (Zemkova et al. 1982). The exact mechanism underlying this change is not known although stimulation of the sodium pump or effects on intracellularly bound potassium were proposed as possibilities. The former possibility was unlikely however, due to the lack of effect of removal of extracellular potassium, although ouabain inhibited the above changes.

In rat adipocytes, Shechter and Karlsh (1980) and Degani

et al. (1981) have demonstrated a stimulation of glucose oxidation by extracellularly applied vanadate ($10^{-4}M$). Although vanadate is known to inhibit Na^+/K^+ -ATPase from many cells and inhibition of this enzyme results in a stimulation of glucose oxidation, the stimulation seen in adipocytes was due not to vanadate but to the vanadyl formed by the intracellular reduction of vanadate (Shechter and Karlsh 1980, Degani et al. 1981). In adipocytes the rate of glucose oxidation is thought to be limited by glucose uptake and it appears that vanadyl exerts its action on the glucose uptake. In view of the known inhibitory actions of vanadyl on alkaline phosphatase (Lopez et al. 1976), it was suggested that these effects were due to an inhibitory action of vanadyl (and/or insulin) on a cellular phosphatase which in turn altered the degree of phosphorylation of proteins involved in sugar transport (Shechter and Karlsh 1980). An alternative proposal, based on the fact that in adipocytes at least, GSH is involved in the reduction of vanadate, is that the effects of the vanadate/vanadyl are related to the intracellular redox potential (Degani et al. 1981). If vanadate was to induce a peroxidative metabolism coupled to GSH oxidation which leads finally to an increase in cellular NADP and a decrease in NADPH, this would explain the accelerated flux through the hexose monophosphate shunt. This would not explain however, the stimulation of glucose oxidation also seen via the Embden-Meyerhof pathway (Degani et al. 1981).

As a result of the actions of vanadate/vanadyl illustrated above, it is conceivable that vanadate treatment may produce alterations in the cellular energy charge, and that this in turn

may be at least partially responsible for some of the toxicity seen in Chapter 2. Additionally, significant alterations in nucleotide concentration may also affect ion transport. These possibilities have been investigated here in HeLa cells using high performance liquid chromatography (HPLC) to measure intracellular nucleotide concentration.

4.I.iv. Quantification of nucleotides

Quantification of nucleotides can be achieved using enzymatic techniques such as the bioluminescent luciferin-luciferase reaction for ATP (Strehler 1974). Such assays are highly sensitive and specific but it is only possible to measure one nucleotide in any single assay. With HPLC it is possible to obtain complete or nearly complete profiles of intracellular nucleotides in each analysis, relatively quickly and free from any interfering substances present in cell extracts. A brief introduction of the principles of HPLC follows before the chromatographic separation of nucleotides is presented. Further information can be found in many standard texts, (e.g. Parris 1976, Knox et al. 1978, Pryde and Gilbert 1979, and Simpson 1976).

4.I.v. Historical development of high performance liquid chromatography (HPLC)

In 1941, Martin and Synge published a paper which laid the foundations of gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC). This led to the rapid development of GLC but it was not until the late 1960's that the

first high pressure liquid chromatograms were produced; by operating at higher pressures (5000 psi) the effects of high liquid viscosities relative to gas velocities were overcome and analysis times obtained which were comparable with GLC. The ability to analyse thermally labile compounds together with improvements in HPLC column packing materials leading to greater versatility in application and lower operating pressures, has ensured that HPLC is now used as widely as GLC.

4.1.vi. Chromatographic system

The components of a typical HPLC system are shown in Figure 4.2. Basically the eluent or "mobile phase" from the solvent reservoir is filtered by a short silica-containing precolumn (which also serves to saturate the eluent with silica and hence protect the silica backbone of the main column from erosion) and pumped under pressure through the main column - the "stationary phase". A mixture of solutes injected is separated into individual components on travelling down the column, monitored as they pass through a detector and recorded as peaks on a chart recorder. Peaks can be identified by co-chromatography with standards, comparison of retention times (time taken to elute) with standards under the same conditions, or by enzyme reactions e.g. hexokinase quantitatively converts ATP to ADP and hence both peaks can be identified. The most widely used detector is the UV detector which measures the change in UV absorption as the solute passes through the flow cell in a UV transparent solvent. Other detection systems include refractive index detectors, electrochemical detectors, radiochemical detectors, electron

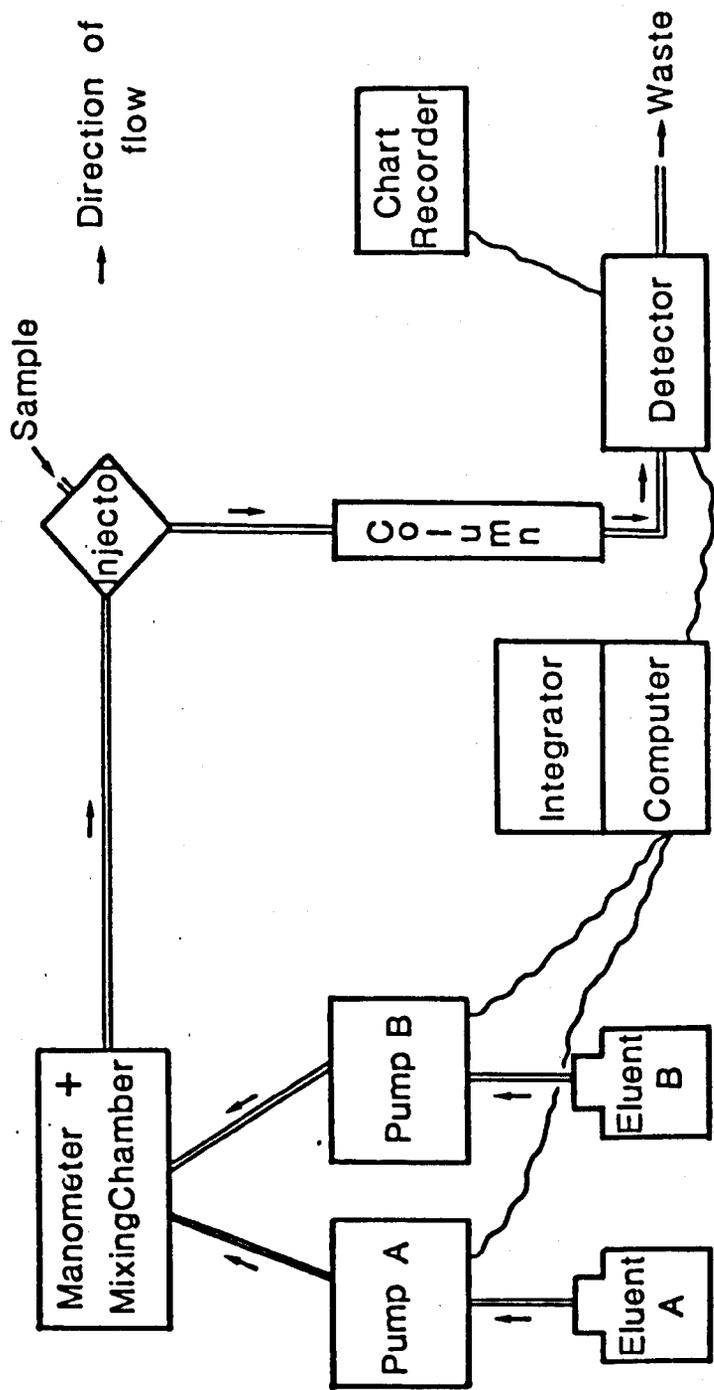


Figure 4.2. A "typical" HPLC system

capture detectors and fluorimetric detectors.

4.I.vii. Principles of chromatographic separation

The chromatographic separation of two components depends on their having different distribution ratios between the stationary and mobile phases with separation being primarily achieved by modifications of the mobile phase. Solvent molecules compete with solute molecules for polar adsorption sites. The stronger the interaction between the mobile and stationary phases the weaker the solute adsorption will be and vice-versa. Solvents are rated according to their strength of adsorption in the "elutropic series". Table 4.1 shows a shortened form of an elutropic series with alumina as the adsorbent but similar series hold for other polar adsorbents such as silica. E° is referred to as the "solvent strength parameter" and has been defined by Snyder as "the adsorption energy per unit area of standard adsorbent" (Simpson 1976). The greater the polarity of the solvent, the greater its adsorption energy. Thus the polarity of an eluent mixture and hence the chromatographic separation achieved can be altered by changing the ratios of the eluent components or by substituting one component for a more or less polar solvent. Secondary solvent factors such as pH are also important. HPLC separations may be run isocratically i.e. with a constant eluent composition, or they may be run in a gradient mode where the mobile phase composition, pH or ionic strength is varied during the run. There are also a number of different types of stationary mode which allow the division of HPLC into distinct modes although separations achieved are often

Table 4.1. Eluotropic series for alumina (from Pryde and Gilbert 1979).

<u>Solvent</u>	<u>E^o</u>
n-pentane	0.00
Isooctane	0.01
Cyclohexane	0.04
Carbon tetrachloride	0.18
Xylene	0.26
Toluene	0.29
Benzene	0.32
Diethyl ether	0.38
Chloroform	0.40
Methylene chloride	0.42
Tetrahydrofuran	0.45
Acetone	0.56
Methyl acetate	0.60
Aniline	0.62
Acetonitrile	0.65
Isopropanol	0.82
Ethanol	0.88
Methanol	0.95
Ethylene glycol	1.11
Acetic acid	large
Water	large

as a result of a combination of modes.

The four main modes are:

a). Adsorption chromatography which is useful for separating non-polar or slightly polar organic molecules. The solutes are eluted with an organic mobile phase e.g. hexane, down a column packed with an adsorbent material e.g. silica. Retention and selectivity are caused by hydrogen bonding of polar groups on the solute molecules with the surface silanols on the stationary phase. The retention time is dependent upon the competition between solute and solvent molecules for the adsorption sites and hence an increase in polarity of the eluent will tend to elute the solutes more quickly.

b). Reverse-phase chromatography which is especially useful for the separation of highly polar molecules which would give rise to long retention times in adsorption chromatography. Basically the same principles apply but the roles are reversed with a non-polar stationary phase and a polar mobile phase. The most common combination is a hydrocarbon bonded surface e.g. octadecylsilyl groups ($C_{18}H_{37}Si-$) bound to silica, with a polar eluent such as a methanol/water mixture. The hydrophobic nature of the stationary phase ensures a greater affinity for the non-polar solutes and thus polar molecules are eluted first.

c). Ion-exchange chromatography where charged solutes are separated by interacting with ionic sites bonded or coated onto a packing surface e.g. bonded quarternary ammonium groups for the separation of anions. Solute ions having a greater charge or charge density are attracted more strongly by the stationary

charges of the opposite sign. Mobile phases are always aqueous and by modifying the ionic strength or pH of the eluent, the separation of the components can be modified. This mode is not used as often as other modes due to the relative instability of the currently available packing materials.

d). Ion-pair chromatography is an alternative to the above for the separation of polar molecules. This is based on the principle of extracting a hydrophilic ionised sample from an aqueous into an organic phase by "neutralising" its net charge with a pairing ion of opposite charge. Normally the reverse-phase mode is used. Examples of suitable ion-pairing reagents include, for example, basic quarternary ammonium ions for the separation of acidic anions, and sulphonic acid ions for the separation of basic cations. The exact mechanism of separation is not known but is thought to be a combination of i). the formation of ion-pairs in the mobile phase and the resulting increase in hydrophobic interaction with the stationary phase causing retention, and ii). the coating of the stationary phase with the ion-pairing agent and the adsorbed ions acting as an ion exchanger (Tomlinson et al. 1978). Prime parameters which can be varied to alter the retention and selectivity of the separation are given in Table 4.2..

4.I.viii. Quantification in HPLC

Peaks can be quantified either by measurement of their heights or areas. With an integrator the measurement of areas is more accurate. Obviously a reproducible volume must be injected

Table 4.2. Adjustable variables in reverse-phase ion-pair HPLC

<u>Variable</u>	<u>Effect</u>
Nature of pairing ion	Generally the more hydrophobic the pairing ion, then the greater the retention.
Concentration of pairing ion	An increase in concentration will increase retention to a limit and further increases will cause a decrease in retention.
Type of organic modifier in the mobile phase	Retention will decrease as the modifier becomes more non-polar.
Concentration of organic modifier	Retention decreases with increasing modifier concn.
Stationary phase	A more hydrophobic stationary phase results in greater retention.
pH	As pH changes, resulting in more solute ionisation, the retention increases.
Temperature	Retention decreases as the temperature increases.
Ionic strength and counter-ion concentration	Increased ionic strength and counter-ion concn. will lead to a reduction in retention.

every time and this is fairly easy with the current loop injection systems. However variations can still occur, e.g. due to injection of an air bubble. The effects of variation in column performance and in injection volume can be overcome by using an internal standard. Such a compound should be well resolved, of comparable peak dimensions, affected by any injection or column variables to the same extent as the sample, recovered to the same extent during extraction as the compounds of interest, and stable and not naturally occurring in the samples being analysed. Thus if all the samples are spiked with the same volume of internal standard, the peak height ratio i.e. the peak height of x divided by the peak height of the internal standard, should be constant for any given concentration of x, whatever the injection volume. Usually a calibration curve is constructed by analysing standards, each spiked with the same amount of internal standard, and plotting their concentration against peak height ratio. Thus when an unknown sample is analysed in the same way, the peak height ratios can be ascertained and hence by direct comparison with the calibration curve, the concentration of the unknown can be determined.

4.I.ix. HPLC separation of nucleotides

The nucleotides are strong acids and are ionised at pH values of 3 or higher, due to the presence of the phosphate group(s) (Brown et al. 1980). Obviously the number of negative charges per nucleotide molecule is dependent upon the number of phosphate moieties e.g. the triphosphates have three. At pH values greater than 7 the nucleotides gain an additional negative charge

due to secondary phosphate dissociation (Zakaria and Brown 1981). Separation of nucleotides has been accomplished using anion-exchange chromatography (D'Souza and Glueck 1977, Brown et al. 1982, Pogolotti and Santi 1982). Although satisfactory in terms of separation, this mode of chromatography suffers from the disadvantages of long retention times (40-120 minutes for the triphosphates), lengthy equilibration periods and a limited column life. Later separations involved the use of ion-pair reverse-phase chromatography, allowing total analysis times of less than 40 minutes (Brown et al. 1982, Ingebretsen et al. 1982). In both these chromatographic modes the nucleotides were extracted from cells or tissues using perchloric acid to precipitate the proteins (so preventing column clogging and enzymatic degradation of the nucleotides), and either tri-N-octylamine/Freon mixture, dipotassium hydrogen phosphate or potassium hydroxide was used to neutralise the extract and precipitate the perchlorate. These methods of extraction were found to result in good recoveries and stable, when frozen, protein-free samples. The method used here is an ion-pair reverse-phase method modified from that developed by Brown et al. (1982).

4.II. MATERIALS AND METHODS

4.II.i. Reagents

Reagents used were purchased from the following:-

Aldrich Chemical Co. Ltd. (Gillingham, Dorset):- Na_3VO_4 .

BDH Chemicals (Poole, Dorset):- perchloric acid (PCA), tetrabutylammonium hydroxide (TBA), KH_2PO_4 , KOH and K_2CO_3 (Analar grade), and methanol (HPLC grade).

Capital HPLC (Edinburgh):- Hypersil 5 ODS 15 cm x 4 mm i.d. column fitted with zero dead volume fittings.

Gibco-Biocult (Paisley, Scotland):- all tissue culture supplies as detailed in Chapter 2.

HPLC Technology (Macclesfield, Cheshire):- Microsorb C18 15 cm x 4 mm i.d. column.

Sigma (Poole, Dorset):- AMP sodium salt, ADP sodium salt, ATP disodium salt, CTP sodium salt, GDP sodium salt, GTP sodium salt, β -NAD, β -NADH disodium salt, β -NADP sodium salt and β -NADPH tetrasodium salt.

Water used for solutions was produced using a Milli-Q water system (Millipore S.A., France). All solutions were dispensed using Gilson adjustable pipettes during experiments.

4.II.ii. Chromatographic apparatus

The following apparatus comprised the chromatographic system. Operating parameters are as described in the following "Methods" sections.

Pumps: Gilson Model 302 dual pumps with 5 ml pump heads.

Manometer: Gilson Model 802 manometric module and mixing chamber.

Detector: Gilson UV Holochrome detector, fitted with an 11 ul flow cell. Wavelength setting was 254 nm and sensitivity setting was 0.1 AUFS. A Rikadenki chart recorder was used to monitor the appearance of peaks.

Gradient control and peak analysis: Apple IIe computer, Kaga screen and dual disc drives, linked to a Gilson Model 620 Data Master and NEC PC8023BE-N printer.

Injection: Rheodyne 7125 injection valve with 100 ul loop. Hamilton injection syringes.

Columns: Initially a Microsorb C18 15 cm x 4 mm i.d. column was used (HPLC Technology - Macclesfield, Cheshire). Later changed to a Hypersil 5 ODS 15 cm x 4 mm i.d. column fitted with zero dead volume endings (Capital HPLC - Edinburgh). Pre-column packed with Lichroprep used in the early stages.

4.II.iii. Separation of nucleotide standards

Separation of aqueous AMP, ADP and ATP standards (10 uM) was

initially accomplished using the following format.

Flow rate: 1.5 ml/min.
Eluent A: 0.1M KH_2PO_4 , 5 mM TBA, pH to 6.4 with 10M KOH.
Eluent B: 0.1M KH_2PO_4 , 5 mM TBA, 30% methanol (v/v), pH to 6.4 with 10M KOH.

<u>Time (mins.)</u>	<u>%B</u>
0.00	0
27.00	75
30.00	100
32.00	0
45.00	0

Reproducibility of the system was checked by repeated injection of a solution of 10 μM ATP, ADP and AMP and examining the peak areas and retention times. Sample stability and day to day variation of the system was checked by freezing down a number of aliquots of 10 μM ATP, ADP and AMP solution and injecting then at intervals over a 3 week period. The detection limits of the system were found by injecting progressively smaller concentrations of nucleotides until integration of peak area was no longer possible.

Calibration was achieved by chromatographing mixtures of nucleotides at various concentrations, in triplicate. Maximum concentrations used were: ATP - 100 μM , ADP - 20 μM , AMP - 20 μM , NAD - 40 μM and NADP - 40 μM . The higher maximum level of ATP was chosen in order to reflect the higher anticipated cellular levels compared with the other nucleotides. Peak areas were then plotted against concentration for each nucleotide in turn, and response factors calculated by regression analysis

through the origin using the "GLIM" statistical package on the VAX mainframe computer.

Due to the difficulties involved in finding a stable internal standard which was not normally present in the biological extracts and was well resolved from other peaks in the samples, coupled with the inherent errors involved in spiking samples with an insignificant volume of internal standard, it was decided to adopt an alternative standardisation procedure. The consistency of the system response and the sample stability were considered to be such that system performance could be adequately monitored by injecting an aliquot of a 10 μ M solution of ATP, ADP and AMP daily before any sample analysis. If the results were in agreement with the previous calibration, analysis was begun. If not in agreement, the system was recalibrated. Recalibration was also carried out if any system parameter was altered, e.g. new column, UV lamp etc.

4.II.iv. Chromatography of biological samples

In order to improve nucleotide separation, the gradient profile was altered slightly as follows. The flow rate and composition of the individual eluents A and B remained unchanged.

<u>Time (mins.)</u>	<u>%B</u>
0.00	0
6.75	20
9.50	20
19.00	52.5
23.00	52.5
24.00	100
26.00	0
40.00	0

HeLa cells were cultured as described in Section 2.II.iii. (0.33 x 10⁶ cells/5 cm plate - day 0). Twenty-four hours later, half the plates were used to determine cell numbers and plate water. The medium was rapidly aspirated from the remaining plates which were then placed on ice. 500 ul of 6% PCA (ice-cold) was added to each plate in turn. The plates were regularly tilted to prevent drying of any areas. After 30 minutes, 400 ul samples were removed from each plate and neutralised by adding to 65 ul of 3M K₂CO₃. The samples were whirlmixed and left for 10 minutes before being centrifuged at 3,000 rpm, 4°C, for 15 minutes (MSE Coolspin - Fisons). The resulting supernatant from each sample was then decanted and frozen until analysed, usually within one week of preparation. Plates containing medium without cells were extracted in a similar manner and used as blanks. Before being injected, samples were thawed out and filtered through 0.22 um Millipore filters.

Identification of peaks from biological samples was accomplished by co-chromatography with known nucleotide standards and by comparison of retention times with standards. Stability of the various nucleotides during the extraction process was examined by extracting aqueous solutions of each nucleotide in turn and analysing the resulting chromatograms.

Percentage recoveries of the nucleotides were determined by extracting plates of cells which had been spiked with known concentrations of nucleotide standards (10 ul), and chromatographing the resulting samples. By subtracting the peak areas obtained when the plates were spiked with 10 ul water, from the

peak areas when similar plates of cells were spiked with nucleotides, the peak areas due to the nucleotide spikes were determined. Using the response factors found from the calibration graphs, the areas were converted to concentrations and corrections made for the dilution of the plate water during the extraction process. Comparison of the resulting values with the theoretical values expected from each spike, yielded the percentage recovery for each nucleotide over a range of concentrations. The samples for recovery studies were extracted on several days, chromatographed at random and the results pooled. Significance testing of the results was performed by Student's t-test (unpaired) using the "Minitab" statistical package on the VAX mainframe computer.

4.II.v. Chromatographic analysis of HeLa cells treated with vanadate

HeLa cells were plated as described in Section 2.II.iii. (0.33×10^6 cells/5 cm plate - day 0), and the medium replaced with fresh medium \pm Na_3VO_4 (10^{-9} - 10^{-4} M) on day 3, with 6 plates for each treatment condition. Twenty-four hours later, half the plates in each treatment condition were used to determine the cell numbers and plate water. The remaining plates were extracted as described in the previous section and frozen until subsequent analysis. Suitable blanks were also run consisting of cell-free plates containing similar medium and extracted as above. Cellular concentrations of nucleotides were calculated by converting the peak areas to concentrations using the current response factors, and making the appropriate corrections for

recovery and sample dilution. Results were analysed by Student's t-test (unpaired) using the "Minitab" statistical package on the VAX mainframe computer.

4.III. RESULTS

4.III.i. Equipment performance and separation of nucleotide standards

The linear gradient profile of Brown et al. (1982) - see Section 4.II.iii., proved to be adequate for the separation of AMP, ADP and ATP in standard solutions but failed to resolve the AMP peak from other, initially unidentified peaks, present in biological extracts. Resolution was improved by modifying the gradient as detailed in Section 4.II.iv. The separation of nucleotide standards achieved using this gradient is illustrated in Figure 4.3.. No peaks were seen in the blank sample runs.

Following the identification of the NAD, AMP, ADP and ATP peaks in biological extracts, the system was calibrated for the measurement of these four nucleotides. A good linear correlation between peak area and injected amount was seen up to at least the maximum concentrations injected in this study, i.e. ATP - 100 μ M, ADP - 20 μ M, AMP - 20 μ M and NAD - 40 μ M (see Figures 4.4., 4.5., 4.6. and 4.7.), with standard errors of the gradient of the fitted line ranging from \pm 0.53% in the case of ATP to 1.1% in the case of ADP ($n = 3$ for each point on the lines). Detection and integration limits for the four nucleotides were all approximately 0.5 μ M with a 100 μ l injection loop, i.e. 50 picomoles on column. Further increases in equipment sensitivity could be achieved but only at the expense of increasing the baseline noise, thus making the detection of peaks more difficult.

Reproducibility of the system, as assessed by the repeated

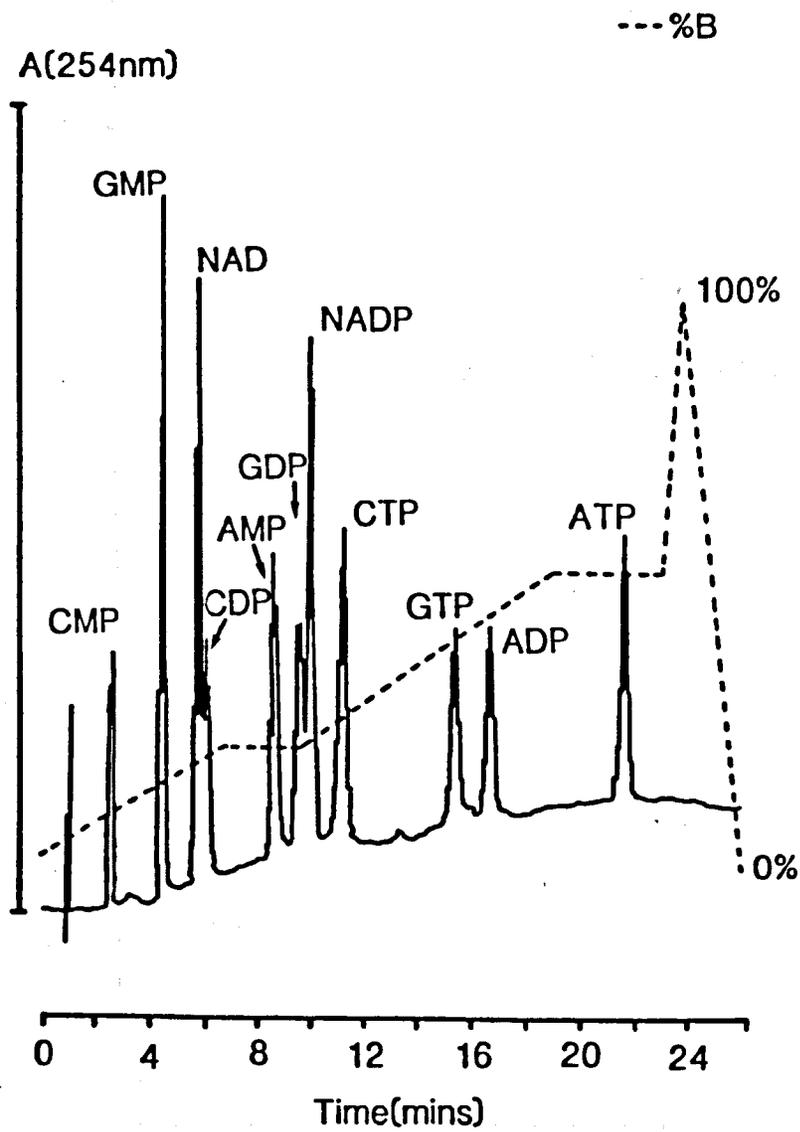


Figure 4.3. Separation of nucleotide standards

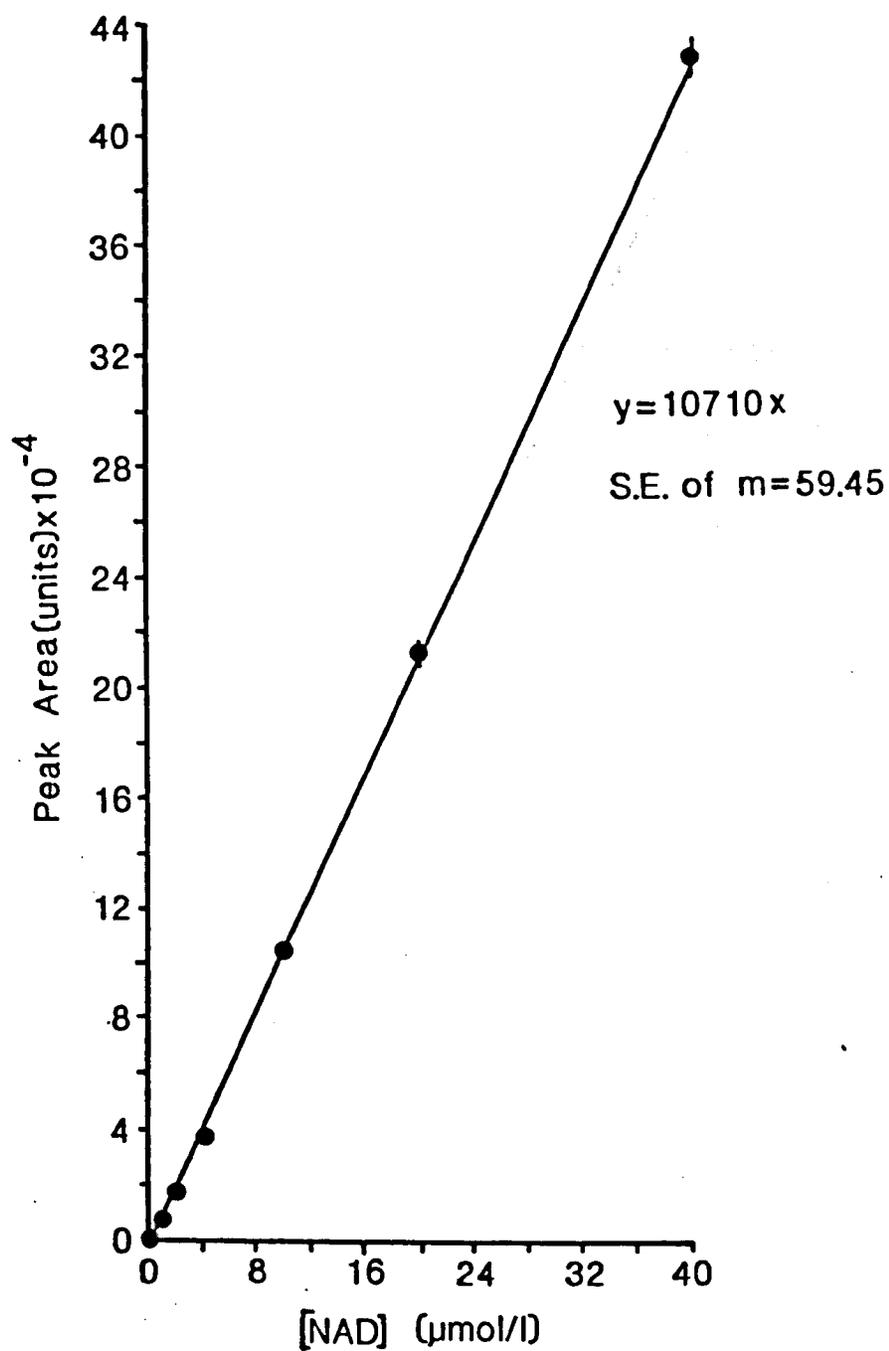


Figure 4.4. Calibration graph for NAD

Each point represents the mean \pm S.E. of 3 observations.

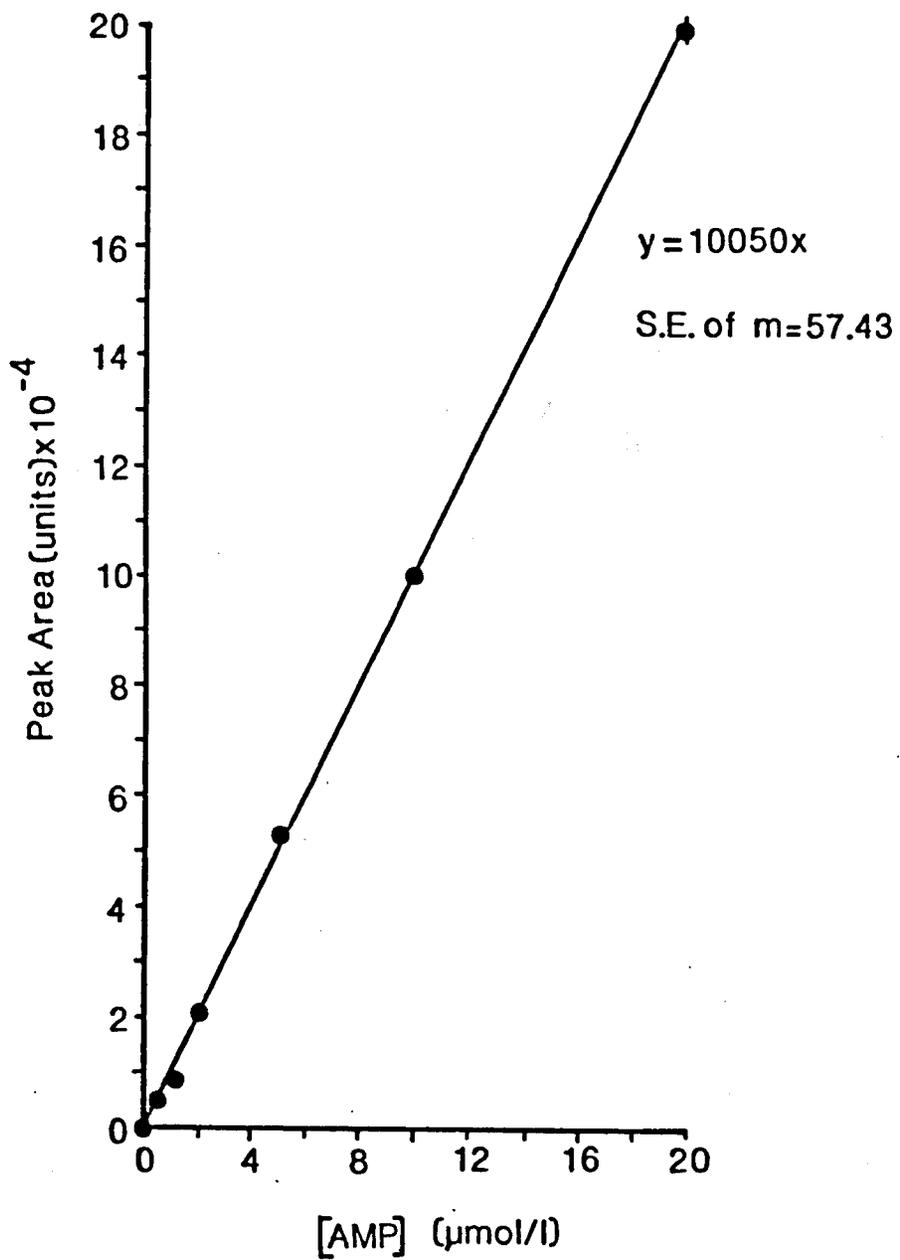


Figure 4.5. Calibration graph for AMP

Each point represents the mean \pm S.E. of 3 observations.

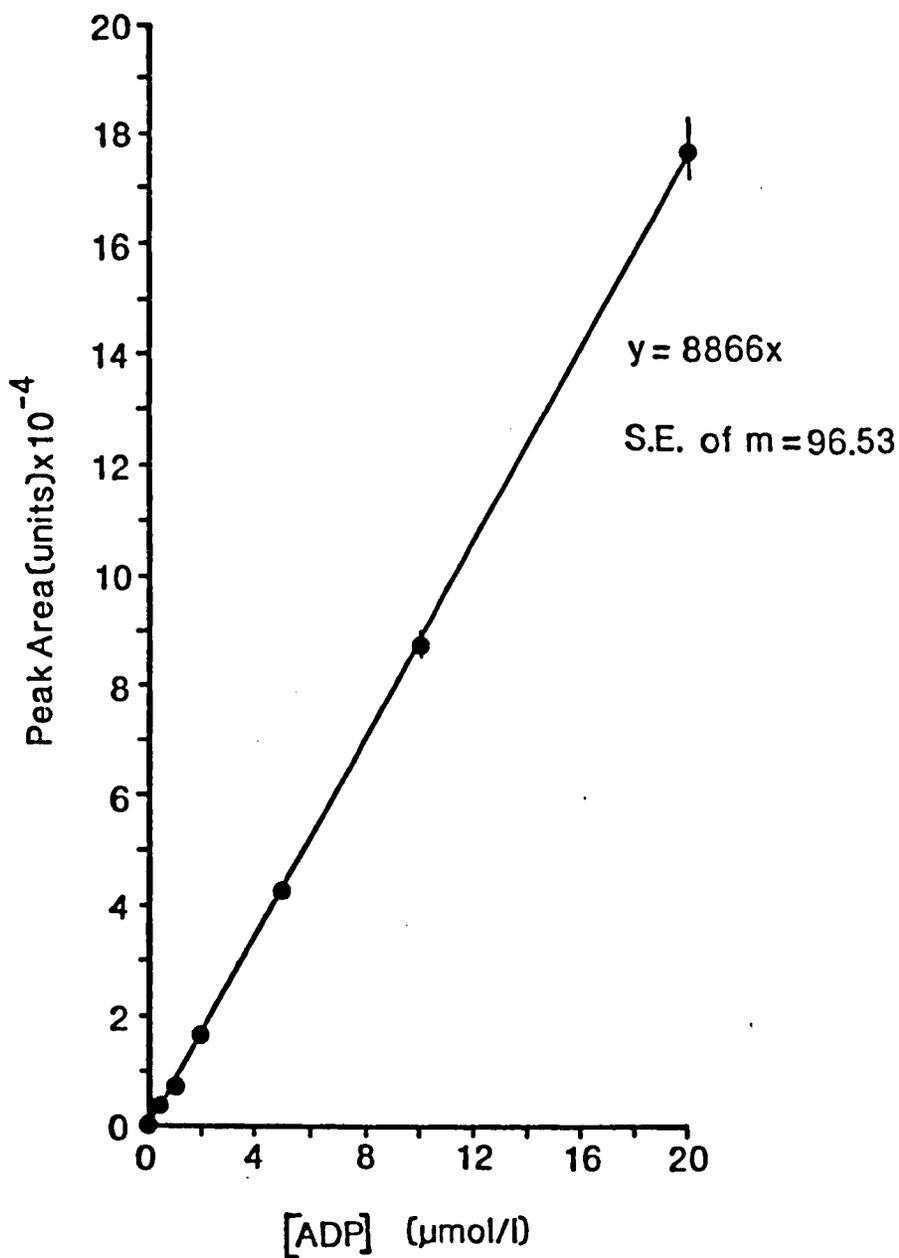


Figure 4.6. Calibration graph for ADP

Each point represents the mean \pm S.E. of 3 observations.

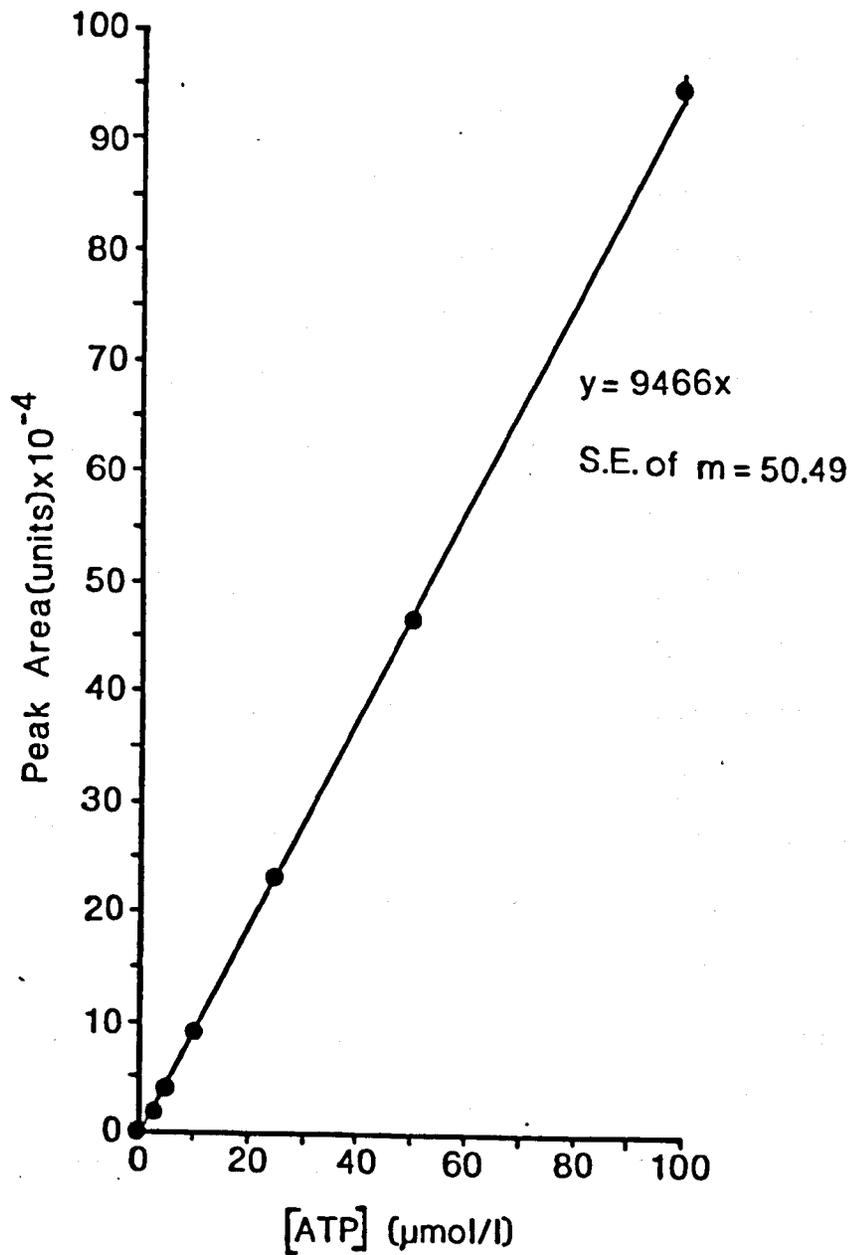


Figure 4.7. Calibration graph for ATP

Each point represents the mean \pm S.E. of 3 observations.

injection (n = 5) of a solution of 10 uM AMP, ADP and ATP and the examination of the resulting peak areas and retention times, was very good. Maximum standard errors of the mean of these peak parameters were $\pm 1.4\%$ and 1.1% respectively. Results of the three week sample stability study, which also incorporated any day to day variation in machine performance, were similar with maximum values of $\pm 2.2\%$ for the standard error of the mean of the peak areas (n = 10). These results illustrate the consistent machine performance both between- and within- days, and also the stability of aqueous nucleotide samples when frozen. Similar results were later obtained for frozen biological extracts when assayed over a week long period.

4.III.ii. Extraction and chromatography of biological samples

Acid extraction of standard solutions of AMP, ADP, ATP, NAD and NADP followed by chromatography yielded a single peak for each solution, similar to those obtained upon chromatographing unextracted standard solutions. NADH and NADPH however, were acid-labile yielding several peaks after extraction with acid, including a minor peak of ADP. As the major breakdown products were not seen in biological extracts in this study, it was concluded that the acid decomposition of NADH and NADPH made a negligible contribution to the ADP pool in biological extracts. The major break-down products of NADH have previously been identified as adenosine and ADP-ribose (Brown et al. 1982).

A chromatogram of the nucleotide profile of HeLa cells is shown in Figure 4.8. with all the peaks which were identified, labelled. Despite the gradient modifications AMP, GDP and NADP

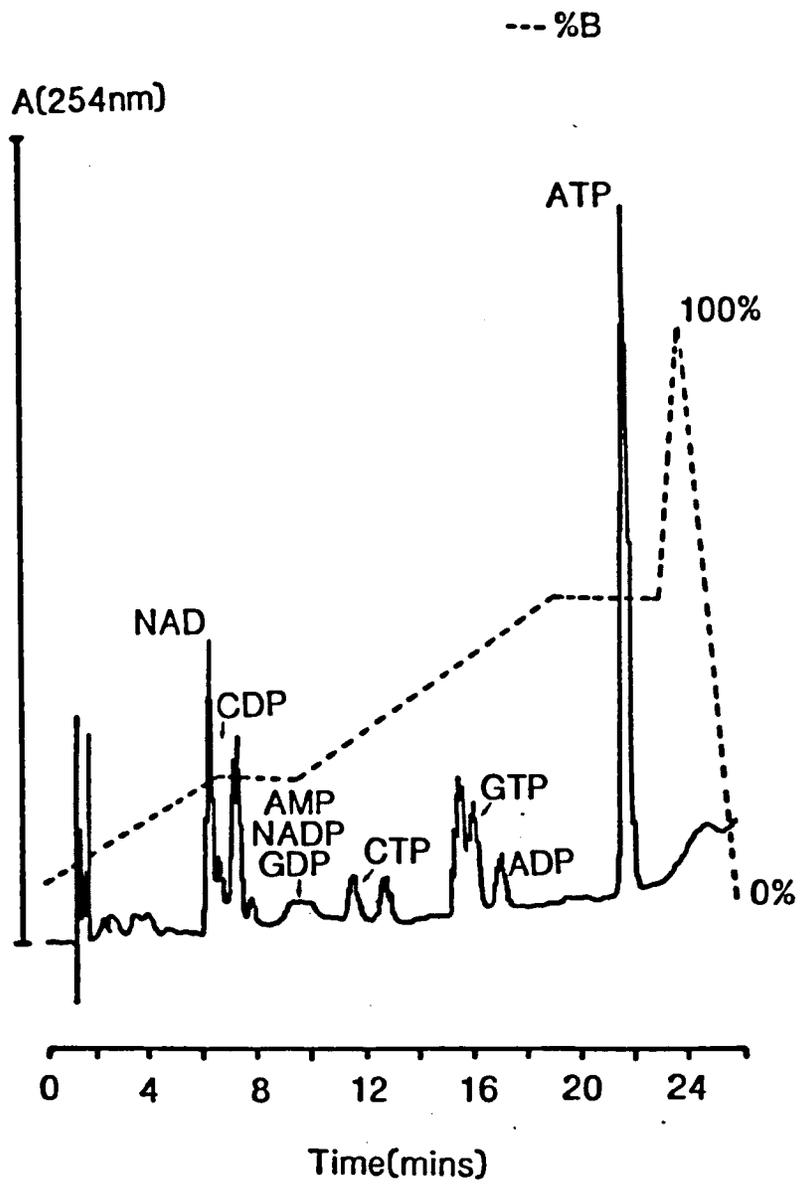


Figure 4.8. Chromatographic profile of nucleotides in HeLa cells

were still not satisfactorily resolved on every occasion, partly due to progressive column deterioration but largely due to the small amounts present in extracted samples.

Nucleotide recoveries during extraction are shown in Table 4.3.. No significant difference was found between the percentage recovery and concentration of spike for each nucleotide, so the results for the various spike concentrations were pooled to give an overall mean % recovery for each nucleotide. Due to their presence in low concentrations, NADP was never resolved from GDP in chromatograms of biological extracts. For this reason NADP was not included in the recovery studies. Due to the low concentrations of AMP present in biological extracts, combined with later resolution problems as a result of column deterioration, measurement of AMP was also largely not possible. Recovery figures are available for AMP however, as these studies were performed when the column was new, although resolution was still occasionally lost even at this time. As recovery studies were not performed for the remaining identified nucleotides present in biological extracts, these nucleotides can only be assessed qualitatively rather than quantitatively. Some peaks still remain to be identified.

4.III.iii. Effects of vanadate upon nucleotide concentrations in HeLa cells

The chromatographic profile of nucleotides from HeLa cells treated for 24 hours with 10^{-4} M vanadate can be seen in Figure 4.9.. When comparing this chromatogram with the control chromatogram i.e. Figure 4.8, it must be appreciated that the

Table 4.3. Nucleotide recoveries during extraction

Nucleotide	[Spike] (mmol/l)	Mean % recovery ± S.E.M.	n	Overall mean %recovery ± S.E.M.
NAD	0.1	72.6 ± 1.3	4	82.5 ± 2.8 (n = 25)
	0.2	76.4 ± 2.7	5	
	0.4	88.6 ± 3.9	6	
	1.0	88.0 ± 3.4	6	
AMP	0.02	87.6 ± 12.1	3	88.4 ± 3.1 (n = 16)
	0.04	90.9 ± 5.3	3	
	0.08	90.8 ± 5.8	5	
	0.2	85.0 ± 4.6	5	
ADP	0.05	92.1 ± 3.5	3	91.6 ± 2.0 (n = 21)
	0.1	90.6 ± 6.5	6	
	0.2	90.2 ± 2.4	6	
	0.5	93.8 ± 1.5	6	
ATP	0.5	83.6 ± 4.8	6	80.0 ± 1.5 (n = 24)
	1.0	78.9 ± 2.7	6	
	2.0	76.6 ± 1.3	6	
	5.0	81.1 ± 1.6	6	

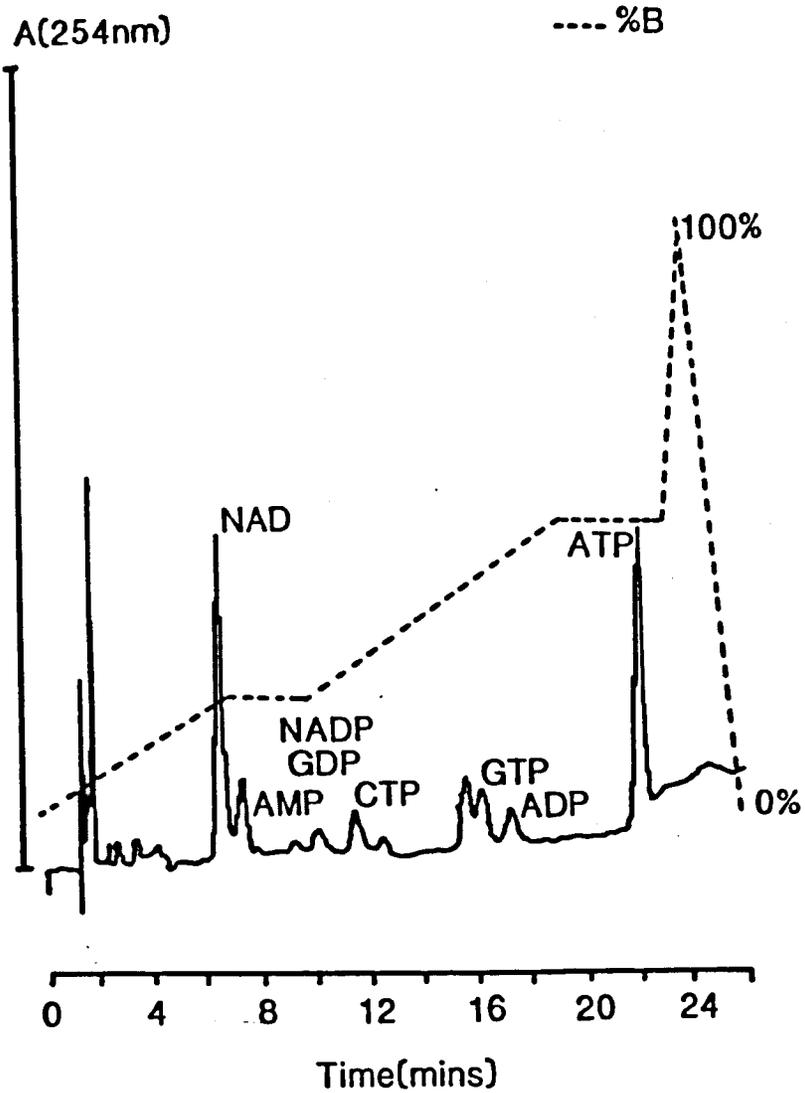


Figure 4.9. Chromatographic profile of nucleotides in HeLa cells treated with 10^{-4} M vanadate for 24 hours

chromatogram in Figure 4.9. is the result of the extraction of a plate containing at least 40% fewer cells. The effects of vanadate upon cell numbers have been discussed in Chapter 2. These effects have been taken into account when calculating cellular nucleotide levels at various vanadate concentrations.

Treatment of HeLa cells with varying concentrations of vanadate for 24 hours produced no significant changes in NAD or ADP levels (Tables 4.4. and 4.5. and Figure 4.10.). The absence of NAD data at the higher vanadate concentrations in one experiment was due to the loss of resolution of CDP and NAD, as illustrated in Figure 4.9. (c.f. Figure 4.8.). Significant decreases in cellular ATP levels were seen at $[V^{5+}]_0$ greater than $10^{-5}M$ (Table 4.6. and Figure 4.10.), i.e. at those concentrations where 24 hour vanadate treatment has previously been shown to significantly decrease cell numbers (see Chapter 2). No significant change was seen at lower vanadate concentrations. These results are expressed as per litre of cell water. Implicit in this statement is the assumption that the nucleotides are freely distributed within the cell which may not be the case as compartmentalisation may occur. If expressed per cell, the results appear similar.

By comparing Figure 4.8. with 4.9., it is possible to obtain qualitative data on the other identified nucleotides, taking into account the different numbers of cells in each chromatographed extract. It appears that there is little or no change in the AMP, NADP, GDP or GTP levels with vanadate treatment, and possibly a slight increase in CTP levels.

Although it was not possible to calculate exact values for AMP

Table 4.4. Effect of 24 hours growth in vanadate on NAD levels
in HeLa cells

The values are the mean of 3 experiments (* = 2 experiments), with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test.

$[V^{5+}]_0$ (M)	Mean [NAD] \pm S.E.M. (mmol/lcw)	Level of Significance
0	1.34 \pm 0.12	-
10^{-9}	1.35 \pm 0.09	N.S.
10^{-8}	1.27 \pm 0.11	N.S.
10^{-7}	1.36 \pm 0.06	N.S.
10^{-6}	1.25 \pm 0.01	N.S.
3.2×10^{-6}	1.30 \pm 0.02	N.S.
10^{-5}	1.37 \pm 0.07	N.S.
2.1×10^{-5}	1.30 \pm 0.08*	N.S.
4.6×10^{-5}	1.16 \pm 0.27*	N.S.
10^{-4}	1.67 \pm 0.38*	N.S.

Table 4.5. Effect of 24 hours growth in vanadate on ADP levels in HeLa cells

The values below are the mean of 3 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test.

$[V^{5+}]_0$ (M)	Mean [ADP] \pm S.E.M. (mmol/lcw)	Level of Significance
0	0.36 \pm 0.02	-
10^{-9}	0.39 \pm 0.03	N.S.
10^{-8}	0.38 \pm 0.03	N.S.
10^{-7}	0.40 \pm 0.01	N.S.
10^{-6}	0.38 \pm 0.02	N.S.
3.2×10^{-6}	0.38 \pm 0.04	N.S.
10^{-5}	0.40 \pm 0.01	N.S.
2.1×10^{-5}	0.34 \pm 0.04	N.S.
4.6×10^{-5}	0.37 \pm 0.04	N.S.
10^{-4}	0.38 \pm 0.07	N.S.

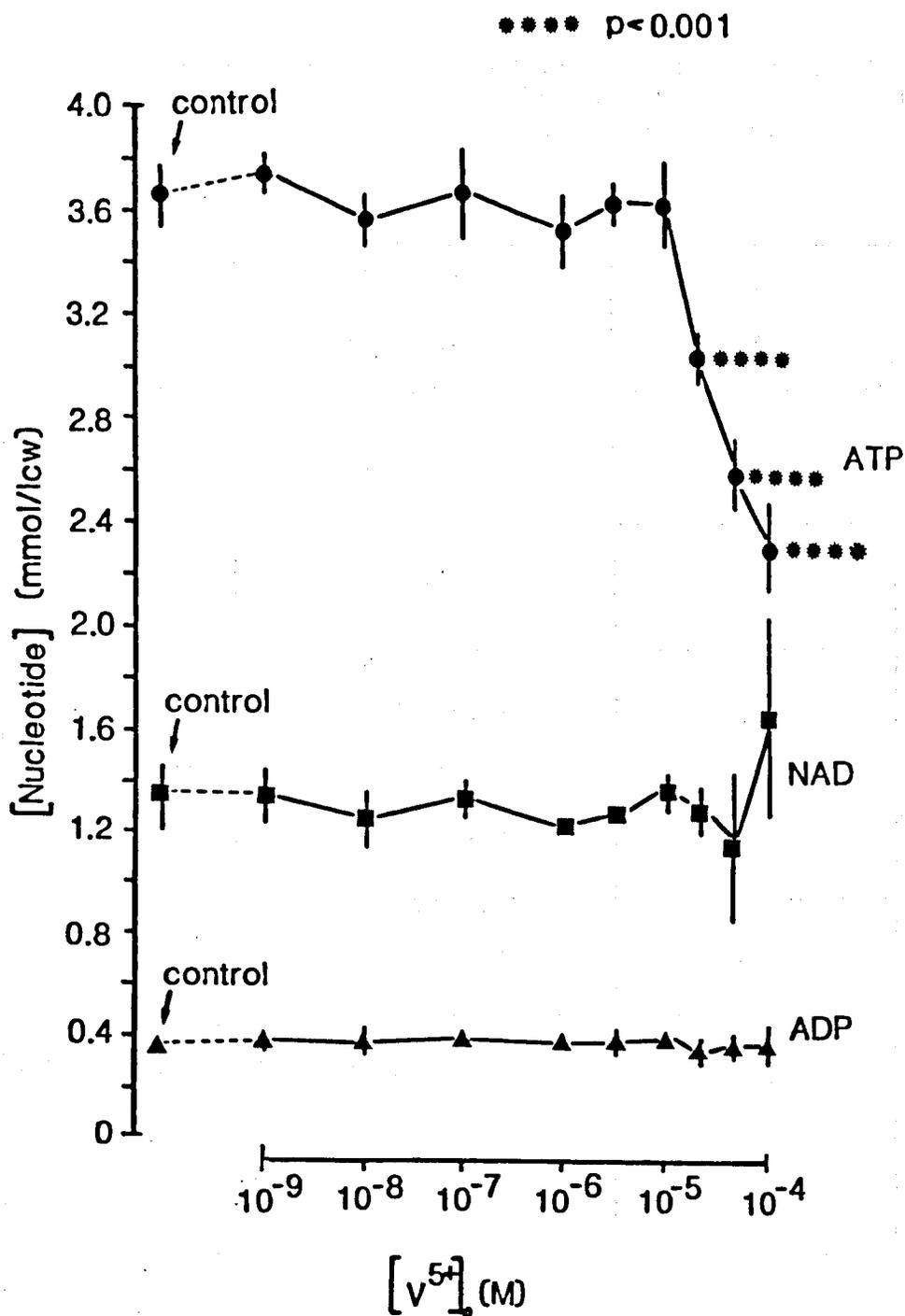


Figure 4.10. Effect of 24 hours growth in vanadate on HeLa cell nucleotide concentrations

Each point represents the mean \pm S.E. of triplicate data from 3 experiments. Significance testing relative to the control group is by Student's t-test.

Table 4.6. Effect of 24 hours growth in vanadate on ATP levels in HeLa cells

The values are the mean of 3 experiments, with each condition being triplicated within each experiment. Significance testing relative to the control group is by Student's t-test.

$[V^{5+}]_0$ (M)	Mean [ATP] \pm S.E.M. (mmol/lcw)	Level of Significance
0	3.67 \pm 0.12	-
10 ⁻⁹	3.76 \pm 0.06	N.S.
10 ⁻⁸	3.57 \pm 0.07	N.S.
10 ⁻⁷	3.68 \pm 0.17	N.S.
10 ⁻⁶	3.52 \pm 0.13	N.S.
3.2 x 10 ⁻⁶	3.64 \pm 0.06	N.S.
10 ⁻⁵	3.63 \pm 0.17	N.S.
2.1 x 10 ⁻⁵	3.05 \pm 0.08	p < 0.001
4.6 x 10 ⁻⁵	2.60 \pm 0.13	p < 0.001
10 ⁻⁴	2.31 \pm 0.17	p < 0.001

levels in the extracts, an upper estimate can be made as the peak size appears to be just below the detection and integration limits of 0.5 $\mu\text{mol/litre}$. Using dilution factors of approximately 110 for the control cells and 160 for the cells treated with 10^{-4}M vanadate, (different factors due to different cell numbers), together with a recovery figure of 88.4% (Table 4.3.), upper limits of 0.06 mmol/lcw and 0.09 mmol/lcw respectively can be calculated. Using these figures as upper limits and 0 mmol/lcw as a lower limit, a maximum and minimum value for the energy charge can be calculated using the equation given in Section 4.I.1.. Thus HeLa cells treated with 10^{-4}M vanadate for 24 hours can be assumed to have an energy charge in the range 0.91 to 0.93 compared with the control cells where the range is 0.93 to 0.96.

4.IV. DISCUSSION

The HPLC method finally used to separate nucleotides in this study is both sensitive and reproducible. It is comparable, both in terms of retention times and resolution, with the reverse-phase ion-pairing methods of Ingebretsen et al. (1982) and Brown et al. (1982), but achieves better resolution overall due to its slightly longer running time. When biological samples were run, it was not always possible to resolve AMP, GDP and NADP, partly due to the close proximity of their respective retention times but also due to their low levels in the extracts. Significant improvements were obtained by using a column with zero dead volume fittings and by introducing a plateau into the gradient profile. Further improvements in the method would be realised if the samples were lyophilised in order to concentrate them before being applied to the column, and also possibly if the gradient was modified further. Problems in resolving CDP and NAD were also encountered occasionally.

The % recovery of the nucleotides varied depending upon the nucleotide. This was also seen by Brown et al. (1982) who reported higher recovery values following neutralisation of the acid-extract with tri-N-octylamine in Freon, compared with the precipitation method used here. This is presumably due to adsorption of the nucleotides onto the precipitate to varying extents. It should also be noted that any recovery study such as this assumes that the spiked sample is recovered to the same extent as the biological sample to which it is added. In the case of nucleotides at least, this assumption is probably

justified as the acid protein precipitation procedure would be likely to release any compartmentalised/protein-bound nucleotides that may be present in the cells. The extraction method can not be used for NADH or NADPH however as they are unstable in the acidic extraction conditions employed here. It would be necessary to perform a separate extraction procedure in order to quantify these compounds.

As already discussed in the "Introduction" to this chapter, the direct dependence of the sodium pump upon ATP is widely recognised (e.g. Baker 1966, Glynn 1968). More recently it has also been shown that the diuretic-sensitive cotransport pathway is also dependent upon ATP albeit indirectly probably via a protein kinase-mediated reaction (Saier, Jr. et al. 1984). It is possible therefore that vanadate/vanadyl may exert effects on these transport systems by depleting ATP levels. This discussion is mainly confined to the sodium pump flux as little information is available concerning the effects of nucleotides on the diuretic-sensitive fluxes.

A depletion of ATP (depending on its magnitude) would decrease the pump flux directly and would also augment any inhibitory action of vanadate (if present) on the sodium pump, as ATP antagonises the vanadate-induced inhibition of the sodium pump (Josephson and Cantley, Jr. 1977). As it is difficult to investigate the kinetics of the activation by ATP of ion transport via the Na^+/K^+ -ATPase molecule in intact cells, most of the studies have used either isolated enzyme systems (e.g. Robinson 1976) or vesicular preparations and the results extrapolated to intact cells. Whether the existence of both

high and low affinity ATP binding sites is dependent upon the tissue or depends upon the limitations of the method employed is not clear. For example a single K_m value for ATP of 0.21 mM has been reported for a canine cardiac sarcolemmal vesicular Na^+/K^+ -ATPase preparation (Philipson and Nishimoto 1983) whereas K_m values for ATP of 1 μM and 0.5 mM were reported for an isolated enzyme preparation from rat brain (Robinson 1976). If similar values are assumed to be valid in intact cells, the depletion of ATP seen here at $[\text{V}^{5+}]_o$ greater than 10^{-5}M would probably have little if any effect directly on the pump. However the augmentation of the inhibitory action of vanadate upon the pump is very much an unknown quantity as the majority if not all of the intracellular vanadium has been shown to be in the vanadyl form (Chapter 2), which is only inhibitory if highly purified isolated enzyme preparations are used (North and Post 1984). It is possible that very low concentrations of vanadate are present in which case the ATP depletion may result in an increase in any vanadate-induced sodium pump inhibition. As already discussed in Chapter 3, it is not clear whether a specific inhibition of flux per pump site is present anyway, due to the toxic effects present coupled with a concomitant decrease in pump number and a postulated stimulation of pump flux by increased intracellular sodium levels.

Inhibitory actions of ADP upon the Na^+/K^+ -ATPase active transport system have been described by Apell et al. (1986). Using Na^+/K^+ -ATPase from rabbit kidney outer medulla incorporated into artificial lipid vesicles, Apell et al. (1986) demonstrated an inhibition of the active transport by ADP with a K_i of

approximately 0.1 mM. The inhibition was virtually independent of ATP concentration indicating that it was not likely to be due to a competitive effect on nucleotide binding. Within the framework of the Albers-Post model (described in Chapter 3), the remaining possibility was that the inhibition was due to an increase in the rate of the backward reaction $\text{NaE}_1\sim\text{P} + \text{ADP} \longrightarrow \text{NaE}_1\text{ATP}$, caused by the increased ADP levels (Apell et al. 1986). However a competitive effect of ATP on the inhibition by ADP has been described by Robinson (1976) for example, using an isolated enzyme preparation from rat brain.

In this study no changes were seen in the intracellular ADP concentration with vanadate treatment. However if the K_i values reported above for ADP are equally applicable to intact cells it would appear that in HeLa cells at any rate, ADP is normally exerting a significant inhibitory effect on the sodium pump activity. This is postulated as being the normal case in cells, with ADP being part of a negative feedback system regulating pump activity (Apell et al. 1986). A possible slight increase in CTP levels was seen with vanadate treatment but this would have to be quantified by performing appropriate recovery studies and hence it is not clear to what extent this would affect the pump flux. An increase in the peak eluting directly before GTP seems to occur in the vanadate-treated cells. As the identity of this peak is not known however, possible ramifications of an increase can not be discussed.

It is also interesting to note that the decrease seen in ATP was not associated with a concomitant increase in ADP and/or AMP. The reason for this is not obvious. Whether the decrease in ATP

concentration occurred as a result of a decreased production or an increased utilisation, increased levels of ADP and AMP would have been expected. It is possible that the lack of change in AMP and ADP is due to a vanadate-induced increase in membrane permeability which would allow the diffusion of AMP and ADP out of the cell, but not the more polar ATP. This is unlikely however, especially in view of the decrease in passive ion fluxes seen at similar vanadate concentrations (Chapter 3).

It is also possible that the ATP levels may actually be no different from the control values but appear to be reduced due to a decrease in the percentage recovery. It is known for example, that both vanadate and vanadyl form strong complexes with ATP (Ramasarma and Crane 1981). If the complexed vanadate/vanadyl subsequently bound strongly to intracellular proteins in such a way that acidification did not release the complex, this would also yield lower ATP recoveries. This is highly unlikely as acidification (with HCl rather than perchloric acid) has been shown to release intracellular vanadyl (Fitzgerald and Chasteen 1974). Formation of such a complex though raises the possibility that whether or not any decrease in ATP levels is present, the ATP may not be as available for utilisation if bound to vanadate/vanadyl intracellularly to any significant extent. This may contribute towards the toxicity.

The decrease in ATP levels was only seen at $[V^{5+}]_o$ greater than $10^{-5}M$. Although the biggest changes in cell number and K^+ influx pathways were also seen at similar vanadate concentrations, smaller changes, which were in some cases significant, were also seen at lower vanadate concentrations.

It would therefore appear that ATP may not be responsible for the changes seen, but whether the effects on ATP are specific actions of vanadate/vanadyl or occur secondarily to some other change is not clear. The decrease in ATP does seem to occur slightly after the decrease in cell numbers and therefore may be a consequence of the toxicity. Further research in order to clarify the situation should include studies involving time courses of vanadate treatment to see in what order the changes occur.

The lack of a significant change in the energy charge with vanadate treatment despite the decrease in ATP levels is due to a lack of concurrent change in ADP and AMP levels. The reason for this, as discussed above, is not clear. On the basis of the high energy charge, vanadate/vanadyl would be assumed to have had no effect on the viability of the cells. In view of the marked toxicity seen in terms of decrease in cell numbers, it is unlikely that only some cells are susceptible to the toxic actions of vanadate/vanadyl and the rest remain totally healthy. Indeed in terms of changes in flux, ion contents and ATP levels, there is clear indication that the cells remaining appear to be showing signs of toxicity at the higher extracellular vanadate levels. It is likely therefore that as demonstrated in cultured fibroblasts following exposure to heat, there is little or no correlation between cellular energy charge and viability (Calderwood et al. 1985).

Whether the decrease in cell numbers (Chapter 2) seen with vanadate treatment is due to cell death or decreased cell division is not known. Since nucleotides are important as

precursors of RNA and DNA and the intracellular concentrations of nucleotides appear relatively unaltered with vanadate treatment, it can be concluded that the decrease seen in cell numbers does not occur as the result of a depletion of the precursors important in cell division. Other possible causes of the reduction in cell numbers with vanadate treatment have been discussed in Chapter 2, in the light of the known biological actions of vanadate and vanadyl. The inhibitory actions of vanadate (and to some extent vanadyl) on enzymes involved in phosphoryl transfer have been discussed in the "Introduction" to this chapter. It is conceivable that such actions may be partly responsible for the decrease in ATP levels, and/or play a role in the toxicity. However it is predominantly vanadyl which is present in the cell and the actions of vanadyl on such enzymes are less well documented.

The likelihood that at least some of the reduction of vanadate seen in HeLa cells is non-enzymatic has been discussed in Chapter 2. If this was due to the non-enzymatic oxidation of NADH for example, as seen in mouse liver plasma membranes (Ramasarma et al. 1981), although at high levels of vanadate and at low rates, an increase in NAD levels and a decrease in NADH levels may be expected. This would also be the case if a vanadate-stimulated NADH oxidase is present in HeLa cells, similar to that seen in mouse liver plasma membranes (Ramasarma et al. 1981). Such an increase in NAD levels in vanadate-treated HeLa cells was not apparent until an extracellular $[V^{5+}]$ of $10^{-4}M$ and even then this was not significant. As already discussed it is not possible to measure NADH levels using this method. If such

effects on NAD and NADH had been present, this may have at least partly explained the decreased ATP levels, as NADH/NAD is important in ATP production via both glycolysis and the citric acid cycle. Additionally such vanadate-stimulated NADH oxidase activity has been postulated to involve the production of free radicals in mouse liver plasma membrane (Ramasarma et al. 1981) and if a similar sort of mechanism was to occur in HeLa cells, free radical production may account for the toxicity. However decavanadate was probably responsible for these changes and there are no reports on the production of free radicals by vanadyl which appears to be the main species of vanadium present in HeLa cells.

If, as occurs in erythrocytes (Macara et al. 1980), reduction of vanadate in HeLa cells is due to GSH, there may be a reduction in GSH levels. GSH is important in the prevention of intracellular free radical formation (Stryer 1981). If GSH is depleted free radical damage may result. Free radical formation could also account for the decrease in ATP levels as non-specific ATP hydrolysis can be caused by free radicals (Ramasarma and Crane 1981). Further research should involve the measurement of GSH in vanadate-treated cells and the investigation into possible free radical formation.

In summary, the results in this chapter demonstrate little effect of vanadate/vanadyl on nucleotide levels with the exception of ATP which is reduced by approximately 40% at 10^{-4} M vanadate. Again it is impossible to conclude with certainty whether vanadate or vanadyl is responsible for the changes, although for reasons discussed in the previous chapter it is

likely to be vanadyl. Whether changes are secondary to other mechanisms such as possible free radical formation is also not clear. Because of the virtual lack of change in nucleotide concentrations, it is unlikely that nucleotides play a role in accounting for any of the changes seen in ion fluxes or cell growth with vanadate treatment.

CHAPTER 5. LONG-TERM CULTURE OF LYMPHOBLASTOID CELL LINES FROM
BIPOLAR MANIC-DEPRESSIVE PATIENTS AND CONTROLS; TRANSFORMATION,
GROWTH AND MORPHOLOGY

5.1. INTRODUCTION

5.1.1. Establishment of long-term lymphoblastoid cell lines

The fortuitous observation that phytohemagglutinin (PHA) caused the transformation of peripheral lymphocytes to "blast-like" mitotic cells (Nowell 1960), facilitated the investigation of many genetically-determined diseases. However investigations were still limited by the short life-span of such cells.

With the first successful long-term culture of lymphoid cells from solid tumour and tumour fluid aspirates of patients with Burkitt's lymphoma, thus defining suitable culture conditions (Epstein and Barr 1964, Pulvertaft 1964), many cell lines from patients with various lymphoproliferative disorders were established, using tissue from tonsil, spleen, lymph node or peripheral blood as the source of cells (reviewed by Glade and Beratis 1976). Long-term cell lines were also established from patients with infectious mononucleosis (glandular fever), measles, mumps and herpes simplex. These cell lines, with the exception of some of those derived from malignant tissue but with the inclusion of those derived from Burkitt's lymphoma patients, were all subsequently shown to harbour either Epstein-Barr virus or its genome (Glade and Beratis 1976, Povey et al. 1980). This virus was later shown to be responsible for the proliferation of at least the majority of human B lymphoblastoid cell lines in long-term culture. Consequently it was possible to culture lymphocytes from healthy people with no evidence of previous viral infection by cultivating the cells in the presence of viable Epstein-Barr virus (Henle et al. 1967, Miller et al. 1971,

Steel 1972).

The value of such cell lines in genetic research is now well established with the demonstration of abnormal giant lysosomes in cell lines from a patient with Chediak-Higashi syndrome and his heterozygous father, and the persistence in culture of the aetiological biochemical and/or chromosomal abnormalities in cell lines from patients with e.g. Lesch-Nyhan syndrome, citrullinemia, and maple-syrup urine disease (reviewed by Glade and Beratis 1976). More recently, long-term lymphoblastoid cell lines have been used to investigate a possible genetic abnormality in beta-adrenergic receptors in patients with manic-depressive psychosis (Wright et al. 1984).

5.I.ii. Characteristics of long-term lymphoblastoid cell lines

Lymphocytes consist of two major populations of cells which have different properties and functions. B lymphocytes appear to be bone-marrow derived and thymus-independent and are involved in antibody and immunoglobulin production, whereas T lymphocytes are bone-marrow derived but are thymus-dependent and are involved with cell-mediated immunity. Based on the above properties together with other membrane-associated differences, it appears as though the lymphoblastoid cells in long-term culture are B cell derived (Schneider and zur Hausen 1975, Glade and Beratis 1976). However the evidence is not clear-cut and it has been suggested that the cultured cells represent an earlier stage of differentiation with potential for both T and B cell function (Glade and Beratis 1976).

The cells grow in suspension and resemble the immature "blast-

like" PHA-stimulated peripheral lymphocytes. However a spectrum of cell forms may be seen resembling e.g. lymphocytes, lymphoblasts and stem cells. In most human B lymphoblastoid cell lines, regardless of source, 0.1 to 5% of cells contain the Epstein-Barr virus detectable by electron-microscopy or immunofluorescence. Those lines with no morphologically detectable virus contain the viral genome as shown using, for example, nucleic acid hybridisation techniques (reviewed by Glade and Beratis 1976).

The doubling time of the cells is generally between 24 to 48 hours with an apparently infinite life-span providing that nutrient supplies are adequate. Most of the cells remain diploid with more than 90% of cells in lines established from chromosomally normal donors maintaining the normal karyotype (Steel 1972, Glade and Beratis 1976). The majority of the cells in lines established from patients with chromosomal abnormalities have also maintained their karyotype in culture although slight changes have been reported to occur sometimes in both normal and abnormal karyotypes with time (reviewed by Glade and Beratis 1976). In 101 cell lines derived from individuals with no lymphoproliferative malignancy and cultured for an average of over 3 years, there was no evidence of a change in electrophoretic phenotype at a single locus for many cytoplasmic enzymes examined (Povey et al. 1980).

Interestingly, there is also evidence that enzymes which are limited to certain internal organs and are not present in peripheral lymphocytes may be present in cultured lymphoblastoid cell lines e.g. cystathionase (Glade and Beratis 1976). This may

allow their use in the detection of enzymatic defects within internal organs, thus alleviating the need for organ biopsies.

Advantages of using lymphoblastoid cell lines in genetic research include:- a). they can be established from small quantities of blood which is the most accessible peripheral tissue; b). the cells are available in large numbers and are genetically representative and stable; c). they have an apparently unlimited lifespan; d). they can be easily stored in liquid nitrogen; and e). they are free from any medication or neuroendocrine influences. The major disadvantage is that these cell lines are infected with Epstein-Barr virus. The role of this virus in human disease is not entirely clear at present and therefore the cell lines must be used with caution.

5.II. MATERIALS AND METHODS

5.II.1. Reagents

Reagents used were purchased from the following:-

BDH Chemicals (Poole, Dorset):- ethanol, NaCl, Na₂HPO₄.12H₂O, and NaH₂PO₄.2H₂O (Analar grade).

Edinburgh Cameras (Edinburgh, Scotland):- Agfachrome 1000 ASA film.

EMscope Laboratories Ltd. (London):- all electron microscope supplies as detailed in Chapter 2.

Gibco-Biocult (Paisley, Scotland):- RPMI 1640 (HEPES-buffered) tissue culture medium, foetal calf serum, L-glutamine (200 mM), and penicillin/streptomycin (5000 U/ml).

Sigma (Poole, Dorset):- ethidium bromide and acridine orange.

Surgikos Ltd. (Livingston, Scotland):- Presept disinfectant tablets.

Water used for solutions was produced using a Milli-Q water system (Millipore S.A., France).

5.II.ii. Setting-up of lymphoblastoid cell lines

The cell lines used in this project are Epstein-Barr virally transformed B lymphocytes which were provided by Dr. A. Wright of the MRC Clinical and Population Cytogenetics Unit, Edinburgh (control cell lines from non-psychiatrically ill subjects), Professor T. Reich of Washington University, USA (cell lines from manic-depressive patients and family members), and Dr. G. J.

Naylor of the Psychiatry Dept., Ninewells Hospital, Dundee (1 control and 2 manic-depressive cell lines). The manic-depressive patients in the USA from whom the cell lines were established (probands), all fulfilled the criteria of severe illness of early onset and a family history of affective disorder in at least two generations. A complete family history was recorded for each proband; however the clinical status of the family members was unknown to me as part of a blind study. An example of a "typical" family tree from a proband is shown in Figure 5.1. and details of the cell lines are given in Table 5.1..

The cells had been originally transformed by co-cultivation with Epstein-Barr virus as described by Steel (1972) and Wright et al. (1984). Essentially, blood samples were centrifuged, the plasma removed and the lymphocytes isolated on a Ficoll-Hypaque gradient. These were then co-cultivated in a suitable medium with either lethally-irradiated or sonicated cells infected with Epstein-Barr virus. Transformation could be monitored by the appearance of a pH change in the medium and usually occurred within 4-8 weeks (Wright et al. 1984).

5.II.iii. Sub-culturing of lymphoblastoid cell lines

All procedures were carried out in a Gelaire laminar air flow cabinet using aseptic techniques as described in Chapter 2. Gloves were always worn and all media and glassware etc. was discarded into Presept - a chlorinated disinfectant.

Cells were routinely grown at 37°C in 125 ml medicine bottles containing HEPES-buffered RPMI 1640, pH 7.3-7.4, supplemented

UNIPOLAR

BIPOLAR

PROBAND

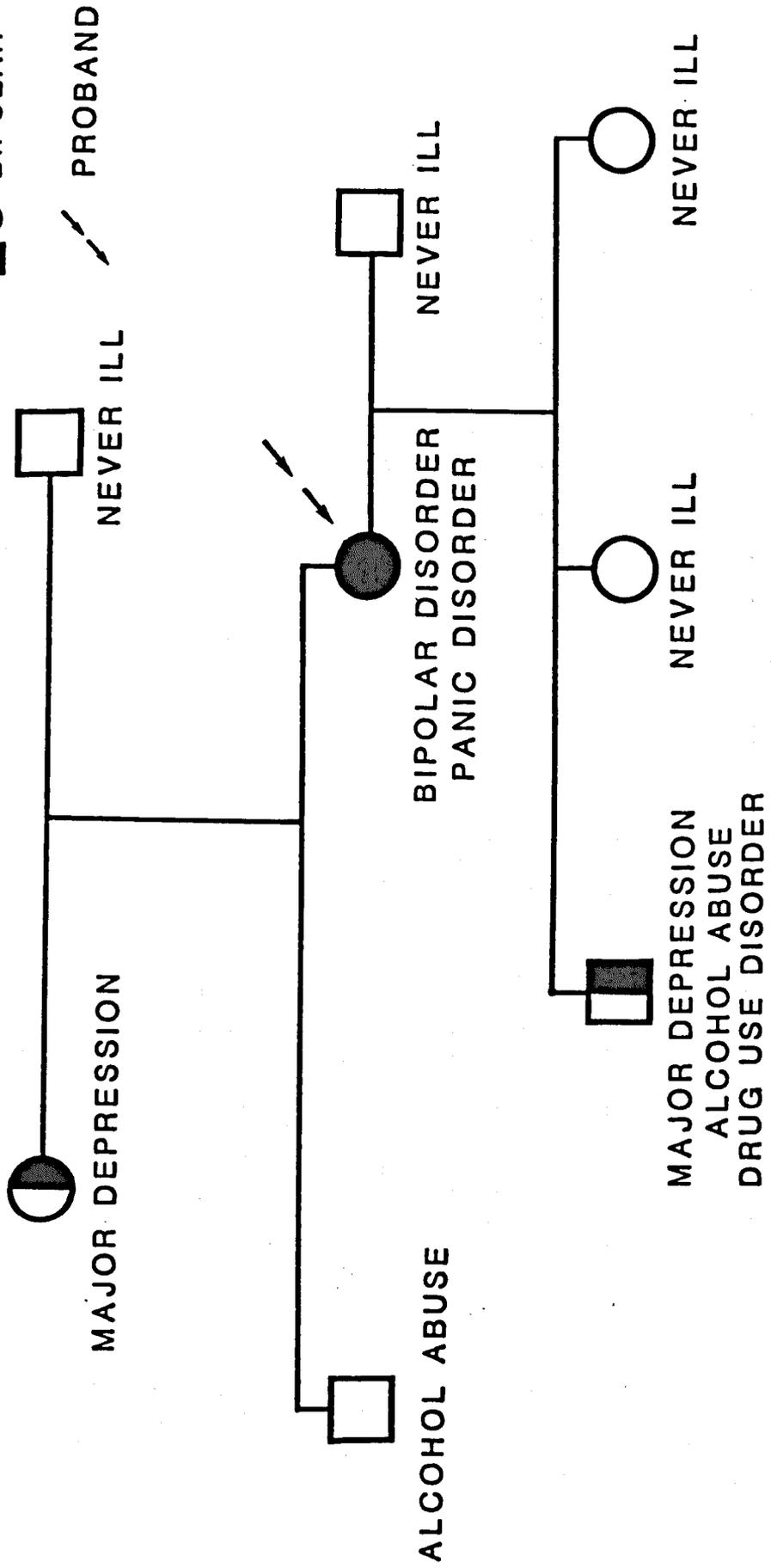


Figure 5.1. Example of a "typical" family tree of a manic-depressive proband

Table 5.1. Details of the lymphoblastoid cell lines used in this study

<u>Cell Line</u>	<u>Year of Birth</u>	<u>Sex</u>	<u>Status</u>	<u>Source</u>	<u>Established</u>
Ale	1938	M	Control	E	1983
C1b	1940	F	Control	E	1983
D11	1936	M	Control	E	1983
Fran	1965	M	Control	E	1984
Gavi	1969	M	Control	E	1984
Ged	1947	M	Control	E	1983
J11	1938	M	Control	E	1983
Mib	1915	F	Control	E	1983
Sep	1971	M	Control	E	1978
Tony	1909	M	Control	E	1984
Twel	?	F	Control	E	1984
Wib	1944	M	Control	E	1983
01-01	1951	F	Proband	U	1984
02-01	1941	M	Proband	U	1984
05-01	1921	F	Proband	U	1984
06-01	1947	F	Proband	U	1984
07-01	1921	M	Proband	U	1984
08-01	1961	M	Proband	U	1984
08-02	1932	(P)F	Unknown	U	1985
08-03	1932	(P)M	Unknown	U	1985
08-04	1959	(S)F	Unknown	U	1985
09-01	1938	F	Proband	U	1984
10-01	1944	F	Proband	U	1984
10-02	?	(C)M	Unknown	U	1984
10-03	1914	(P)M	Unknown	U	1984
10-04	1948	(S)M	Unknown	U	1984
10-05	1939	(H)M	Unknown	U	1984
10-06	1923	(P)F	Unknown	U	1984
13-01	1963	(?)F	Unknown	U	1984
14-01	1961	M	Proband	U	1984
15-01	1933	F	Proband	U	1984
17-01	1915	F	Proband	U	1984
Fleming	1960	F	Control	D	1982
Cox	1922	F	Proband	D	1982
Greig	1929	F	Proband	D	1982

Members of the same family have the same prefix e.g. 08-01, 08-02 etc. Relationship to the proband is given in (). i.e. (S)=Sibling, (H)=Husband, (P)=Parent, and (C)=Child. For the source, U=USA, E=Edinburgh, and D=Dundee, and for sex, M=male and F=female.

with 10% (v/v) foetal calf serum, 2 mM L-glutamine and 100 U/ml of penicillin/streptomycin. The exact composition of this growth medium is given in Table 5.2.. The cells grew in suspension and could be seen on the base of the bottle. Usually the bottles contained between $5-15 \times 10^6$ cells in approximately 30 mls of medium. Optimum densities for growth appeared to be within the range $0.1-1 \times 10^6$ cells/ml as outside these levels growth appeared to slow or stop. Every 3 days the medium was removed using pipettes and replaced with fresh medium. When the change in medium pH became more rapid than normal, some cells were discarded in order to maintain the cells at an optimum density and under optimum conditions for growth. When larger quantities of cells were required, cells were grown in 500 ml bottles containing approximately 200 mls of medium and normally about $30-60 \times 10^6$ cells. For experimental purposes, bottles of cells were seeded at exact densities depending upon the experiment as detailed in later chapters.

Cell viability was assessed regularly using the nucleic acid-specific dyes, ethidium bromide and acridine orange (Becton-Dickinson FACS Systems information leaflet). Acridine orange freely enters viable cells producing a green fluorescence whereas ethidium bromide is excluded from viable cells but stains non-viable cells fluorescent orange (Parks et al. 1979). By using acridine orange/ethidium bromide the viable cells can be distinguished from the non-viable cells.

A 100x stock solution of ethidium bromide/acridine orange was prepared by dissolving 50 mg ethidium bromide and 15 mg acridine orange in 1 ml of 95% (v/v) ethanol. This was then mixed with

Table 5.2. Composition of RPMI 1640 (HEPES-buffered) tissue

culture medium

<u>Inorganic Salts</u>	<u>mg/l</u>
Ca(NO ₃) ₂ ·4H ₂ O	100.00
KCl	400.00
MgSO ₄	48.84
NaHCO ₃	2000.00
Na ₂ HPO ₄ (anhyd.)	800.00
NaCl (varied slightly to adjust the osmolarity)	6000.00
<u>Other Components</u>	
D-Glucose	2000.00
Glutathione (reduced)	1.00
Phenol Red	5.00
HEPES	5957.50
<u>Amino Acids</u>	
L-Arginine (free base)	200.00
L-Asparagine	50.00
L-Aspartic Acid	20.00
L-Cystine (2 HCl)	65.15
L-Glutamic Acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine (free base)	15.00
L-Hydroxyproline	20.00
L-Isoleucine (Allo free)	50.00
L-Leucine (Methionine free)	50.00
L-Lysine HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline (Hydroxy L-Proline free)	20.00
L-Serine	30.00
L-Threonine (Allo free)	20.00
L-Tryptophan	5.00
L-Tyrosine (Na salt)	28.83
L-Valine	20.00
<u>Vitamins</u>	
Biotin	0.20
D-Ca Pantothenate	0.25
Choline Cl	3.00
Folic Acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
Para-aminobenzoic Acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B ₁₂	0.005

In addition, the medium was supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine and 100 U/ml penicillin/streptomycin before use.

49 mls of water and stored frozen in 1 ml aliquots. To make a working solution, a 1 ml aliquot was thawed and diluted 1/100 in phosphate buffered saline (0.291g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 4.524g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 8.767g NaCl - made up to 1 litre with water and the pH adjusted to 7.4). This had a working life of 1 month when stored at 4°C in an amber bottle. Equal volumes (25 ul) of cell suspension ($1-5 \times 10^6$ cells/ml) and stain solution were mixed and a drop of this placed on a clean glass slide. This was covered with a glass coverslip and examined on a Leitz Dialux 20 microscope with Ploemopak 2.4 fluorescence vertical illuminator and camera attachment. The microscope was equipped with an I2 filter block for fluorescein (excitation 450-490 nm and emission above 515 nm). Specimens were routinely viewed using a x25 objective lens with x12.5 eyepieces or a x50 oil-immersion lens if photographs were being taken. Photographs were taken using Agfachrome film, ASA 1000. Only those cell lines with a viability greater than 95% were used in experiments.

Scanning and transmission electron micrographs of these cells were also taken solely to illustrate their morphology and ultra-structure. The method of preparation and examination was as described for HeLa cells in Chapter 2 with the exception that for scanning electron microscopy purposes, the lymphocytes were not grown on glass coverslips but were sucked onto millipore filters using a millipore filter holder and syringes. By carefully introducing the cell suspension into one side of the filter holder and simultaneously drawing the fluid out at the opposite side, the cells were drawn onto the filter. Using this technique, the cells were fixed and dehydrated in the holder

using the syringes to introduce and withdraw the appropriate solutions. The filter containing the cells was then removed from the holder and treated in the same manner as the HeLa cell-covered coverslip.

5.II.iv. Growth curves of lymphoblastoid cell lines from manic-depressive and control subjects

Cell lines from 5 manic-depressive probands and 5 controls were taken at random as representative samples and seeded at approximately 0.2×10^6 cells/ml in 30 mls of fresh medium. 1 ml samples were taken at daily intervals over the next 6 days and used to determine cell numbers and volumes using the Coulter counter as described in Chapter 2. Replacement of 15 mls of the medium with 15 mls of fresh medium was carried out after the first 72 hours.

5.III. RESULTS

Figure 5.2. is a fluorescent micrograph showing the easily distinguishable viable (green) and non-viable (orange) cells. Within the cytoplasm of the viable cells can be seen fluorescent orange inclusions; these are the lysosomes which stain orange with acridine orange (Poole 1977). A cytoplasmic protrusion from one of the cells may possibly be the start of the formation of a pseudopod involved in locomotion of these cells (McFarland 1969). Similar structures have been observed in these cultures during routine examination. Figure 5.3. is a phase-contrast micrograph illustrating the diverse range of sizes and shapes of the cultured cells and also the clumping which often occurs during culture.

Transmission and scanning electron micrographs illustrating the general morphology of the cells are shown in Figures 5.4. and 5.5. respectively. The cells appear to have an irregular outline and characteristic polarity with a large irregular nucleus at one end and most of the cytoplasmic organelles at the opposite end, as reported by Chandra et al. (1968). There is a high nuclear to cytoplasmic ratio. The oval-shaped mitochondria contain few cristae and a few arrays of endoplasmic reticulum can be seen dispersed throughout the cytoplasm. Polyribosomes are also present. In the scanning electron micrograph numerous microvilli can be seen projecting from the surface of the cells. Some of these appear to be connecting adjacent cells.

The graph showing the growth rates of some of the manic-depressive and control cell lines is given in Figure 5.6.

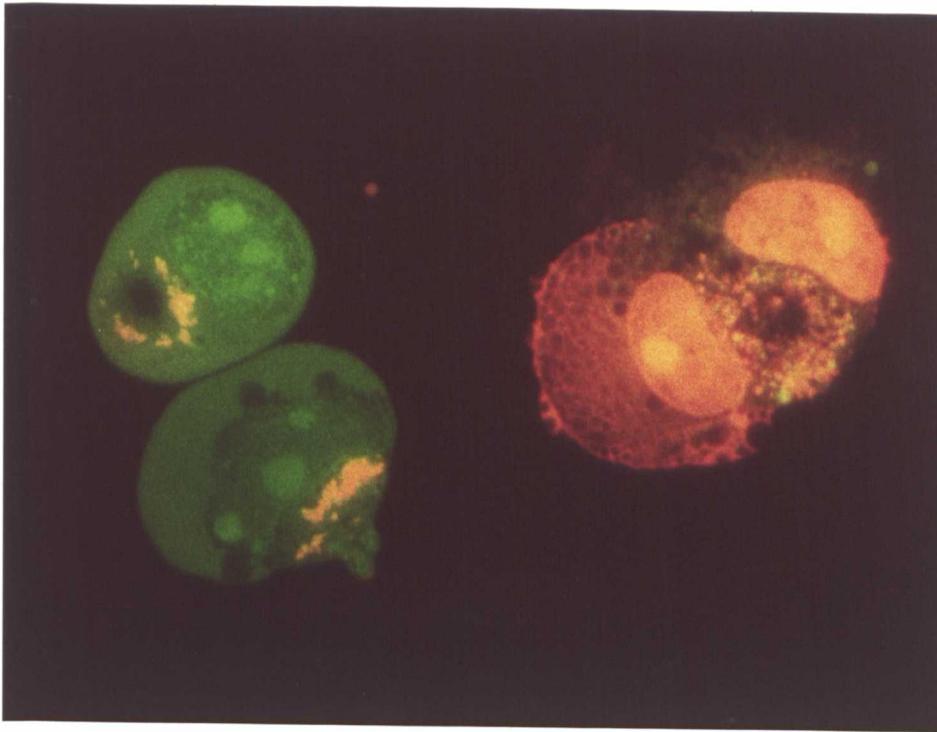


Figure 5.2. Fluorescent micrograph of virally-transformed lymphoblastoid cells stained with ethidium bromide/acridine orange. Green = viable, orange = non-viable.

10um

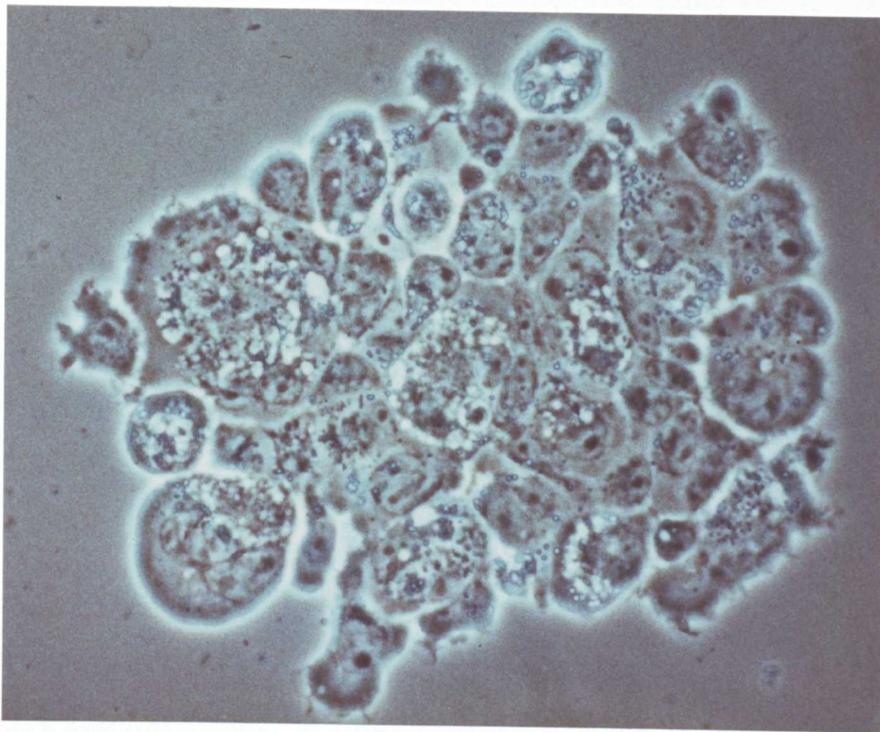


Figure 5.3. Phase-contrast micrograph of virally-transformed lymphoblastoid cells

30um

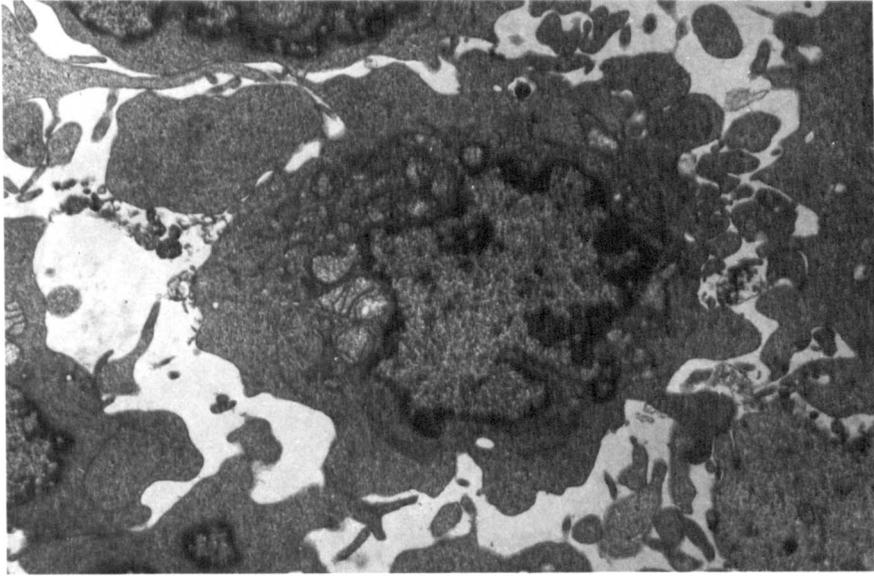


Figure 5.4. Transmission electron micrograph of a virally-
transformed lymphoblastoid cell

2.5um

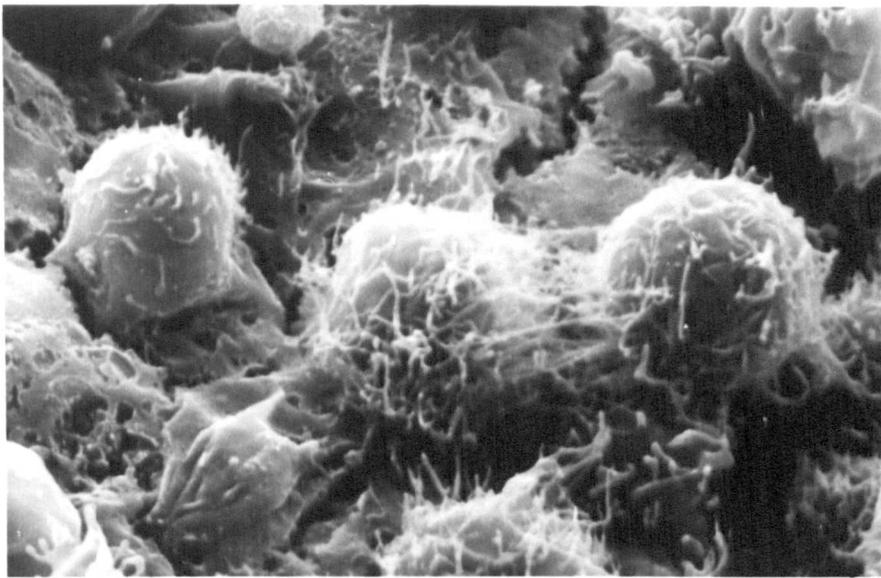


Figure 5.5. Scanning electron micrograph of virally-transformed
lymphoblastoid cells

5um

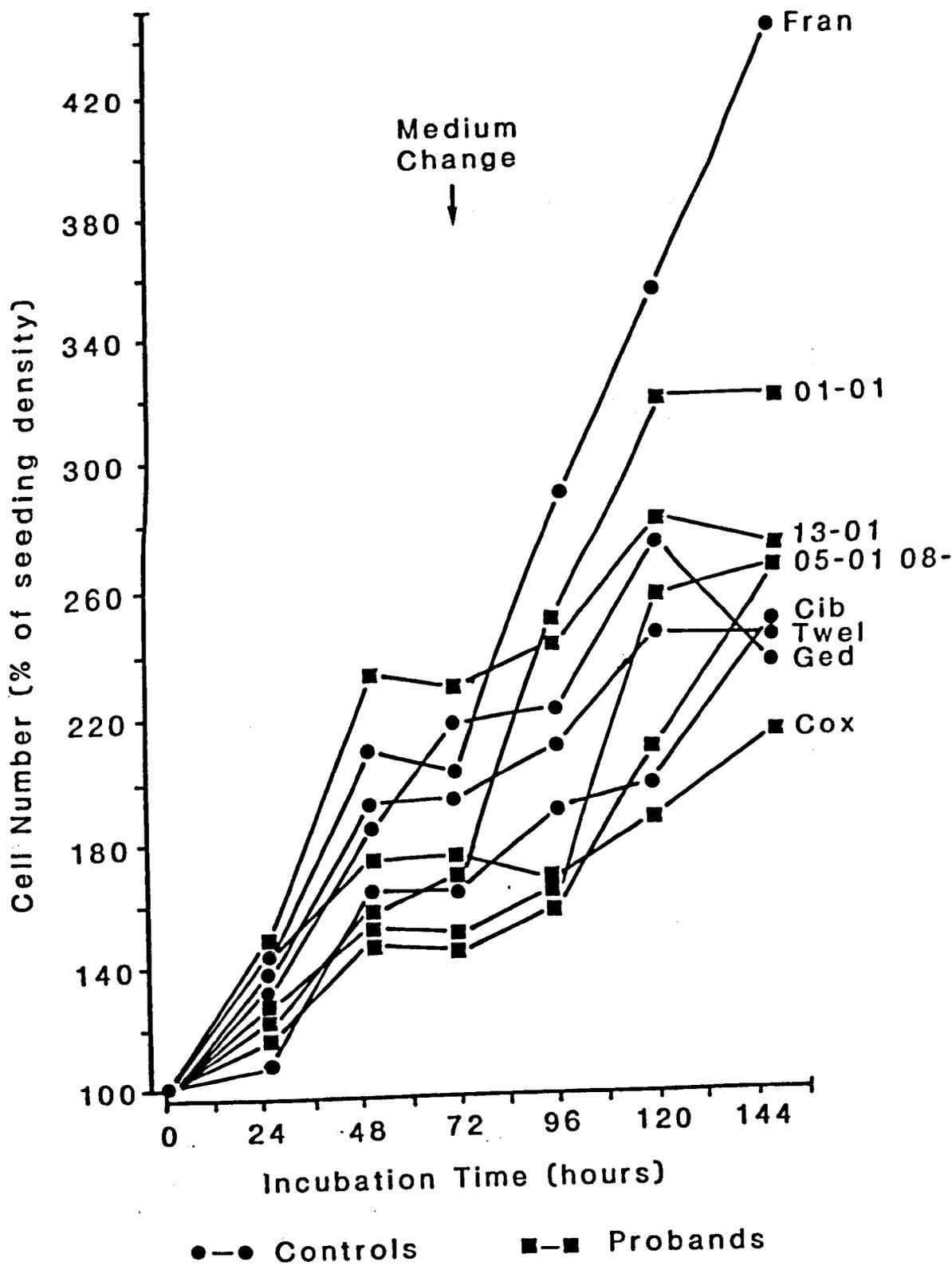


Figure 5.6. Growth rates of lymphoblastoid cell lines from 5 manic-depressive and 4 control subjects

Although the study commenced with 10 cell lines, only the results for 9 cell lines are presented as one was lost due to infection. The majority of the cell lines appear to follow a pattern of a slow initial growth during the first 24 hours after the starting medium change, probably due to a time-lag in the early part of this period which is inherent in many culture systems (Paul 1970). This is followed by a faster logarithmic phase over the next 24 hours and then a slower or stationary phase as nutrients are depleted. Essentially the same pattern is repeated after the subsequent medium change although variations can be seen in some cell lines e.g. Fran. There appear to be no obvious differences in the growth patterns between the manic-depressive and control groups, or based on age or sex, although variation can be seen between individual cell lines with absolute population doubling times ranging from 36 to 132 hours.

5.IV. DISCUSSION

Ideally the cell lines used in this study would have all been established in the same laboratory, at the same time, and with age and sex-matched controls. However as we have neither the facilities for transformation nor access to appropriate clinical material, we have used the cell lines kindly supplied to us, interpreting any results with the necessary caution and taking into account any known differences. Although the cell lines had been established in different laboratories, essentially the same transformation technique was used in each case, as previously described. In addition the cell lines had all been in culture approximately the same length of time.

There seems to be no apparent difference between the growth patterns of the manic-depressive and control cell lines used here, as discussed in the previous section, although slight differences do exist between individual cell lines. The reason for this is not known but potential effects of these differences must be borne in mind when interpreting any results. The range of population doubling times of 36 to 132 hours is longer than the 24 to 48 hours reported by Glade and Beratis (1976). This may be due to either shorter periods of logarithmic growth and/or slower rates of growth during the logarithmic phase, in the cell lines used here. The actual reason for this is unknown but may be due to differences in the composition of the medium used, particularly with regard to serum concentration or composition.

Another point to take into account is that growth curves do not provide exact information about the proliferative fraction of

a cell population. A more accurate picture may be obtained if growth curves are carried out concurrently with measurements of the percentage of cells incorporating radioactive thymidine (Macieira-Coelho 1973). The proliferative fraction of a cell population is also influenced by the split ratio i.e. the ratio of the number of cells per volume of medium after sub-culturing, to the number of cells per volume of medium before sub-culturing (Macieira-Coelho 1973). Such factors may explain the discrepancies between the population doubling times in this study where the initial split ratio was approximately 1:2 and the 72 hour split ratio was essentially 1:1, and those reported by Glade and Beratis (1976) where the split ratio is not indicated. The slight differences between the growth patterns after the initial medium change and after the 72 hour medium change may also possibly be explained on this basis.

CHAPTER 6. K^+ FLUXES AND SODIUM PUMP NUMBERS IN LYMPHOBLASTOID
CELL LINES FROM BIPOLAR MANIC-DEPRESSIVE PATIENTS AND CONTROL
SUBJECTS; EFFECTS OF CHRONIC INCUBATION WITH OUABAIN, LITHIUM OR
VANADATE

6.I. INTRODUCTION

6.I.i. Cation transport in bipolar manic-depressive psychosis

The possible involvement of a perturbation in cation transport in the aetiology of manic-depressive psychosis, together with the postulated role of vanadium, have been reviewed in Chapter 1. This section discusses in greater detail those studies which have measured cation transport in manic-depressive illness. The majority of these studies have used erythrocytes.

In a study by Naylor et al. (1973), the Na^+/K^+ -ATPase activity and ouabain-sensitive potassium influx of erythrocytes from psychotically depressed patients, were found to increase significantly upon recovery, in some but not all patients. This study was based on 58 female patients with various types of depressive illness. Since no control group was included in this study, it was not possible to compare remission values with control values. Similar findings have been reported for Na^+/K^+ -ATPase, $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase and Mg^{2+} -ATPase activities upon recovery from depression, with the depressed values being 70%, 88% and 76% respectively of remission values and the remission values being similar to those of normal controls (Rybakowski et al. 1981). Lower ouabain-sensitive K^+ and Na^+ fluxes have subsequently also been reported in manic patients compared with a control group, although the two groups were not sex-matched (Rybakowski et al. 1983). Thakar et al. (1985) also found no difference in sodium pump activity between euthymic patients and controls. In some studies however, it appears that people susceptible to manic-depressive psychosis may have lower sodium

pump activities even when recovered, if compared with controls. For example, the mean sodium pump activity as measured by active sodium extrusion was found to be lower in bipolar patients during the manic phase, and during remission, relative to healthy individuals (Hokin-Neaverson et al. 1974).

Sengupta et al. (1980) however, found a higher total Na^+/K^+ -ATPase activity in erythrocyte membranes and platelets from manic or depressed patients when compared with controls, although their activity values were much different to values reported in other studies. No alteration in the lithium-sodium exchange, diuretic-sensitive cotransport or passive lithium, sodium or potassium permeabilities was observed by Dagher et al. (1984) in erythrocytes from either unipolar or bipolar patients. Sodium pump activity however was greatly reduced both in lithium-treated and non-treated bipolar patients compared with controls. Whether this was due to lower pump numbers or activity per pump was not ascertained. MacDonald et al. (1982) found that the best correlation with mood was with the Ca^{2+} -ATPase activity and no correlation between sodium pump activity and mood was found by Linnoila et al. (1983). However, this latter study used only a small group of patients and drug therapy was not controlled between the groups.

Despite differences in experimental methods, the results of the majority of studies are consistent with a lower total sodium pump flux in mania and depression compared to remission (Naylor 1986), although whether remission values are also lower than controls is less clear.

The change in total Na^+/K^+ -ATPase activity appeared to be due

to a change in activity per sodium pump molecule rather than to a change in the number of transport sites per cell in both manic and depressed patients (Naylor et al. 1980). This study however included only female patients who were not used as their own controls but were compared with a recovered group, due to patient non-compliance. Although differences existed between the groups with respect to medication, it seemed unlikely that this was responsible for the differences in pump activity seen. The fact that the changes in total Na^+/K^+ -ATPase activity were found to be due to changes in activity of individual pump sites rather than number is to be expected, both from the fact that mature erythrocytes no longer possess the capacity to synthesise proteins, and from the rapidity and magnitude of the changes in some cases (Naylor 1983). The changes therefore, were not likely to be explained by the production of new erythrocytes with a different number or activity of pump sites (Naylor 1983).

In erythrocytes from bipolar patients on lithium therapy (commenced at a predetermined time independent of the mood state), a significant increase in Na^+/K^+ -ATPase activity together with a non-significant increase in the active potassium influx was seen, when compared with the same patients taking a placebo (Naylor et al. 1974). There was no significant change in intracellular sodium. This increase in sodium pump activity was not seen in either controls or rats taking lithium (Naylor et al. 1977), but an increase in pump site numbers was seen in HeLa cells when treated with lithium at a concentration 10-50x that of therapeutic levels (Boardman et al. 1975a). Similar changes in Na^+/K^+ -ATPase and Mg^{2+} -ATPase activity, but not in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -

ATPase activity, have also been reported in a lithium-treated group of patients with affective disorder when compared with non-lithium treated patients (Hesketh 1976).

If the increase in sodium pump activity brought about by lithium was crucial to its therapeutic role, simultaneous treatment with an inhibitor of the enzyme such as digoxin should block the therapeutic effect. Chambers et al. (1982) showed that this was indeed the case in manic patients. There were, however, age differences between the lithium/digoxin and lithium/placebo groups, but these were not thought to account for the changes seen. However the results regarding effects of lithium on Na^+/K^+ -ATPase activity are equivocal since it has also been reported that lithium treatment has no effect in vivo upon Na^+/K^+ -ATPase activity in patients (Linnoila et al. 1983). Many other studies have been carried out in this area but there is still no consensus of opinion.

6.I.ii. Postulated defects in cation transport in bipolar manic-depressive psychosis

The possibility that the reduced level of activity per pump site in manic and depressive patients is due to higher concentrations of a "modifying factor", tentatively identified as vanadium, has been reviewed in Chapter 1. It has been postulated that if vanadium is an aetiological factor in manic-depressive psychosis, affected subjects must have some defect in their sodium pumps, probably genetic, which renders them more susceptible to the actions of vanadium, since vanadium given to normal subjects does not invariably produce depression of mood

(Naylor 1983). Naylor and Smith (1981) showed that lymphocytes from female bipolar patients had a significantly reduced ability to synthesise new pump sites in response to raised intracellular sodium levels, when compared with control subjects, and proposed that this is where the genetic defect lies, increasing the susceptibility of affected individuals to the actions of vanadium. The oxidation state of vanadium has not been considered in these studies. The finding of a reduced activity per pump site in erythrocytes from manic-depressive patients (Naylor et al. 1980) would also point to a further defect which, in view of the intracellular reduction of vanadate to the less biologically active vanadyl, may be a defect in the reducing capability of manic-depressive patients. Such hypotheses can not totally explain the illness however, as they fail to account for either the difference between mania and depression, or the onset and spontaneous remission of the illness.

The problem with the studies described here is the possible effects of medication, hospitalisation, diet etc. on the results. In addition the research has been carried out on various types of blood cell and results extrapolated to the brain. The use of long-term lymphoblastoid cell lines as described in the previous chapter will circumvent the first of these problems but there is no (ethical) solution to the second. However there is considerable evidence (reviewed in Chapter 1) that the illness is genetic in origin. The use of lymphoblastoid cell lines in research into genetically-determined diseases has been reviewed in the previous chapter and as they possess the genetic material of the individual it is possible that under stressed conditions

any defect present in the brain cells may be manifest in these cell lines grown in vitro. The remainder of this chapter will be devoted to the examination of the ion contents, sodium pump numbers, and K^+ influx pathways in cell lines established from control subjects and bipolar manic-depressive patients, under basal and treated conditions. The treatment conditions include growth in, a). lithium because of its therapeutic value in the illness and its postulated effects on Na^+/K^+ -ATPase; b). ouabain in order to stress the ion transport systems of the cells through its effects on both sodium pump number and ouabain-sensitive flux per pump site; and c). vanadate because of its postulated involvement in the aetiology of the illness through effects on the sodium pump, as already reviewed. With regard to the treatment with vanadate, the previous chapters utilising HeLa cells may be used to aid the interpretation of the results in this chapter, although it is recognised that these are different cell types and that a far from clear picture has emerged from the study of the cellular actions of vanadate in the relatively well-defined HeLa cells.

6.II. MATERIALS AND METHODS

6.II.1. Reagents

Reagents used were purchased from the following:-

Aldrich Chemical Company Ltd. (Gillingham, Dorset):- Na_3VO_4
(99% pure).

Alfa Chemicals Ltd. (Wokingham, Berks.):- Versilube F50.

Amersham International (Amersham, Bucks.):- ^{86}Rb (200-240
mCi/mmol initially).

BDH Chemicals (Poole, Dorset):- all the salts (Analar grade)
used in the Krebs solution, LiCl, sucrose, and "Cocktail T"
scintillant.

Coulter Electronics Ltd. (Luton, Beds.):- Isoton.

Gibco-Biocult (Paisley, Scotland):- all tissue culture supplies
as detailed in Section 5.II.1..

New England Nuclear (W. Germany):- ^3H -ouabain (20.0 Ci/mmol).

Sigma (Poole, Dorset):- Ouabain.

Bumetanide was a kind gift of Leo Laboratories, UK. Water used for solutions was produced using a Milli-Q water system (Millipore S.A., France). All solutions were dispensed using Gilson adjustable pipettes during experiments.

6.II.1i. Cell treatments

The rationale behind investigating the effects of ouabain, lithium and vanadate on cation fluxes has already been discussed in the "Introduction" to this chapter. The doses were

established both from preliminary experiments and from the literature. Lithium was used at a concentration of 10^{-3}M which is in the middle of the range of therapeutic plasma levels in man (0.6-1.5 mM; Bowman and Rand 1980). In this study LiCl was used rather than the clinically prescribed Li_2CO_3 as the solubility of LiCl is greater. Ouabain was used at a concentration of 10^{-8}M since in preliminary experiments with the lymphocyte cell lines, 10^{-8}M ouabain produced a 30-40% decrease in sodium pump numbers over 24 hours, without affecting the cellular viability. Vanadate was used at a concentration of 10^{-6}M Na_3VO_4 . Although vanadate had no effect on sodium pump numbers until concentrations greater than 10^{-5}M when using HeLa cells, toxic effects were also seen at these vanadate concentrations. 10^{-6}M was chosen as being lower than the toxic levels but higher than normal blood concentrations, thus possibly stressing the cells and exposing any inherent defects in cation transport. The effects of these treatments on cellular viability were checked and no detrimental effects were seen.

6.II.iii. Effect of chronic ouabain, lithium or vanadate treatment on sodium pump numbers (ouabain binding) of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The principles and validity of using ouabain binding to ascertain sodium pump numbers have already been discussed. Preparation of the solutions used experimentally and the routine sub-culturing of the lymphoblastoid cell lines were also as previously described.

Several preliminary studies were carried out in order to establish a viable method for measuring ouabain binding in lymphocytes. The resultant protocol is described first followed by the steps taken to validate it. The following procedure was carried out for each cell line in turn at various times throughout the study. Cells were divided into 4 labelled bottles, each containing $10-15 \times 10^6$ cells at a final density of 0.5×10^6 cells/ml, in either RPMI 1640 or RPMI 1640 containing one of $10^{-3}M$ LiCl, $10^{-8}M$ ouabain, or $10^{-6}M$ Na_3VO_4 . The bottles of cells were incubated at $37^\circ C$ for 24 hours and then the contents of each bottle were divided into 2 x 15 ml disposable plastic tubes. One tube from each bottle was used to measure the total ouabain binding and the other for non-specific binding. The cells were centrifuged at 1000 rpm for 3 minutes ($37^\circ C$; -Fisons MSE Coolspin), the supernatant discarded and the cells resuspended in approximately 10 mls of either K-free or 15K-Krebs ($37^\circ C$) as appropriate. The cells were centrifuged again and the washing procedure was carried out twice more. The cells in each tube were finally resuspended in 3 mls of the appropriate Krebs solution.

From each tube, 3 x 900 ul aliquots were taken and put in RT 30 test tubes (Sterilin) which were then placed in a Techne Dri-block at $37^\circ C$. At 5 second intervals, to each tube in turn, 100 ul of the appropriate 10x radioactive standard solution (i.e. $2 \times 10^{-6}M$ ouabain in either K-free or 15K-Krebs, containing 5 uCi 3H -ouabain/ml) was added, the tube whirlmixed and then incubated for 20 minutes at $37^\circ C$. At the end of the incubation period, 3 mls of ice-cold 15K-Krebs was added to each tube and the tubes

placed on ice. When this procedure had been carried out for all the tubes, they were centrifuged at 1000 rpm for 3 minutes (4°C; Fisons MSE Coolspin), the supernatant discarded and the cells resuspended in a further 3 mls of ice-cold 15K-Krebs. This washing procedure was carried out a further 3x, with the cells in each tube finally being resuspended in 750 ul of Krebs solution.

After whirlmixing, 200 ul of each cell suspension was added to 19.8 mls of Isoton and used for the determination of cell numbers as described in Section 2.II.iv.. 400 ul of each cell suspension was added to scintillation vials containing 5 mls of scintillation fluid. The vials were then capped, shaken and placed in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3255. After allowing 1-2 hours for chemiluminescence to subside, the samples were counted for 10 minutes or 10,000 counts thus keeping the error below 1%.

For standard counts (specific activity), 3 x 100 ul aliquots of a 1 in 10 dilution of each of the 10x radioactive standard solutions were taken (equivalent to 100 ul of the final incubation medium), and added to scintillation vials containing 5 mls of scintillation fluid and 300 ul of Krebs (ensures the same quenching). These vials were cooled and counted as above. Blanks were run to account for any residual radioactivity left after the washing. These consisted of 3 tubes each containing 3 x 900 ul aliquots of one of the cell suspensions. To each tube, 100 ul of one of the radioactive standard solutions was added, immediately followed by the addition of 3 mls of ice-cold 15K-Krebs. These were washed as per the samples above and 400 ul aliquots taken for radioactive counting, also as above. Machine

blanks were run by counting vials containing 5 mls of scintillant and 400 ul of Krebs. Usually the cell blanks were equivalent to the machine blanks indicating the efficiency of the washing procedure.

Total and non-specific binding, and consequently the specific binding, were calculated according to the equation in Appendix 4, and expressed as molecules of ouabain bound/cell. Assuming that one molecule of ouabain binds per pump site (Baker and Willis 1972) this represents the number of sodium pumps per cell. Experiments were repeated for each cell line on several occasions, with each treatment condition in triplicate. Results were analysed by Student's t-test using the "Minitab" statistical package on the VAX mainframe computer.

During the preliminary experiments, cellular viability was monitored using ethidium bromide/acridine orange as described in Chapter 5. No decline in viability was seen in all the cell lines examined during all the procedures carried out.

In order to determine the appropriate incubation time i.e. when binding had saturated, time courses of ouabain binding were run at intervals over 30 minutes using a sample of 3 patient and 3 control cell lines. Once the appropriate time had been decided upon (20 minutes), ouabain binding was measured over 20 and 25 minutes, in a sample of 3 patient and 3 control cell lines, each of which had been divided up and incubated for 24 hours with either LiCl, ouabain or Na_3VO_4 as previously described. This was to check that the treatments had not altered the rate of ouabain binding in such a way that binding was no longer saturated within 20 minutes. Also, in order to

check whether any of the treatment conditions prevented the subsequent binding of ouabain to some pump sites, which may produce the same results for the time course as seen with the untreated condition but with apparently less pump sites, a sample of 3 patient and 3 control cell lines were exposed to the various treatments for 15 minutes before being washed and ouabain binding measured. This assumes that any such change would be apparent during this time period, without being superimposed on any possible treatment-induced changes in sodium pump number seen after longer periods.

In order to see if the seeding density of the cells affected the sodium pump number, several cell lines were put up at various densities and ouabain binding monitored 24 hours later. In the light of the results, it was decided that all cell lines should be seeded at the same density when carrying out ouabain-binding experiments, if direct comparisons were to be made between cell lines.

In the actual experiments, the ouabain binding of many cell lines was measured simultaneously, necessitating some tubes being left on ice for 20 minutes before being washed whilst some tubes were only on ice for 30 seconds. During preliminary experiments this was found to have no effect on ouabain binding.

6.II.iv. Effect of chronic ouabain, lithium or vanadate treatment on the K^+ influx pathways of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The various K^+ influx pathways and the principles involved in their measurement have already been discussed in Chapter 3. The

use of ^{86}Rb as a tracer measuring K^+ fluxes was also discussed and the term K^+ influx is used to here to represent that measured using ^{86}Rb . The preparation of the solutions used experimentally and the routine sub-culturing of the lymphoblastoid cell lines were also as described previously. In order to measure the K^+ influx in lymphocytes, a protocol was devised which allowed the rapid removal of the radioactive medium at the end of the incubation period. Repeated washing and centrifugation steps were of little use since they were too slow relative to the rapid efflux of K^+ across the membrane during the washes. By layering the radioactive cell suspension onto Versilube F50 silicone oil in a microfuge tube and then microfuging (Beckman Microfuge B) for 15 seconds, it was possible to pellet the cells through the oil into isosmotic sucrose (minimising cell lysis) at the bottom of the tube, thus separating the cells from the radioactive solution. By rapidly freezing the tube in liquid nitrogen, ion movements out of the cells were terminated and the tip of the tube containing the cell pellet could be cut off and used to determine the radioactive content of the cells. The optimum relative amounts of Versilube and cell suspension, and the optimum cell density of the suspension were determined during preliminary studies, with samples of the oil and interfaces being checked under the microscope for the presence of cells. The optimum conditions using a 450 ul microfuge tube (Sarstedt) were found to be a 100 ul layer of cell suspension, a 50 ul layer of Versilube and a 50 ul layer of isosmotic sucrose (0.25M). Cell suspensions containing from 0.5×10^6 cells/ml to 5×10^6 cells/ml were tried

and in every case complete passage through the oil of all the cells was found. During preliminary flux experiments, samples of the frozen oil and the sucrose layer above the cell pellet were also taken and the radioactivity determined in order to check for contamination and/or loss of cellular radioactivity.

For the flux experiments, each cell line used was split into 4 labelled bottles at a density of $0.2-0.7 \times 10^6$ cells/ml in 25 mls of either RPMI, or RPMI containing one of $10^{-3}M$ LiCl, $10^{-8}M$ ouabain or $10^{-6}M$ Na_3VO_4 . The following procedure is described for one bottle but was carried out for all of them in turn. After 24 hours incubation at $37^\circ C$, 15 mls of the medium was removed from the bottle by suction. The remaining cell suspension was then poured into an appropriately labelled 15 ml disposable plastic tube and centrifuged at 1000 rpm for 3 minutes ($37^\circ C$; Fisons MSE Coolspin). The supernatant was discarded and approximately 10 mls of Krebs solution ($37^\circ C$) was added to the tube and the cells resuspended. This washing procedure was carried out twice more with the cells finally being resuspended in 4.5 mls of Krebs solution ($37^\circ C$).

From this initial cell suspension, 3 x 250 ul aliquots were taken and added to 19.75 mls of Isoton for the determination of cell numbers as described in Section 2.II.iv. (the cell density of this initial suspension was multiplied by 0.9 to obtain the cell density in the final suspension, due to the dilution incurred by the addition of the radioactive solution). From the remaining cell suspension, 12 x 180 ul aliquots were removed and placed in Steri-G tubes (Sterilin) at $37^\circ C$ in a Techne Dri-Block. At 30 second intervals, 20 ul of the appropriate 10x radioactive

standard solution was added to each tube in turn, the first 3 receiving Krebs containing 20 uCi $^{86}\text{Rb}/\text{ml}$, the second 3 receiving Krebs containing 20 uCi $^{86}\text{Rb}/\text{ml}$ and 10^{-3}M bumetanide, the next 3 tubes receiving Krebs containing 20 uCi $^{86}\text{Rb}/\text{ml}$ and 10^{-2}M ouabain, and the final 3 receiving Krebs containing 20 uCi $^{86}\text{Rb}/\text{ml}$, 10^{-3}M bumetanide and 10^{-2}M ouabain. The $[\text{K}^+]$ of the Krebs had previously been checked by flame photometry (normally 5.7 mM). Immediately after the addition of the radioactive solution, each tube was vortexed and incubated at 37°C for 12 minutes. For each tube in turn, 5-10 seconds prior to the end of the incubation period and at the appropriate time intervals, the contents of the tube were whirlmixed, and 100 ul of cell suspension was layered onto the Versilube layer as previously described (450 ul microfuge tubes containing 50 ul of 0.25M sucrose covered by 50 ul of Versilube F50). The microfuge tube was microfuged (Beckman Microfuge B) for 15 seconds then immediately frozen in liquid nitrogen. This procedure was carried out for each of the tubes in turn. The tips of the frozen microfuge tubes containing the cell pellets were cut off using a razor blade and placed in scintillation vials containing 10 mls of water. After shaking to ensure the cell pellet was dispersed in the water, the samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer 3255 by the Cerenkov method as described in Section 3.II.iv..

Standard counts were determined by taking 3 x 100 ul aliquots from each of a 1 in 10 dilution of the 10x radioactive standards (yields the specific activity of the final incubation medium). These were added to scintillation vials containing 10 mls of

water and the radioactive content determined as previously described. Cell blanks, to account for the radioactivity carried over between the cells, were prepared from each bottle of cells (necessitated by the differing cell densities) by taking 3 x 180 ul aliquots of the initial cell suspensions in Krebs, and placing them in Steri-G tubes (Sterilin) on ice. To each tube in turn, 20 ul of one of the 10x radioactive standard solutions was added and immediately the tube was whirlmixed, 100 ul of cell suspension taken and microfuged through Versilube as for the samples, the microfuge tube frozen, and the tip containing the cell pellet taken for counting as above. Machine blanks were prepared by adding 3 x 50 ul aliquots of sucrose to 10 mls of water in scintillation vials and counting as above. The presence of the microfuge tube tip had no effect on the counting efficiency.

The above procedures were repeated for all the treatment conditions. Results were calculated according to the equation in Appendix 5 and expressed as $\text{mmol K}^+/\text{lcw}/\text{min}$ or as $\text{nmol K}^+/\text{10}^6 \text{ cells}/\text{min}$ for each flux pathway. Experiments were repeated on 3 separate occasions for most cell lines, with each treatment condition in triplicate. Results were analysed by Student's t-test using the "Minitab" statistical package on the VAX mainframe computer.

In order to determine the incubation time for the above experiments, i.e. a time over which the uptake of ^{86}Rb is linear and the efflux is therefore negligible, a sample of 6 cell lines was used and total ^{86}Rb uptake measured at intervals from 1 to 30 minutes. From this data (see the following "Results")

section), the time of 12 minutes was selected as being in the linear portion of the uptake and also a convenient time for handling the tubes generated by two treatment conditions of a cell line.

The problems inherent in using either NO_3^- -Krebs or bumetanide to measure the diuretic-sensitive K^+ influx have already been discussed in Chapter 3. Rather than assuming the equivalence of these two methods for measuring the cotransport pathway in lymphocytes, a sample of 2 cell lines were used and ^{86}Rb influx measured according to the experimental protocol developed, but additionally using NO_3^- -Krebs and NO_3^- -Krebs containing 10^{-3}M ouabain (final concentration) with appropriate washes. From the results (discussed later) it was decided that in lymphocytes, it would be more appropriate to use bumetanide to measure the cotransport pathway. Dose response curves of 3 cell lines to bumetanide were studied using the experimental protocol developed, but with various concentrations of bumetanide (final concentrations = 10^{-7}M to 10^{-4}M). On the basis of these results, a final concentration of 10^{-4}M was chosen.

Four cell lines were also each seeded at various densities for 24 hours and the total ^{86}Rb influx measured. As no significant differences were seen within any cell line in the fluxes at the different cell densities, cells were put up for experiments at a density depending upon the number of cells available for each cell line (usually $0.2\text{-}0.7 \times 10^6$ cells/ml). However, as a precautionary measure, the experimental results were examined to see if any dependence on cell seeding density became apparent later. One cell line was also treated with 10^{-3}M ouabain or

10^{-4} M bumetanide for 12 minutes to see if any volume change occurred during the incubation period, as the cell volumes used in the calculation of flux were determined before the final incubation stage.

6.II.v. Effect of chronic ouabain, lithium or vanadate treatment on the intracellular Na^+ and K^+ contents of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

When measuring ion contents of suspensions of cells, centrifugation and washing steps are usually employed. Such a procedure is successful with erythrocytes for example, due to their relative impermeability and hence small loss of ions during the removal of the extracellular fluid. Although spinning the lymphocytes through oil into sucrose and washing with sorbitol were both tried in attempts to remove the extracellular ions from the cells, they met with little success due to contamination and loss of intracellular ions respectively. Despite modifications of the procedures tried, no progress was made and this line of study had to be abandoned. The possibility of using radioactive tracers equilibrated across the membrane to measure the ion contents was considered but was not pursued further due to the prohibitive expense involved in such studies.

6.III. RESULTS

6.III.1. Development of experimental protocols

The protocols developed and used to measure ouabain binding and K^+ influx in the lymphoblastoid cell lines have been detailed in the preceding "Methods" section. The results of the preliminary experiments upon which these methodologies are based are reported here.

The time courses of ouabain binding for 3 bipolar and 3 control cell lines are shown in Figure 6.1.. Although slight differences may exist in the rate of binding and the maximal binding between the various cell lines, binding appears to saturate within approximately 8 minutes in all the cell lines. An incubation time of 20 minutes should thus allow the comparison of ouabain binding values between the cell lines. The variable length of time for which samples were left on ice (less than 20 minutes) before being washed after termination of their binding, had no effect on either specific or non-specific ouabain binding. Treatment with either ouabain, lithium or vanadate (24 hours) did not appear to affect the time course of binding in the cell lines examined and treatment with ouabain, lithium or vanadate (15 minutes) did not affect the maximal ouabain binding. It can be assumed therefore that any differences seen in ouabain binding with treatment are due to a change in sodium pump number, providing that the dissociation rate of ouabain has not altered. This is unlikely as ouabain binding can be regarded as being practically irreversible in tissues from many species including man. This has been shown to be the case for example, in non-

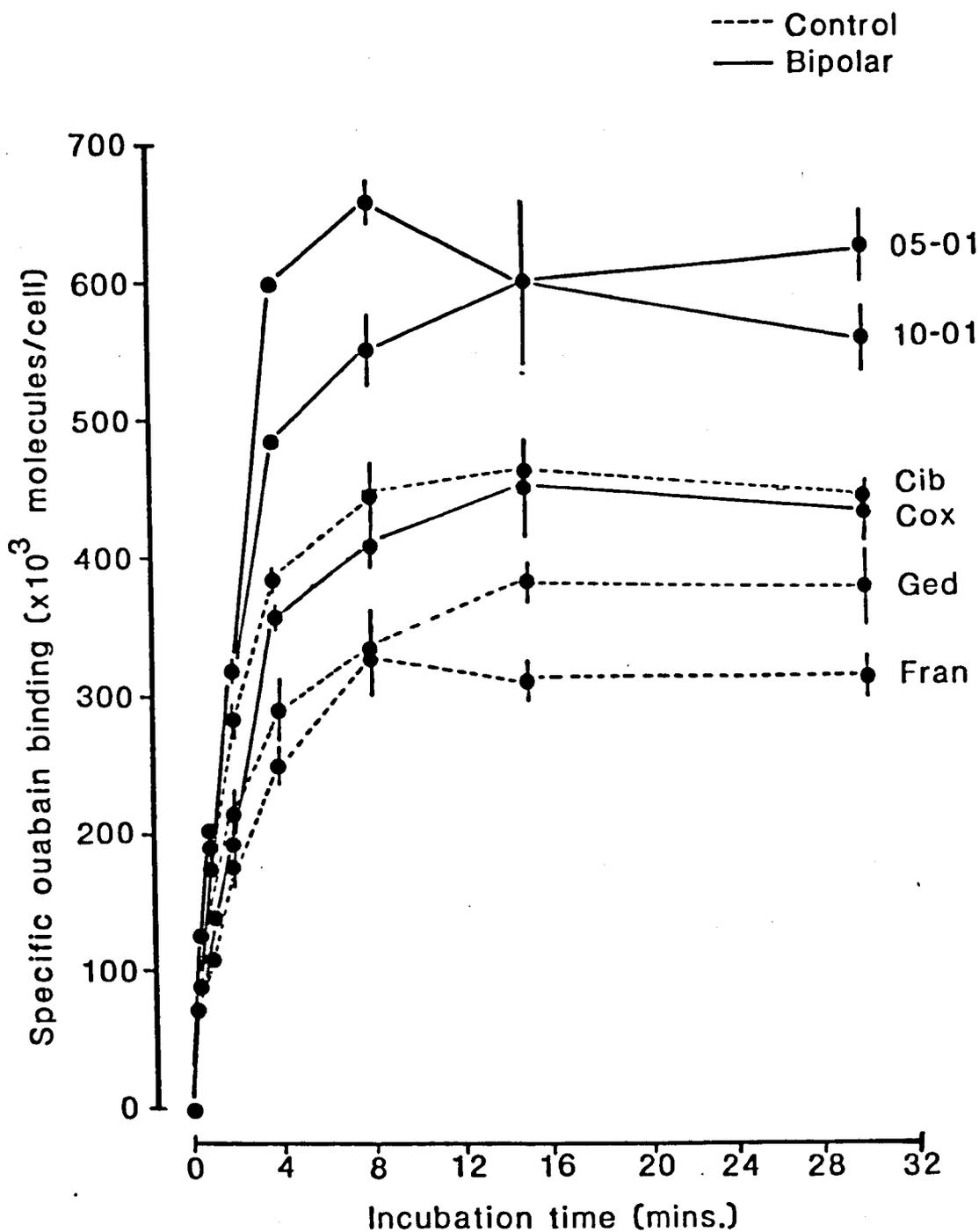


Figure 6.1. Time course of specific ouabain binding in lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Each point represents the mean \pm S.E.M. of triplicate data. [ouabain] = 2.25×10^{-7} M.

transformed lymphocytes (Boon et al. 1984).

The separation of the radioactive incubation medium from the cells in the K^+ influx experiments was made possible by the use of Versilube F50 silicone oil. Although having a similar specific gravity (approx. 1.04g/ml) to dibutylphthalate (which the cells did not spin through), Versilube F50 has different ionic properties and hence different cell surface interactions prevail, allowing the separation. No contamination of the oil or the sucrose by the radioactivity was seen. Time courses of ^{86}Rb uptake by various lymphoblastoid cell lines are shown in Figure 6.2.. Uptake appears to be linear over at least the first 15 minutes for most of the cell lines and upto 30 minutes for some. The lack of linearity over the first 2 minutes is probably due to the blank readings having a proportionately greater effect on the lower time points. It is impossible to obtain exact zero time points for the blanks due to the time taken to layer the cells onto the oil. In effect they represent approximately 10-15 seconds of flux but this is minimised by performing the blanks at $4^{\circ}C$. The effect of such blanks over a 12 minute flux period as used in this study will be negligible.

The cotransport fluxes measured using either bumetanide or nitrate-Krebs were not equivalent, probably due to other Cl^- dependent K^+ fluxes present in these cell lines. For this reason and for the reasons discussed in Chapter 3, it was decided to use bumetanide. A dose-response curve to bumetanide for 3 cell lines is shown in Figure 6.3.. $10^{-4}M$ appeared to be adequate to inhibit the flux and is also a level at which non-specific effects are minimal. K^+ influx appeared to be

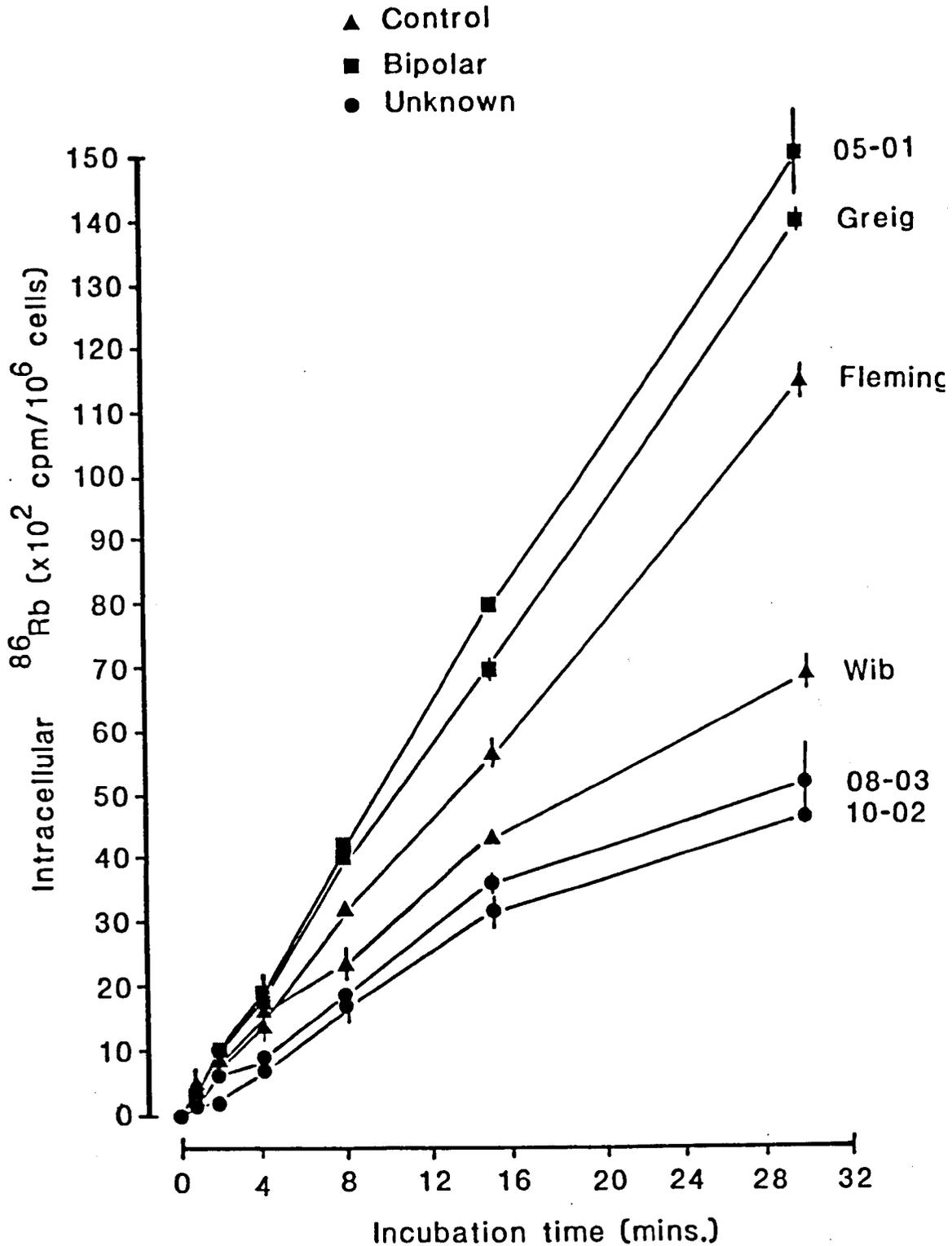


Figure 6.2. Time course of ^{86}Rb uptake in lymphoblastoid cell lines from bipolar manic-depressive patients, relatives, and control subjects

Each point represents the mean \pm S.E.M. of triplicate data.

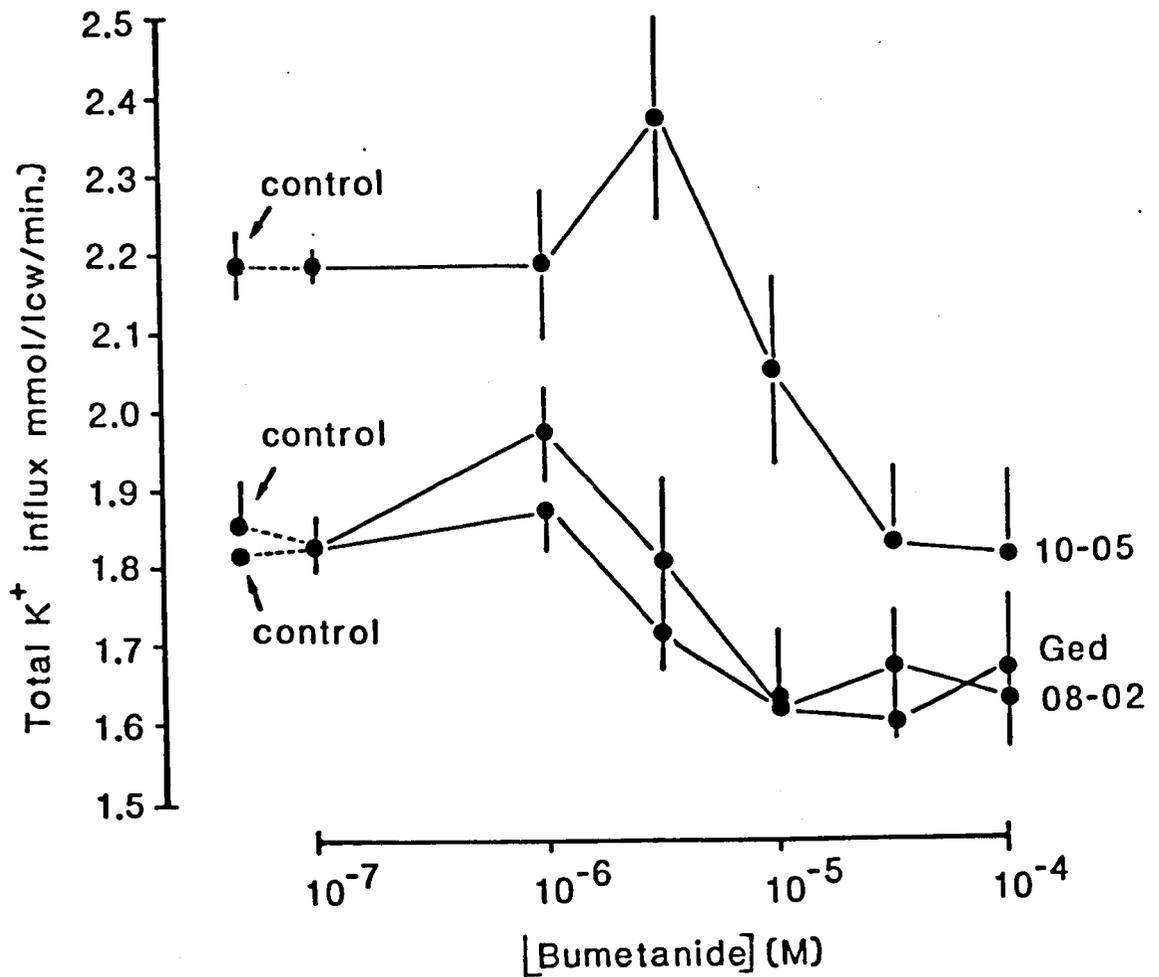


Figure 6.3. Dose-response curve to bumetanide in lymphoblastoid cell lines from relatives of bipolar manic-depressive patients, and control subjects

Each point represents the mean \pm S.E.M. of triplicate data. Incubation time in bumetanide was 12 minutes.

independent of cell seeding density in the cell lines examined and therefore no exact seeding density was used. Any differences found in the results were however examined with respect to cell density as a precautionary measure, in view of the variability in flux values as discussed in the following sections. The use of ouabain or bumetanide to measure the fluxes did not produce any changes in cell volume during the incubation period, validating the use of the cell volumes measured at the start of the incubation, in the calculation of the results.

6.III.ii. Basal values for the ouabain binding and K^+ influx pathways of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The age distribution of the bipolar and control subjects at the time the cell lines were established is shown in Figure 6.4. for all the cell lines used throughout the study. There is no significant difference between the two groups with mean values \pm S.E.M. of 38.3 ± 5.9 ($n = 12$ and 1 unknown) for the control group, and 46.5 ± 4.2 ($n = 13$) for the bipolar patients, although the control group has a greater range of values. Not all the cell lines were used for both the ouabain binding and flux measurements. The mean values for the age of the control group used for ouabain binding was 37.5 ± 7.1 ($n = 10$ and 1 unknown) whilst that for the bipolar group was 46.2 ± 5.5 ($n = 10$). The mean values for the age of the control group used for flux measurements was 35.7 ± 5.7 ($n = 11$ and 1 unknown) whilst that for the bipolar group was 47.3 ± 4.5 ($n = 12$). In both cases

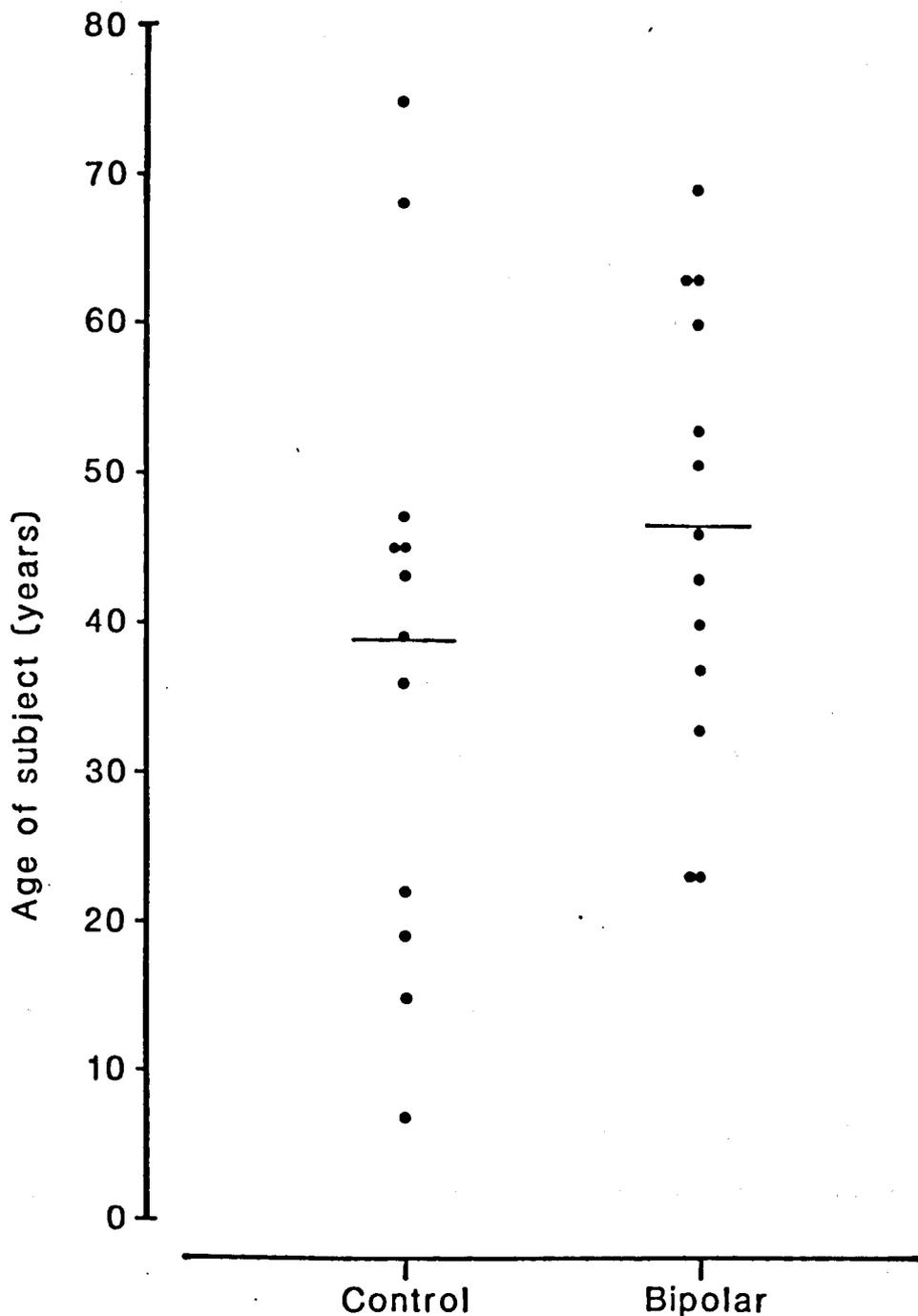


Figure 6.4. Distribution of the ages of the bipolar manic-depressive patients and control subjects from whom lymphoblastoid cell lines were established

The ages represent the age of the subject at the time the cell lines were established. — indicates the mean value for each category of cell line.

the age difference between the groups is not statistically significant. The details of the cell lines with regard to age, date of cell transformation etc. have been presented in the previous chapter (Table 5.1.) and are discussed further in the interpretation of the results in the following "Discussion" section of this chapter.

Extensive day to day variation was seen in the results for each cell line as demonstrated by the high standard errors when the overall mean results for each individual cell line are examined. This was particularly true for fluxes which varied by as much as 100% from day to day. Error bars for the mean experimental data for each cell line are omitted from the distribution plots for the sake of clarity. Within a single experiment they were normally less than 5% of the mean. A summary of the mean values for the two groups of cell lines, of all the parameters measured, is given at the beginning of the individual data, in Table 6.1..

The mean cell volumes for each of the cell lines are shown in Table 6.2. with the distribution of the individual experimental values shown in Figure 6.5.. In both groups there is a considerable spread of values with a higher mean value \pm S.E.M. for the bipolar group ($1772 \pm 166 \text{ u}^3$) compared with the control group ($1582 \pm 97 \text{ u}^3$), but the difference is not statistically significant. The three cell lines established by Naylor's Dundee group (i.e. Fleming, Greig, and Cox) all have anomalously high values, as do the cell lines 15-01, and 17-01. The excess of these cell lines with anomalously high values in the bipolar group compared with the control group largely accounts for the

Table 6.1. Basal values for volume, ouabain binding, and K⁺ influx pathways of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results below are the mean values from 12 control and 12 bipolar cell lines. The mean value for each individual cell line within these groups is derived from (usually) 3 experiments, with each parameter measured in triplicate within an experiment. Significance testing of the results from the bipolar cell lines relative to the control group is by Student's t-test.

Parameter	Mean value \pm S.E.M.		Level of Significance
	Control	Bipolar	
Cell volume (u ³)	1582 \pm 97	1772 \pm 166	N.S.
Total K ⁺ influx (mmol/lcw/min)	1.52 \pm 0.15	1.43 \pm 0.13	N.S.
Bumetanide-sensitive K ⁺ influx (mmol/lcw/min)	0.30 \pm 0.06	0.30 \pm 0.05	N.S.
Ouabain-sensitive K ⁺ influx (mmol/lcw/min)	1.01 \pm 0.11	0.98 \pm 0.08	N.S.
Passive leak K ⁺ influx (mmol/lcw/min)	0.17 \pm 0.02	0.14 \pm 0.02	N.S.
Total K ⁺ influx (nmol/10 ⁶ cells/min)	1.87 \pm 0.17	1.85 \pm 0.13	N.S.
Bumetanide-sensitive K ⁺ influx (nmol/10 ⁶ cells/min)	0.39 \pm 0.07	0.35 \pm 0.05	N.S.
Ouabain-sensitive K ⁺ influx (nmol/10 ⁶ cells/min)	1.24 \pm 0.12	1.28 \pm 0.09	N.S.
Passive leak K ⁺ influx (nmol/10 ⁶ cells/min)	0.23 \pm 0.03	0.19 \pm 0.03	N.S.
Bumetanide-sensitive K ⁺ influx (% of total)	18.20 \pm 3.07	18.38 \pm 2.66	N.S.
Ouabain-sensitive K ⁺ influx (% of total)	67.68 \pm 2.91	70.47 \pm 2.78	N.S.
Passive leak K ⁺ influx (% of total)	11.7 \pm 1.01	9.38 \pm 1.24	N.S.
Specific ouabain binding (x10 ³ molecules/cell)	386 \pm 37 (n=11)	507 \pm 42 (n=10)	p<0.05
Non-specific ouabain binding (x10 ³ molecules/cell)	51 \pm 5 (n=11)	66 \pm 8 (n=10)	N.S.

Table 6.2. Mean cell volumes of lymphoblastoid cell lines from bipolar manic-depressive patients, relatives, and control subjects

The values below are the mean of data from 3 experiments (except for Twel, Wib and 15-01 - 2, 2, and 1 respectively), with volume being measured in triplicate within an experiment. The upper, middle and lower groups represent the cell lines from controls, bipolars and relatives respectively.

Cell line	Mean cell volume \pm S.E.M. (μ^3)
Ale	1765 \pm 122
Cib	1154 \pm 138
Dil	1592 \pm 139
Fleming	2323 \pm 74
Fran	1298 \pm 13
Gavi	1467 \pm 39
Ged	1488 \pm 7
Jil	1268 \pm 33
Sep	2031 \pm 33
Tony	1592 \pm 45
Twel	1327 \pm 100
Wib	1674 \pm 87
01-01	1643 \pm 55
02-01	1129 \pm 155
05-01	1176 \pm 92
07-01	1267 \pm 36
08-01	1609 \pm 61
09-01	1317 \pm 67
10-01	1713 \pm 40
14-01	1445 \pm 130
15-01	2141 \pm 0
17-01	2639 \pm 46
Cox	2473 \pm 118
Greig	2713 \pm 69
08-02	1386 \pm 113
08-03	1270 \pm 73
08-04	1547 \pm 93
10-02	1693 \pm 29
10-03	1538 \pm 169
10-04	1377 \pm 71
10-06	1352 \pm 112

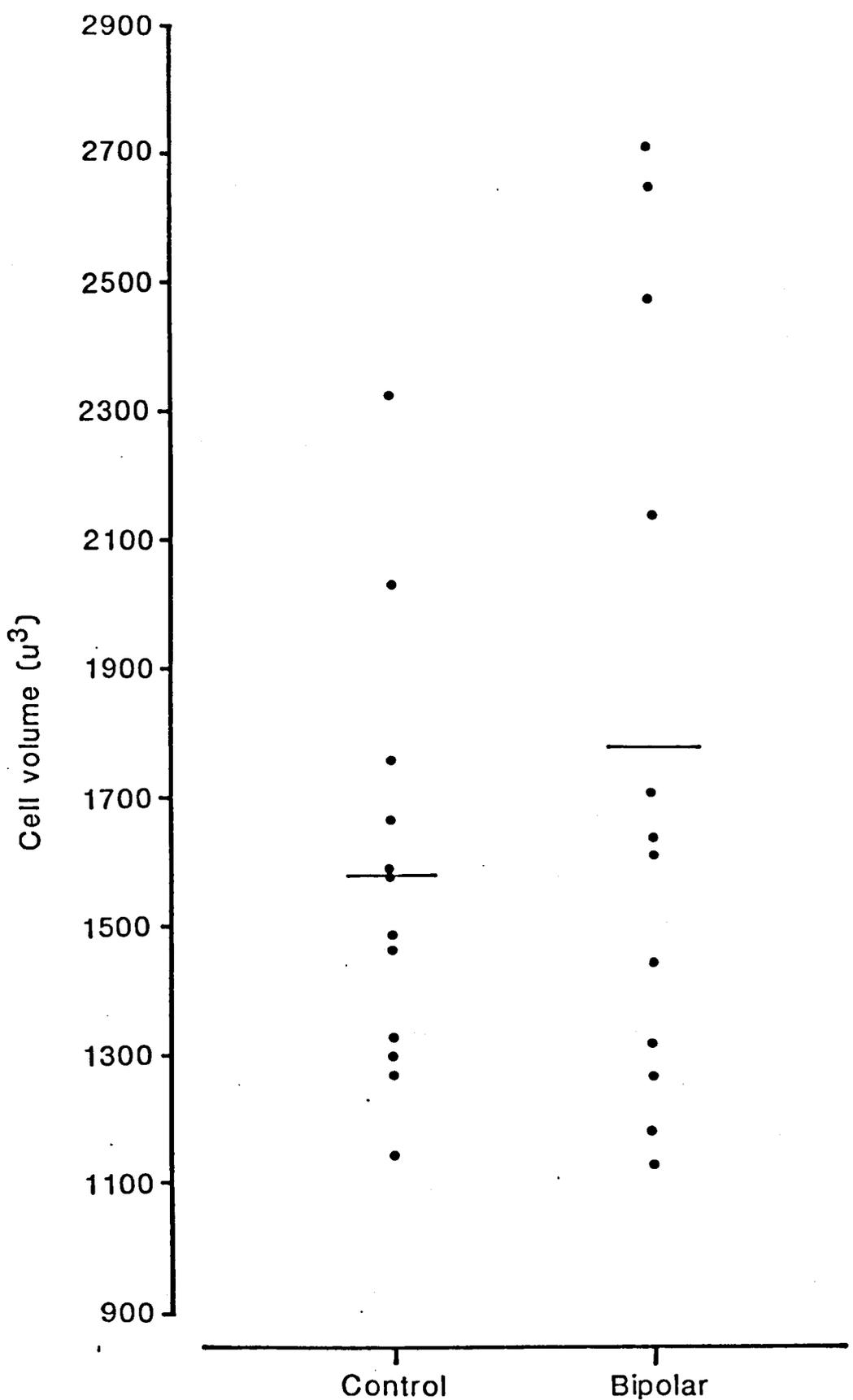


Figure 6.5. Distribution of the mean cell volumes of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 12 control and 12 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

difference seen in the mean volume.

The specific and non-specific ouabain-binding results are given in Table 6.3. and Figures 6.6. and 6.7.. The sizes of the ranges of values for specific binding are similar for both the control and bipolar groups but the mean value is significantly higher for the bipolar group (approx. 130% of the control group value). This could be due to the higher (but non-significant) values seen for the volumes (and hence possibly surface areas) of the bipolar cells compared to the control cells. This is unlikely however as if the approximation is made that the cells are spherical, the mean difference in surface area of the bipolar cells and control cells would only be approximately 7% compared with the 30% difference seen in specific ouabain binding and in addition an increase in the non-specific binding would be expected. The limitations of such an approximation of surface area have been discussed in Chapter 3 with regard to HeLa cells and apply equally to lymphocytes.

Due to the variation in ouabain binding from experiment to experiment coupled with the uncertainty of the cell surface areas, it is impossible to calculate the density of the sodium pumps per cell for each cell line. On the basis of the mean values for each group it would appear that there are more sodium pumps per unit of membrane in bipolar cells than in control cells. It is possible under this analysis however that two distinct populations of cells exist in the bipolar and/or control groups, one of cell lines with more sodium pumps per unit of surface area than the other population. However because of the variability involved and the uncertainty of cell surface areas

Table 6.3. Specific and non-specific ouabain binding values for lymphoblastoid cell lines from bipolar manic-depressive patients, relatives and control subjects

The values below are the mean of data from 3 experiments (except in the case of Ale, Mib, Twel, and 10-05 - 2, 1, 1, and 1 respectively), with each parameter being measured in triplicate within an experiment. The upper middle and lower groups represent the cell lines from controls, bipolars and relatives respectively.

Cell line	Specific ouabain binding \pm S.E.M. ($\times 10^3$ molecules/cell)	Non-specific ouabain binding \pm S.E.M. ($\times 10^3$ molecules/cell)
Ale	350 \pm 38	32 \pm 1
Cib	531 \pm 37	90 \pm 3
Fleming	627 \pm 136	54 \pm 2
Fran	289 \pm 20	34 \pm 3
Gavi	311 \pm 109	45 \pm 4
Ged	405 \pm 50	62 \pm 3
Jil	440 \pm 3	52 \pm 8
Mib	307 \pm 14	53 \pm 3
Sep	460 \pm 53	56 \pm 5
Tony	333 \pm 64	38 \pm 4
Twel	198 \pm 19	43 \pm 3
01-01	549 \pm 100	61 \pm 5
05-01	395 \pm 88	53 \pm 10
06-01	391 \pm 63	46 \pm 6
07-01	330 \pm 58	37 \pm 2
08-01	467 \pm 74	63 \pm 12
10-01	490 \pm 124	50 \pm 4
14-01	763 \pm 125	125 \pm 19
15-01	456 \pm 89	87 \pm 4
17-01	662 \pm 69	81 \pm 12
Cox	566 \pm 134	59 \pm 13
08-02	358 \pm 25	59 \pm 10
08-04	501 \pm 154	72 \pm 17
10-02	550 \pm 124	47 \pm 6
10-03	409 \pm 64	31 \pm 1
10-04	255 \pm 24	33 \pm 6
10-05	365 \pm 9	58 \pm 10
10-06	403 \pm 87	41 \pm 5

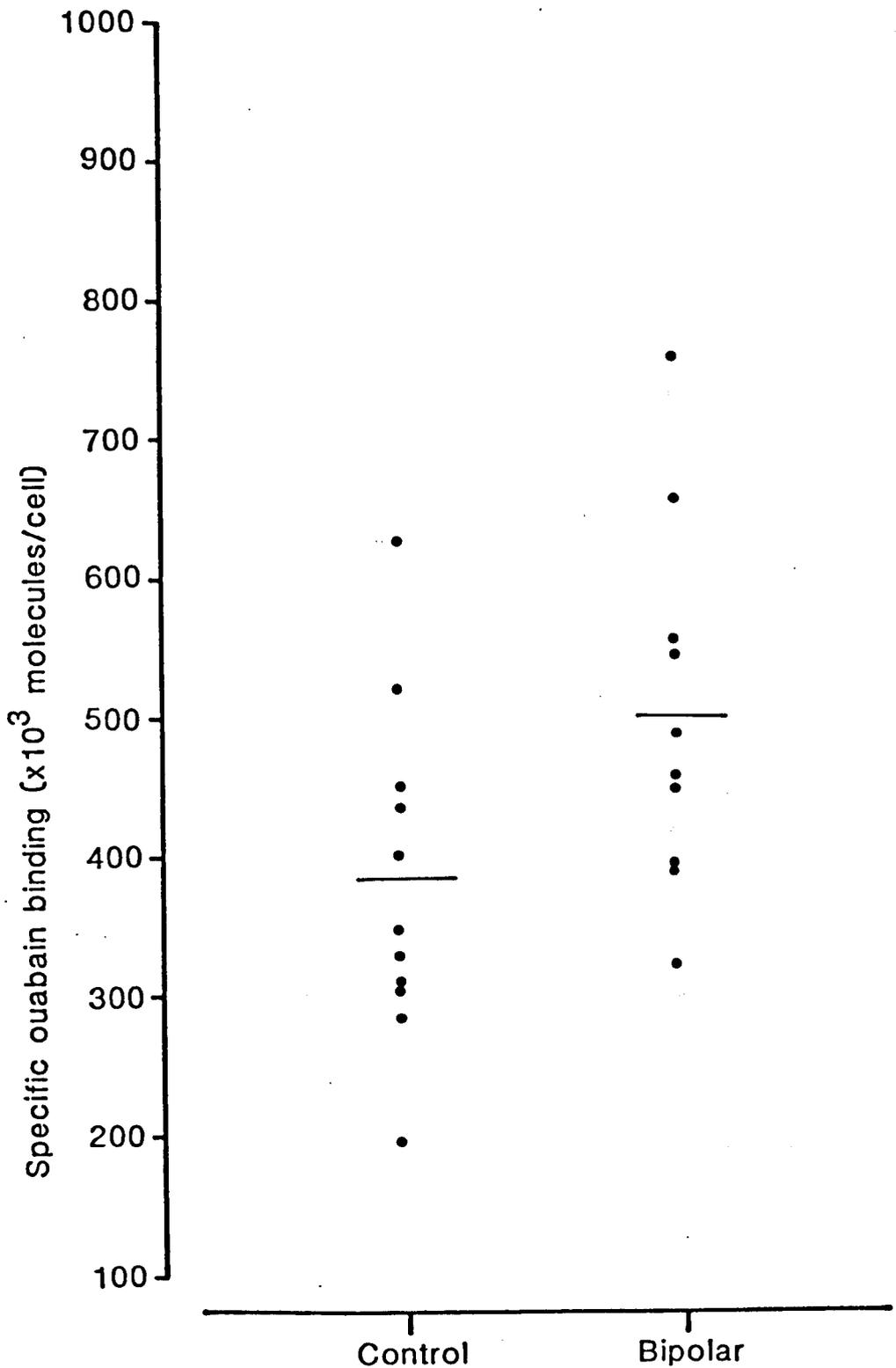


Figure 6.6. Distribution of the specific ouabain binding results for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 11 control and 10 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

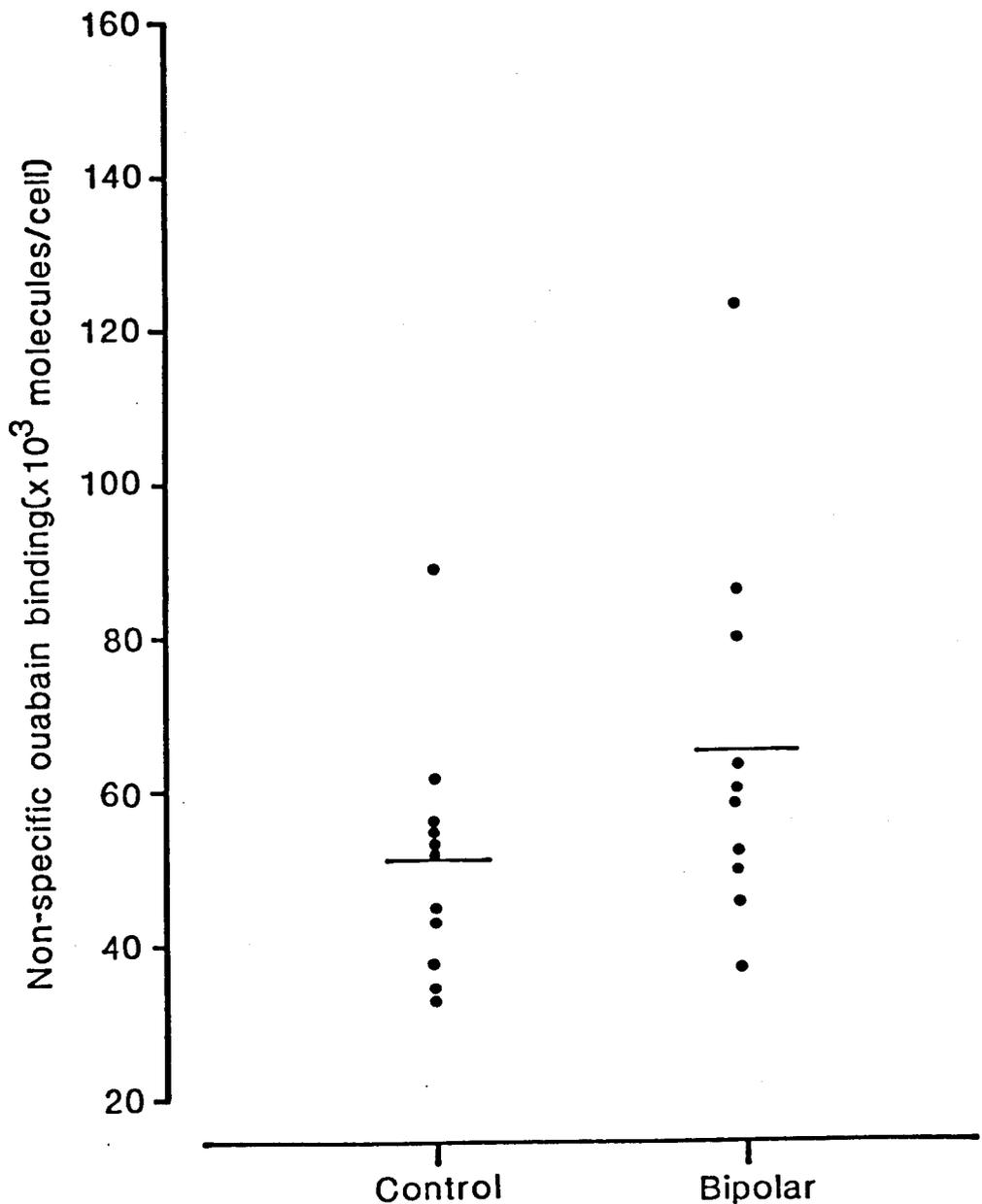


Figure 6.7. Distribution of the non-specific ouabain binding values for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 11 control and 10 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

this is impossible to determine. As already mentioned, the cell lines used for the measurement of the mean cell volumes (and the fluxes) did not include all those used for the ouabain binding studies. For example, the cell lines M1b, and O6-01 were introduced for ouabain binding whilst the cell lines D11, W1b, O2-01, O9-01 and Greig were withdrawn due to infection. These substitutions make little difference to the mean cell volumes of the groups with the exception of the withdrawal of Greig which is one of the cell lines with an anomalously high mean cell volume. This would have the effect of decreasing the difference seen in cell volume between bipolar and control cells however and make the possibility of higher cell volumes being responsible for the larger number of pump sites per cell even less likely. In addition it would be expected that the non-specific ouabain binding would also be increased if the difference in specific binding was solely due to increased cell volumes. There is no significant difference in non-specific ouabain binding however, between the two groups.

Because of the significantly increased values for specific ouabain binding of bipolars compared with controls, the results for the family members of two of the bipolar cell lines were examined (Figures 6.8. and 6.9.). This approach should minimise the influence of the genetic and environmental heterogeneity present in the general population, and examine the possibility that family members fall into sub-groups with differing sodium pump numbers per cell, depending on their clinical status. This is an oversimplification and is discussed further in the following "Discussion" section. Although there is variability

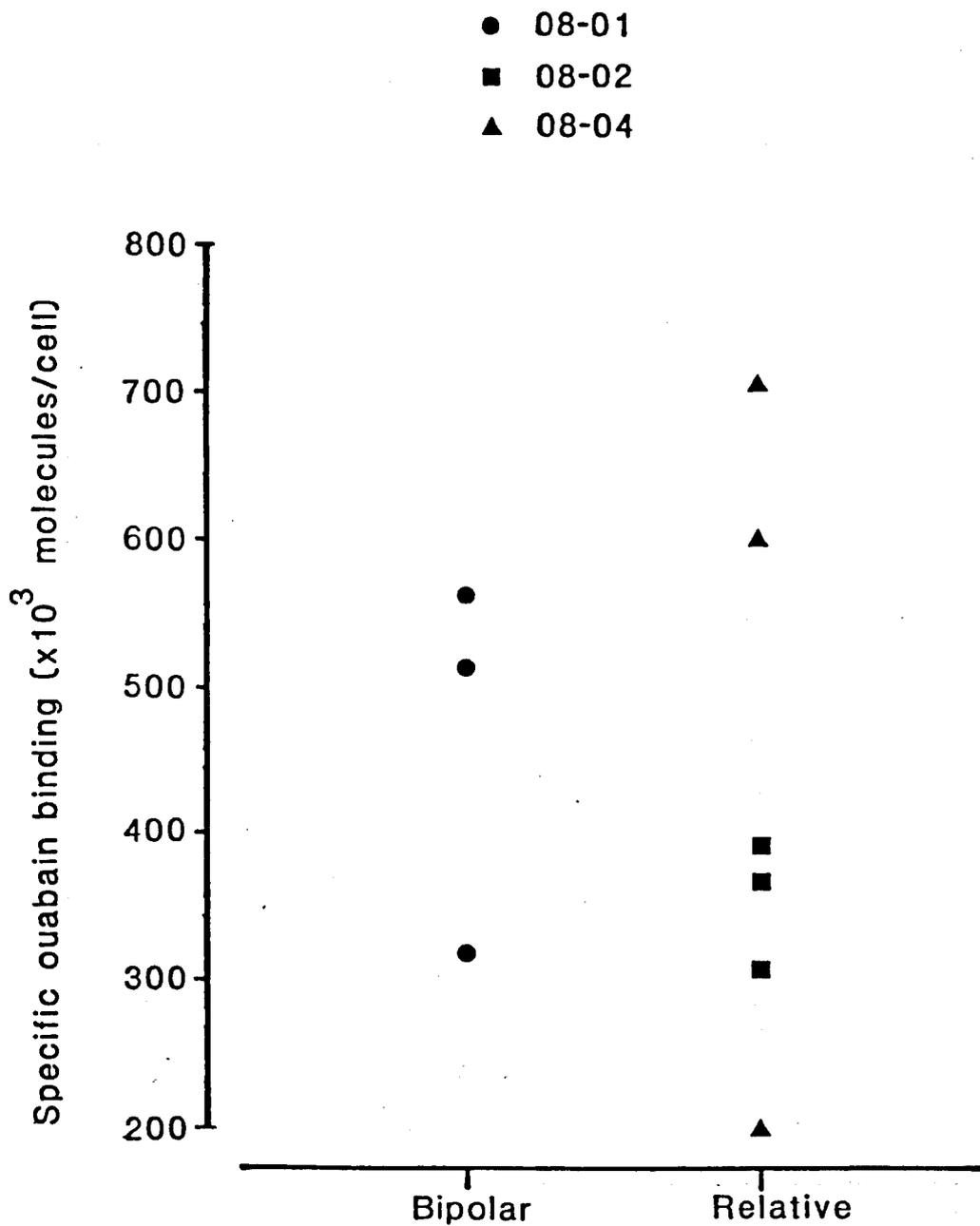


Figure 6.8. Distribution of the specific ouabain binding values for lymphoblastoid cell lines from a bipolar manic-depressive patient and 2 relatives

Each point represents the mean of triplicate data from 1 experiment. The individual results from 3 experiments per cell line are shown.

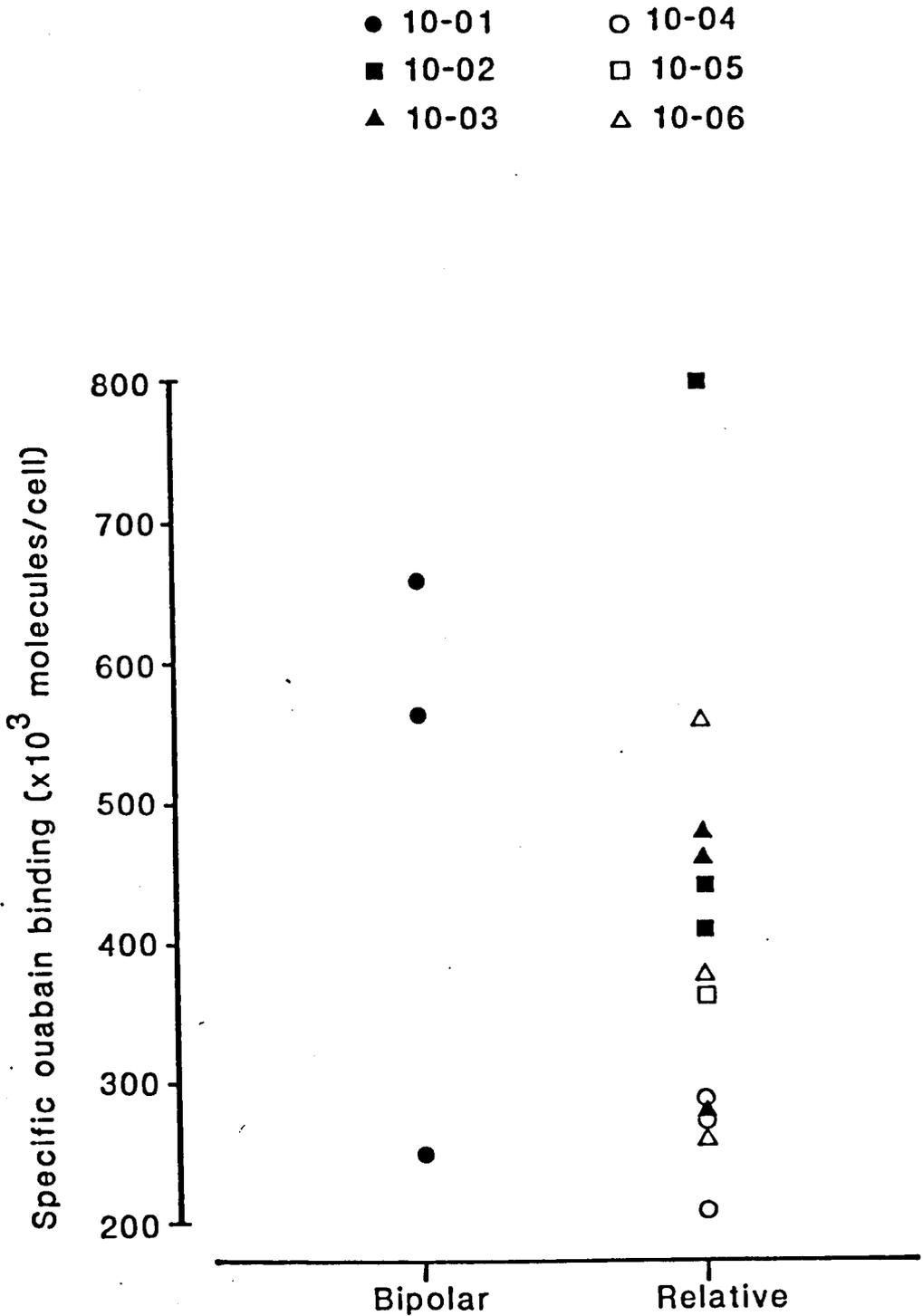


Figure 6.9. Distribution of the specific ouabain binding values for lymphoblastoid cell lines from a bipolar manic-depressive patient and 5 relatives

Each point represents the mean of triplicate data from 1 experiment. The individual results from 3 experiments (1 in the case of 10-05) per cell line are shown.

in the day to day results seen for each cell line it would appear that the values for 08-04 may be distributed relative to 08-01 in a different way to those for 08-02, although one of the three experimental results for 08-04 would not support this. If the mean values are used, the cell lines are not significantly different with respect to specific ouabain binding. For the family members of 10-01 the picture is less clear as more variability is seen in the values for the proband. The values for the family members generally appear to be lower than those for the proband although one very low value for the proband complicates the analysis. Using the mean values, there is no significant difference between 10-01 and the family members with the exception of 10-04 which is significantly lower. The possible relationship of the results to the clinical status of the individual is discussed in the following "Discussion" section.

The values for the various K^+ influx pathways are given in Tables 6.4., 6.5., 6.6., and 6.7. with the relative contributions of the individual pathways to the total flux being given in Table 6.8.. All the results are expressed only as mmol/lcw/min.. A similar picture is seen if expressed as nmol/ 10^6 cells/min., as the distributions are similar with the cells having the highest values when expressed the former way generally having the higher values if expressed the latter way. Expressing the results in this way would also not be affected by the treatments as volumes were unaltered by treatments. The distributions of the mean experimental values per cell line (usually 3 experiments) are given in Figures 6.10., 6.11., 6.12., and 6.13.. There are no

Table 6.4. Total K⁺ influx of lymphoblastoid cell lines from bipolar manic-depressive patients, relatives, and control subjects

The values below are the mean of data from 3 experiments (except for Twel, Wib and 15-01 - 2, 2, and 1 respectively), with the influx being measured in triplicate within an experiment. The upper, middle and lower groups represent the cell lines from controls, bipolars and relatives respectively.

Cell line	Total K ⁺ influx ± S.E.M. (mmol/lcw/min.)
Ale	1.48 ± 0.47
Cib	1.35 ± 0.25
Dil	2.14 ± 0.22
Fleming	0.85 ± 0.13
Fran	2.38 ± 0.16
Gavi	1.91 ± 0.29
Ged	2.01 ± 0.18
Jil	1.81 ± 0.26
Sep	1.22 ± 0.14
Tony	1.22 ± 0.13
Twel	1.29 ± 0.42
Wib	0.62 ± 0.29
01-01	2.05 ± 0.43
02-01	1.31 ± 0.24
05-01	2.09 ± 0.43
07-01	1.60 ± 0.17
08-01	1.77 ± 0.13
09-01	1.83 ± 0.33
10-01	1.27 ± 0.36
14-01	1.29 ± 0.42
15-01	1.14 ± 0.00
17-01	1.21 ± 0.35
Cox	0.88 ± 0.13
Greig	0.67 ± 0.08
08-02	1.67 ± 0.22
08-03	1.57 ± 0.11
08-04	3.07 ± 0.21
10-02	1.60 ± 0.22
10-03	1.59 ± 0.24
10-04	1.16 ± 0.45
10-06	1.42 ± 0.17

Table 6.5. Bumetanide-sensitive K⁺ influx of lymphoblastoid cell lines from bipolar manic-depressive patients, relatives, and control subjects

The values below are the mean of data from 3 experiments (except for Twel, Wib and 15-01 - 2, 2, and 1 respectively), with the influx being measured in triplicate within an experiment. The upper, middle and lower groups represent the cell lines from controls, bipolars and relatives respectively.

Cell line	Bumetanide-sensitive K ⁺ influx ± S.E.M. (mmol/lcw/min.)
Ale	0.45 ± 0.20
Cib	0.16 ± 0.11
Dil	0.15 ± 0.09
Fleming	0.04 ± 0.04
Fran	0.64 ± 0.11
Gavi	0.38 ± 0.11
Ged	0.51 ± 0.06
Jil	0.32 ± 0.12
Sep	0.35 ± 0.01
Tony	0.35 ± 0.09
Twel	0.21 ± 0.03
Wib	0.00 ± 0.00
01-01	0.32 ± 0.22
02-01	0.38 ± 0.09
05-01	0.40 ± 0.18
07-01	0.48 ± 0.10
08-01	0.45 ± 0.11
09-01	0.52 ± 0.11
10-01	0.27 ± 0.09
14-01	0.32 ± 0.31
15-01	0.24 ± 0.00
17-01	0.15 ± 0.04
Cox	0.06 ± 0.05
Greig	0.01 ± 0.01
08-02	0.36 ± 0.16
08-03	0.27 ± 0.13
08-04	0.66 ± 0.13
10-02	0.40 ± 0.12
10-03	0.49 ± 0.17
10-04	0.33 ± 0.20
10-06	0.22 ± 0.04

Table 6.6. Ouabain-sensitive K⁺ influx of lymphoblastoid cell lines from bipolar manic-depressive patients, relatives, and control subjects

The values below are the mean of data from 3 experiments (except for Twel, Wib and 15-01 - 2, 2, and 1 respectively), with the influx being measured in triplicate within an experiment. The upper, middle and lower groups represent the cell lines from controls, bipolars and relatives respectively.

Cell line	Ouabain-sensitive K ⁺ influx ± S.E.M. (mmol/lcw/min.)
Ale	0.99 ± 0.28
Cib	1.06 ± 0.22
Dil	1.51 ± 0.15
Fleming	0.71 ± 0.12
Fran	1.55 ± 0.16
Gavi	1.29 ± 0.24
Ged	1.24 ± 0.09
Jil	1.29 ± 0.22
Sep	0.66 ± 0.13
Tony	0.66 ± 0.07
Twel	0.75 ± 0.24
Wib	0.45 ± 0.21
01-01	1.46 ± 0.24
02-01	1.05 ± 0.13
05-01	1.34 ± 0.36
07-01	1.03 ± 0.04
08-01	1.14 ± 0.10
09-01	1.21 ± 0.24
10-01	0.89 ± 0.23
14-01	0.73 ± 0.27
15-01	0.78 ± 0.00
17-01	0.78 ± 0.19
Cox	0.72 ± 0.07
Greig	0.58 ± 0.07
08-02	1.00 ± 0.08
08-03	0.98 ± 0.08
08-04	2.00 ± 0.19
10-02	1.03 ± 0.11
10-03	1.04 ± 0.18
10-04	0.81 ± 0.25
10-06	1.07 ± 0.11

Table 6.7. Passive leak K⁺ influx of lymphoblastoid cell lines from bipolar manic-depressive patients, relatives, and control subjects

The values below are the mean of data from 3 experiments (except for Twel, Wib and 15-01 - 2, 2, and 1 respectively), with the influx being measured in triplicate within an experiment. The upper, middle and lower groups represent the cell lines from controls, bipolars and relatives respectively.

Cell line	Passive leak K ⁺ influx ± S.E.M. (mmol/lcw/min.)
Ale	0.11 ± 0.02
Cib	0.08 ± 0.04
Dil	0.24 ± 0.06
Fleming	0.12 ± 0.02
Fran	0.24 ± 0.04
Gavi	0.24 ± 0.04
Ged	0.24 ± 0.08
Jil	0.22 ± 0.04
Sep	0.14 ± 0.04
Tony	0.18 ± 0.03
Twel	0.19 ± 0.05
Wib	0.08 ± 0.09
01-01	0.16 ± 0.07
02-01	0.03 ± 0.01
05-01	0.29 ± 0.02
07-01	0.08 ± 0.05
08-01	0.23 ± 0.02
09-01	0.22 ± 0.01
10-01	0.12 ± 0.06
14-01	0.12 ± 0.06
15-01	0.09 ± 0.00
17-01	0.21 ± 0.12
Cox	0.06 ± 0.04
Greig	0.07 ± 0.02
08-02	0.16 ± 0.02
08-03	0.24 ± 0.01
08-04	0.39 ± 0.02
10-02	0.13 ± 0.03
10-03	0.19 ± 0.03
10-04	0.07 ± 0.07
10-06	0.11 ± 0.01

Table 6.8. K^+ influx pathways (% of total K^+ influx) of lymphoblastoid cell lines from bipolar manic-depressive patients, relatives, and control subjects

The values below are the mean of data from 3 experiments (except for Twel, Wib and 15-01 - 2, 2, and 1 respectively), with the influx being measured in triplicate within an experiment. The upper, middle and lower groups represent the cell lines from controls, bipolars and relatives respectively.

Cell line	K^+ influx pathways (% of total K^+ influx) \pm S.E.M.		
	Bumetanide-sensitive	Ouabain-sensitive	Passive leak
Ale	32.7 \pm 11.5	69.4 \pm 5.2	8.1 \pm 1.1
Cib	10.7 \pm 6.0	79.4 \pm 7.7	4.8 \pm 2.5
Dil	8.7 \pm 5.2	70.6 \pm 1.5	10.9 \pm 2.2
Fleming	3.8 \pm 3.8	83.4 \pm 2.4	14.5 \pm 3.4
Fran	26.5 \pm 3.0	64.8 \pm 3.2	9.8 \pm 1.4
Gavi	19.1 \pm 2.5	67.2 \pm 7.0	13.5 \pm 3.7
Ged	25.3 \pm 0.8	62.2 \pm 2.1	11.7 \pm 2.8
Jil	16.7 \pm 3.8	70.8 \pm 2.0	12.4 \pm 2.3
Sep	30.0 \pm 4.2	52.8 \pm 5.5	12.4 \pm 3.7
Tony	27.5 \pm 4.5	54.2 \pm 3.4	14.8 \pm 1.6
Twel	17.4 \pm 3.3	57.1 \pm 4.2	18.2 \pm 10.6
Wib	0.0 \pm 0.0	80.2 \pm 12.2	9.3 \pm 9.3
01-01	12.4 \pm 7.5	72.5 \pm 3.2	7.0 \pm 2.5
02-01	29.3 \pm 4.7	83.9 \pm 11.9	1.8 \pm 1.0
05-01	18.0 \pm 5.7	61.9 \pm 5.5	16.5 \pm 5.0
07-01	30.1 \pm 4.9	65.5 \pm 7.0	4.6 \pm 2.9
08-01	25.6 \pm 6.0	64.3 \pm 1.3	13.0 \pm 0.7
09-01	27.7 \pm 1.8	65.8 \pm 2.4	12.8 \pm 2.7
10-01	20.9 \pm 3.5	70.6 \pm 2.9	7.7 \pm 4.2
14-01	15.6 \pm 14.6	54.9 \pm 2.6	9.2 \pm 5.5
15-01	21.1 \pm 0.0	68.4 \pm 0.0	7.9 \pm 0.0
17-01	12.5 \pm 1.3	67.9 \pm 5.5	13.6 \pm 7.3
Cox	6.0 \pm 4.3	83.9 \pm 7.7	6.1 \pm 3.6
Greig	1.4 \pm 1.4	86.0 \pm 1.5	12.3 \pm 5.7
08-02	19.9 \pm 8.1	61.0 \pm 4.7	10.5 \pm 2.9
08-03	17.3 \pm 8.9	62.0 \pm 1.8	15.7 \pm 1.5
08-04	21.2 \pm 3.1	64.9 \pm 2.9	12.7 \pm 1.0
10-02	24.2 \pm 4.1	65.0 \pm 4.5	8.8 \pm 3.6
10-03	29.2 \pm 6.4	64.6 \pm 2.2	12.4 \pm 1.6
10-04	24.1 \pm 5.8	76.8 \pm 14.4	3.4 \pm 3.4
10-06	15.3 \pm 1.1	75.8 \pm 1.1	8.1 \pm 0.6

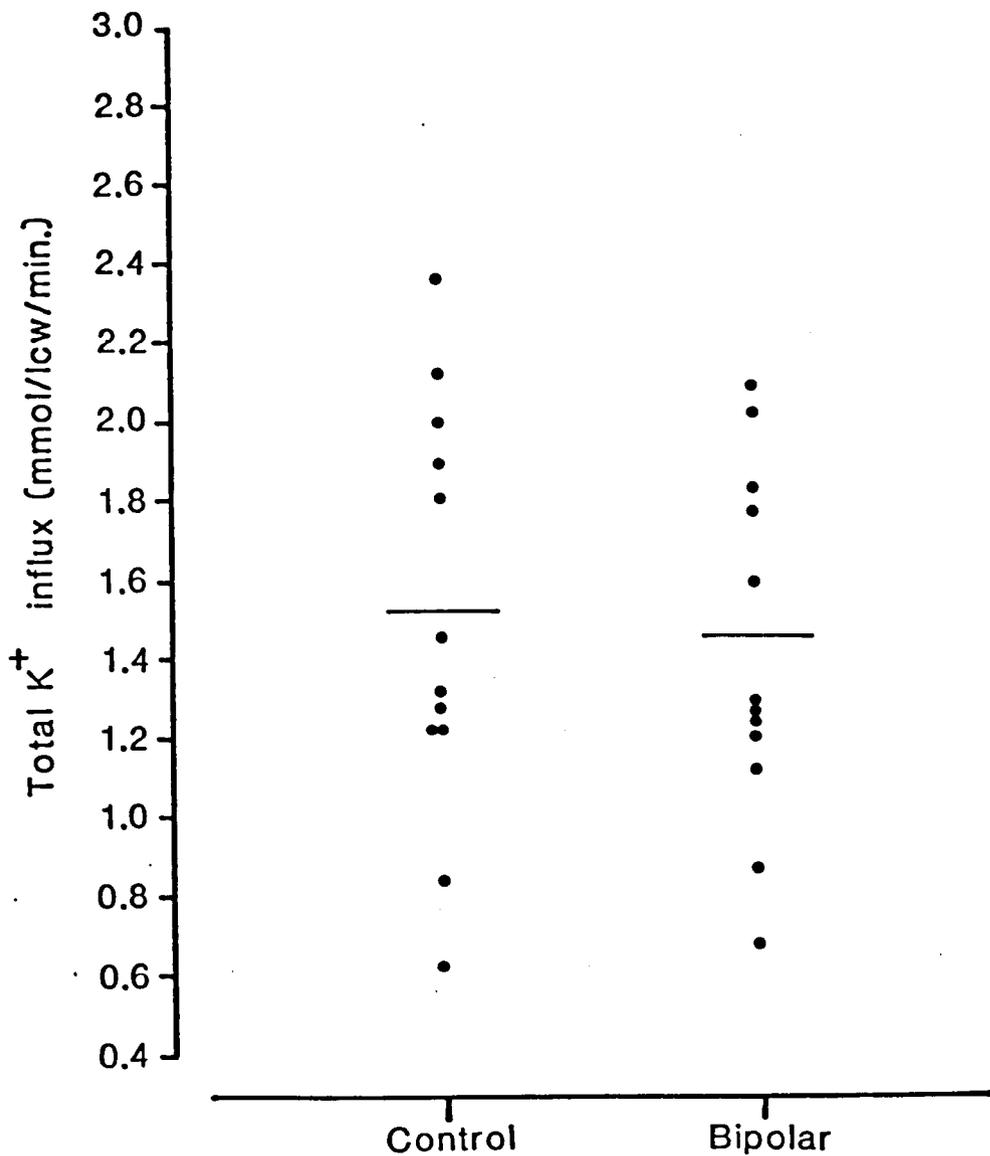


Figure 6.10. Distribution of the total K⁺ influx values (mmol/lcw/min.) for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 12 control and 12 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

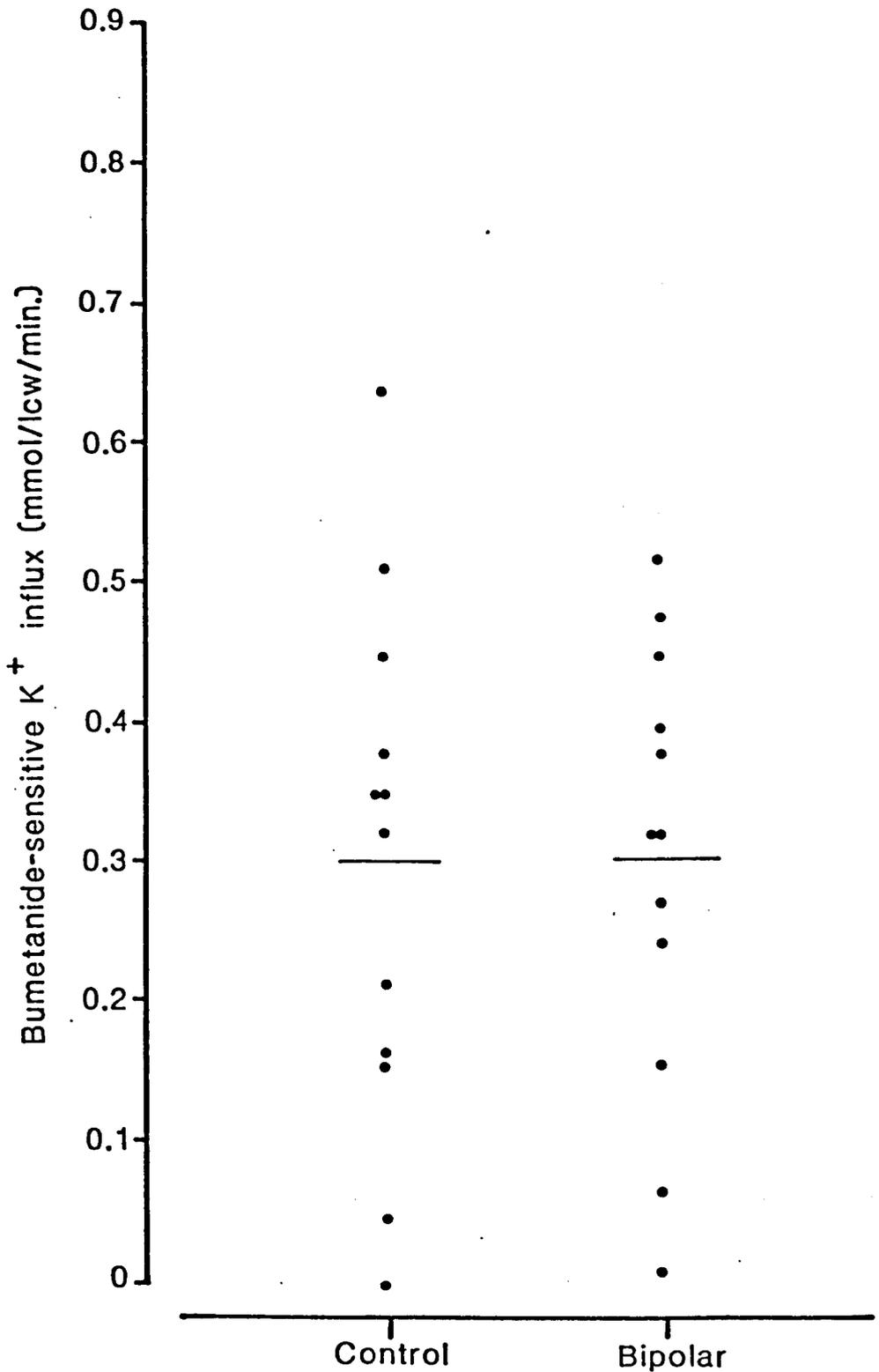


Figure 6.11. Distribution of the bumetanide-sensitive K⁺ influx values (mmol/lcw/min.) for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 12 control and 12 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each

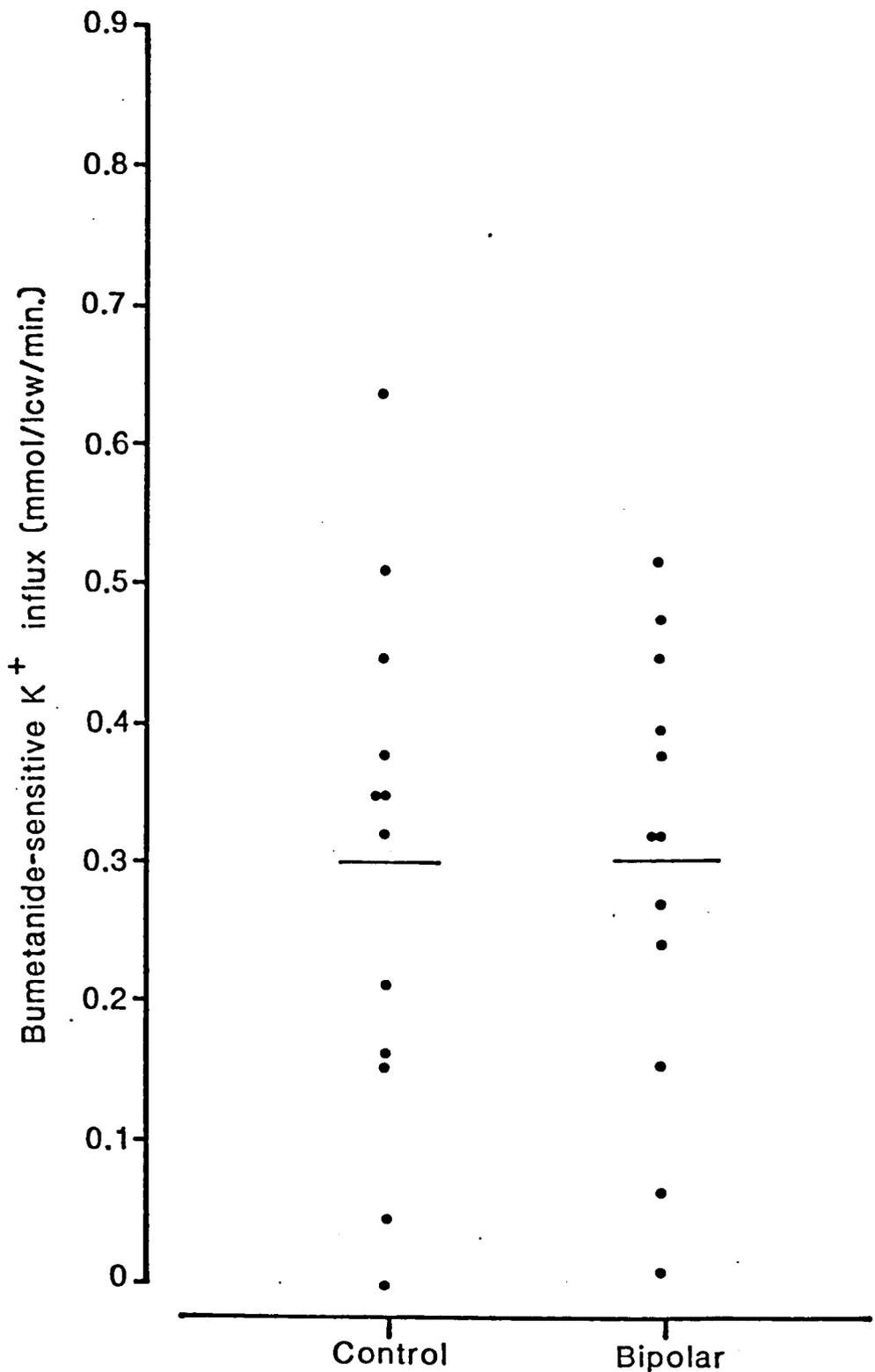


Figure 6.11. Distribution of the bumetanide-sensitive K⁺ influx values (mmol/lcw/min.) for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 12 control and 12 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

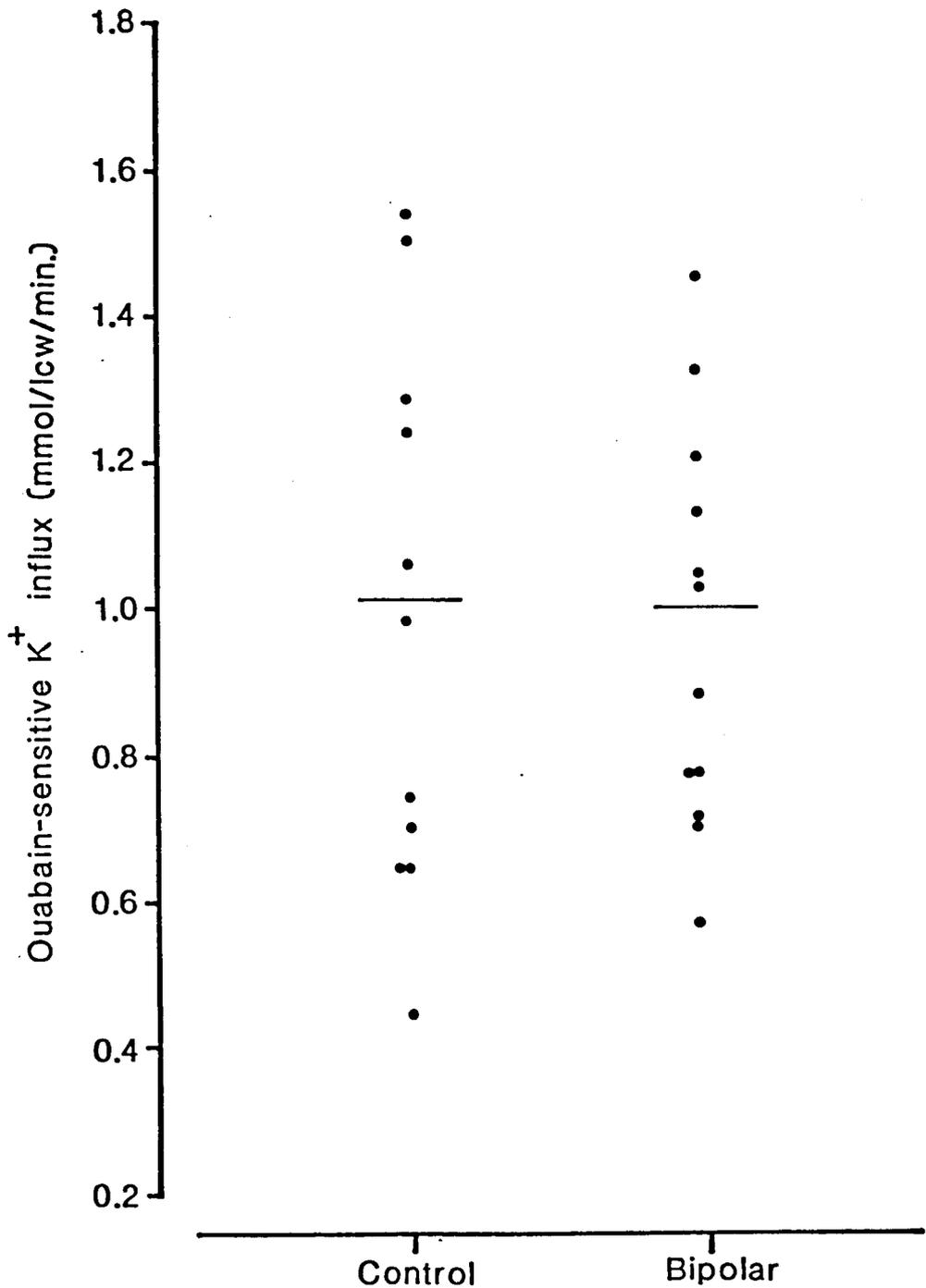


Figure 6.12. Distribution of the ouabain-sensitive K⁺ influx values (mmol/lcw/min.) for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 12 control and 12 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

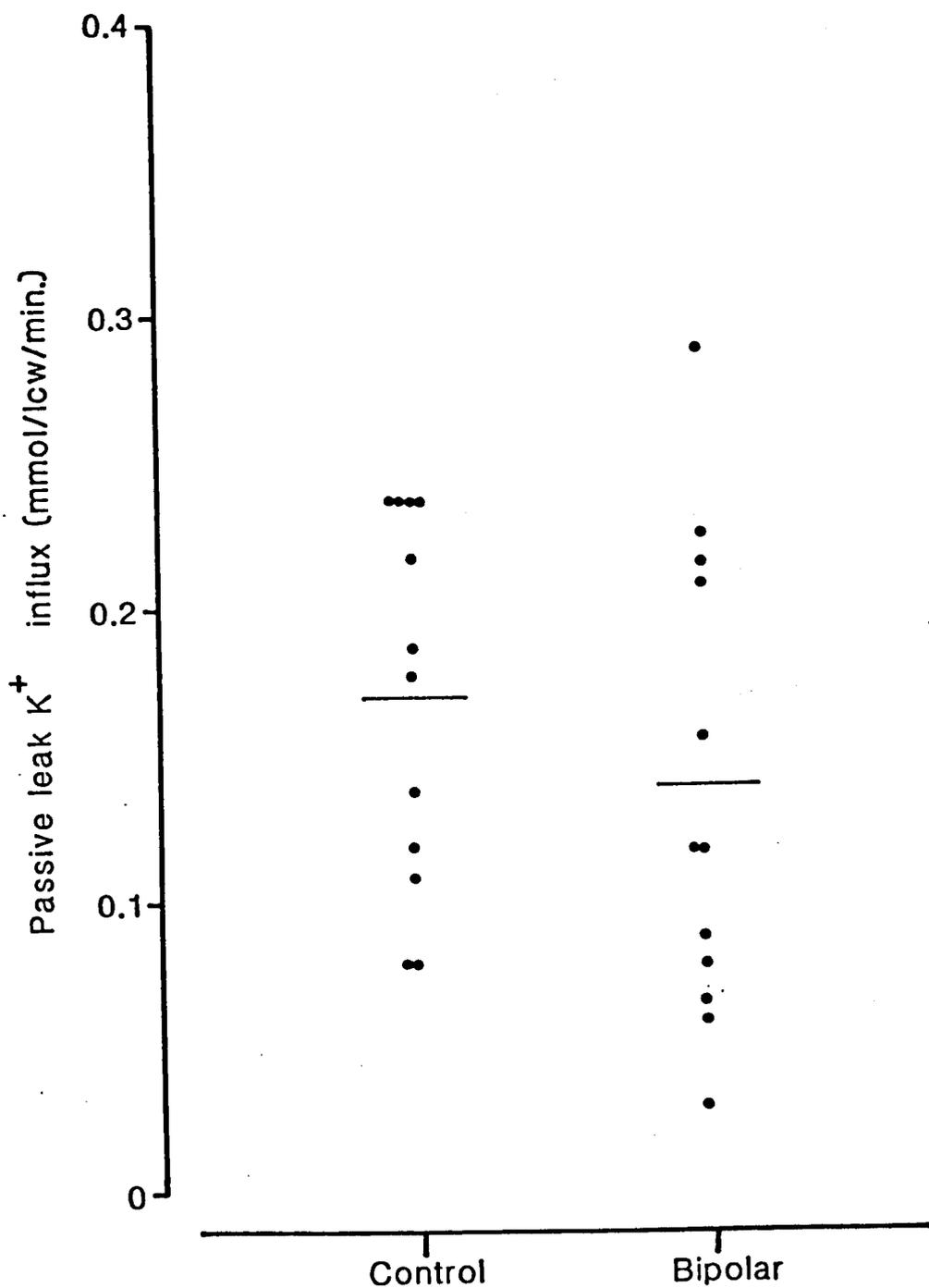


Figure 6.13. Distribution of the passive leak K⁺ influx values (mmol/lcw/min.) for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 12 control and 12 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

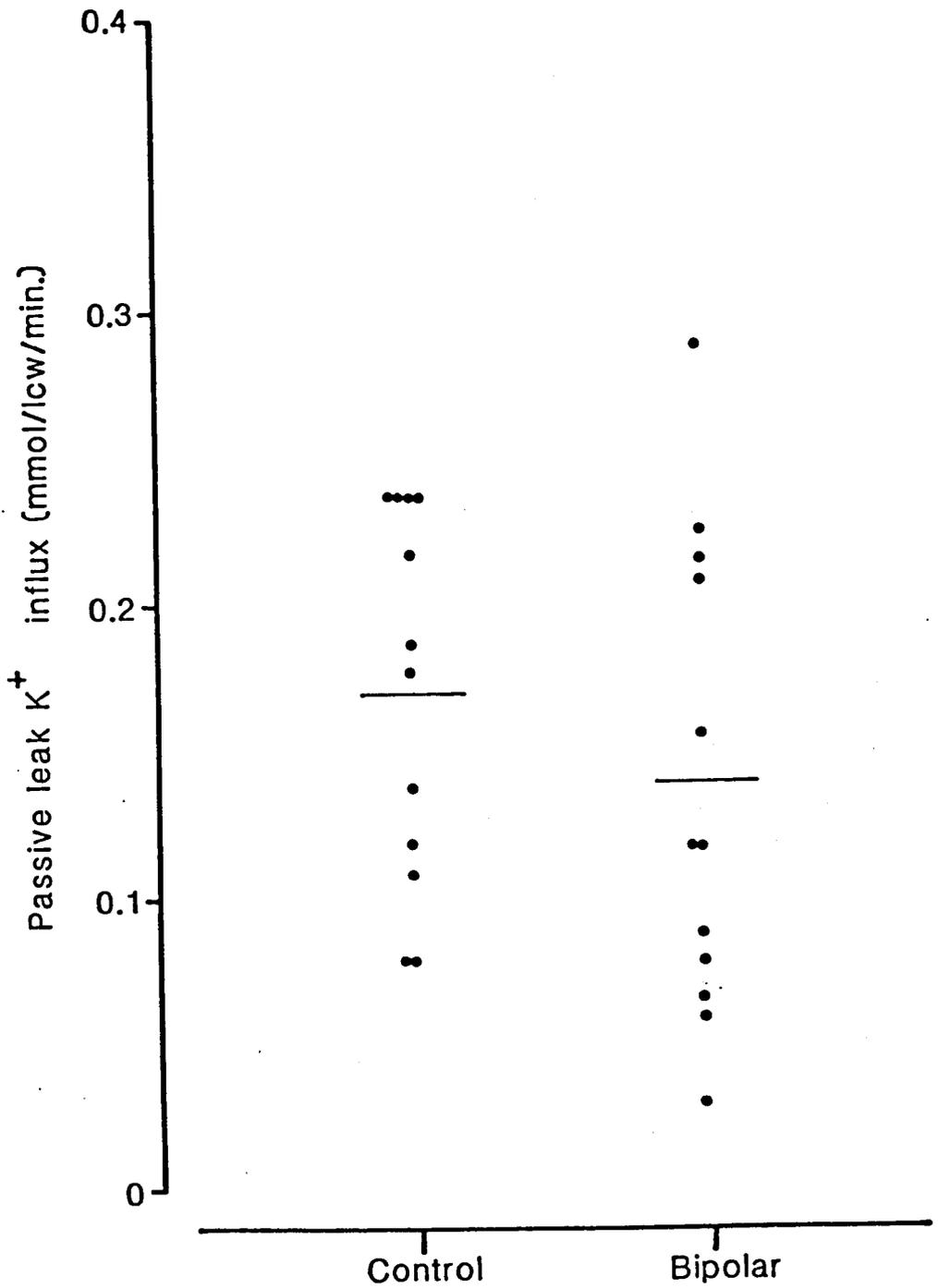


Figure 6.13. Distribution of the passive leak K^+ influx values (mmol/lcw/min.) for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 12 control and 12 bipolar cell lines. Each point represents the mean of results from 3 experiments (usually) per cell line, with each measurement being triplicated within an experiment. — indicates the mean value for each category of cell line.

significant differences between the control and bipolar groups in any of the fluxes. Considerable variation in the actual values of fluxes for the cell lines relative to one another can be seen, together with considerable variation between experiments for each individual cell line. In general the cell lines supplied by the Dundee group tend to have bigger volumes, and lower bumetanide-sensitive fluxes than the remainder of the cell lines.

It is meaningless to calculate the K^+ flux per sodium pump using the mean sodium pump numbers and flux values for each cell line, due to the measurement of the K^+ influx on different days to the ouabain binding, and the variability from day to day within each cell line. This could only be done by measuring the different parameters at the same time. The possibility of the bipolar and control populations of cells containing sub-groups of cell lines with different rates of pumping could also then be examined.

6.III.iii. Effects of ouabain, lithium or vanadate treatment (24 hours) on the ouabain binding and K^+ influx pathways of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results of the treatment studies are summarised in Tables 6.9. and 6.10.. Many cell lines show inconsistent results from experiment to experiment. Little effect of either lithium or vanadate is seen on either ouabain binding or any of the K^+ influx pathways. Similarly, ouabain treatment produces no effects on either the bumetanide-sensitive or passive leak influxes. However, large and significant effects are seen with

Table 6.9. Effects of ouabain, lithium or vanadate treatment (24 hours) on the ouabain binding of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The values below are the mean of data from 3 experiments (except for Ale, Mib, Twel, and 10-05 - 2, 1, 1, and 1 respectively), with each parameter being measured in triplicate within an experiment. Results are expressed as % of basal values. Significance testing relative to the basal values is by Student's t-test.

C Control
 B Bipolar
 R Relative

• p<0.05
 .. p<0.01
 ... p<0.005
 p<0.001

— No significant change
 □ Inconsistent

Cell line	Clinical status	Treatment and ouabain binding (% of basal) ± S.E.M.					
		10 ⁻³ M LiCl		10 ⁻⁸ M ouabain		10 ⁻⁶ M Na ₃ VO ₄	
		Specific	Non-specific	Specific	Non-specific	Specific	Non-specific
Ale	C	—	—	• 52.5 ± 10.1 75.1 ± 3.4	—	—
Cib	C	—	— 59.6 ± 2.9 57.3 ± 4.6	—	—
Fleming	C	—	— 47.8 ± 5.9	—	—	—
Fran	C	—	— 64.1 ± 0.9	• 74.8 ± 8.9	—	—
Gavi	C	—	— 54.4 ± 6.5	—	—	—
Ged	C	—	— 52.9 ± 3.9 55.8 ± 1.7	—	—
Jil	C	—	— 63.5 ± 4.2 58.1 ± 5.6	—	—
Mib	C	•• 86.4 ± 2.8	— 54.8 ± 8.4	• 85.5 ± 4.4	—	—
Sep	C	—	— 59.7 ± 3.2	—	—	—
Tony	C	—	— 48.9 ± 3.1 59.8 ± 7.2	—	—
Twel	C	—	— 39.6 ± 6.5	—	—	• 73.6 ± 10.3
01-01	B	—	—	• 59.8 ± 12.7	—	—	—
05-01	B	—	—	•• 60.2 ± 7.7	—	—	—
06-01	B	—	— 51.2 ± 1.2	—	—	—
07-01	B	—	— 53.7 ± 8.1	—	—	—
08-01	B	—	— 66.4 ± 4.3 70.0 ± 6.1	—	—
10-01	B	—	— 58.0 ± 3.0	—	—	—
14-01	B	—	— 55.3 ± 5.9 69.9 ± 3.2	—	—
15-01	B	—	— 73.6 ± 3.5 69.9 ± 2.0	—	—
17-01	B	—	— 58.0 ± 7.1	—	—	—
Cox	B	—	— 56.1 ± 8.0	—	—	—
08-02	R	—	— 46.2 ± 2.4 64.1 ± 3.7	—	—
08-04	R	—	— 54.7 ± 1.6 62.7 ± 3.2	—	—
10-02	R	—	— 53.6 ± 10.8	—	—	—
10-03	R	—	—	—	—	—	—
10-04	R	—	— 59.8 ± 3.2	—	—	—
10-05	R	—	— 33.5 ± 1.3 52.9 ± 2.5 61.3 ± 2.9	—
10-06	R	—	— 51.8 ± 5.0	—	—	—

ouabain treatment on the sodium pump number in all the bipolar and control cell lines, and the ouabain-sensitive flux in about half of the cell lines. It would appear therefore that with the exception of Ged, about half of the cell lines in both groups show a decrease in flux which is commensurate with the decrease seen in pump numbers. The remainder of the cell lines show inconsistent changes in the ouabain-sensitive K^+ influx e.g. decreasing in one experiment but not the next. The changes in pump numbers are relatively consistent from experiment to experiment unlike the flux results. Based on the 5 control and 4 bipolar cell lines which show a consistent decrease in the ouabain-sensitive flux there does not appear to be a difference between the two groups of cell lines with respect to the magnitude of the decrease (Table 6.11.). There is a small but non-significantly greater decrease seen in sodium pump numbers of control cell lines compared with bipolars (54.3% of basal c.f. 59.5% respectively). No difference is seen between the groups with respect to the magnitude of the decreases in non-specific ouabain binding with ouabain treatment although this is seen in only 3 bipolar cell lines compared with 7 controls.

There does not appear to be a linear relationship however between either the size of the basal ouabain flux or the basal pump number, and the % inhibition of flux for the cell lines of either group. If the individual data for the decrease in specific ouabain binding is examined relative to the basal ouabain binding values (Figure 6.14.), the overall correlation in the control cell lines is positive i.e. the higher the basal sodium pump number, the higher the number remaining per cell

Table 6.11. Summary of the effects of ouabain treatment ($10^{-8}M$ - 24 hours) on the ouabain-sensitive K^+ influx and ouabain binding of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results below are the mean of data from the cell lines which show consistently significant changes with ouabain treatment. The changes were measured in 3 separate experiments with each measurement being triplicated within an experiment. Significance testing of the changes in the bipolar group relative to the control is by Student's t-test.

Category	n	Ouabain-sensitive K^+ influx (% of basal) \pm S.E.M.	Level of Significance
Control	5	55.8 \pm 6.2	-
Bipolar	4	68.9 \pm 9.3	N.S.

Category	n	Specific ouabain binding (% of basal) \pm S.E.M.	Level of Significance
Control	11	54.3 \pm 2.2	-
Bipolar	10	59.5 \pm 2.1	N.S.

Category	n	Non-specific ouabain binding (% of basal) \pm S.E.M.	Level of Significance
Control	7	66.6 \pm 4.4	-
Bipolar	3	69.9 \pm 0.03	N.S.

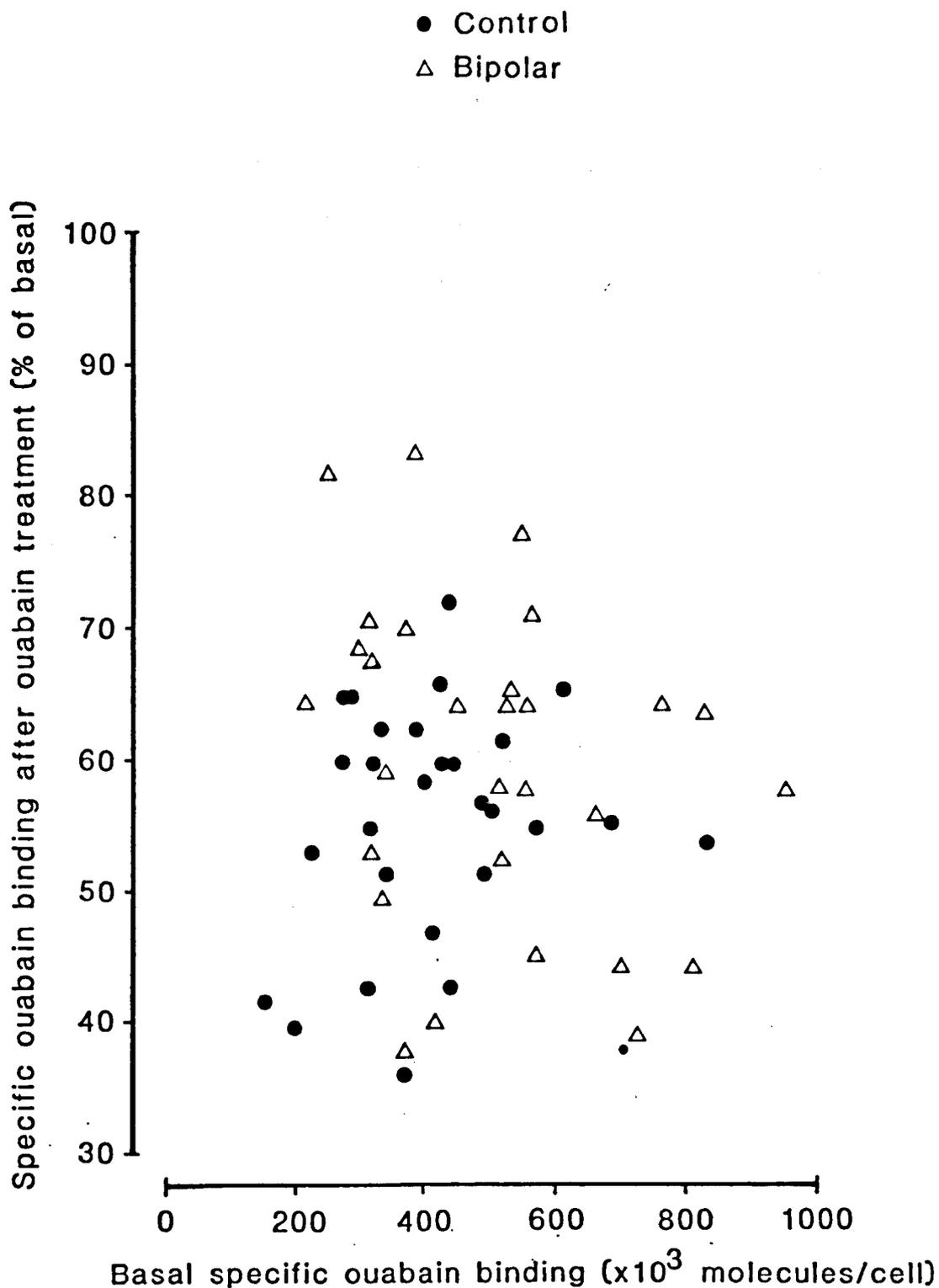


Figure 6.14. Relationship between the specific ouabain binding values before and after treatment with ouabain ($10^{-8}M$ - 24 hours), for lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results are from 10 control and 9 bipolar cell lines. Each point represents the mean of triplicate data from 1 experiment. The individual results from 3 experiments (usually) per cell line are shown.

after treatment. However for bipolar cell lines the correlation is negative. In the case of both the control and the bipolar group however the correlation coefficient is small (0.199, n = 28, and -0.320, n = 29 respectively), and in neither case is there a significant correlation between the parameters.

6.IV. DISCUSSION

This is the first study in which cultured lymphoblastoid cell lines have been used to elucidate the possible role of cation transport in the aetiology of bipolar manic-depressive psychosis. Ideally the cell lines used would all have been established in the same laboratory at the same time, and with age- and sex-matching of the control and patient groups. However this was not possible for the reasons listed in the previous chapter. Although there was no significant difference in the mean age of the control and bipolar groups, the subjects were not matched for age and there were also sex-differences between the groups. The control and bipolar groups of cell lines used for the ouabain binding studies had male:female ratios of 7:4 and 4:6 respectively, whilst the control and bipolar groups used in the flux studies had male:female ratios of 9:3 and 4:8 respectively. The effect of such differences on, for example, ouabain binding, is not clear as although Erdmann and Hasse (1975) reported no effect of age or sex on sodium pump number or affinity of ouabain binding in human erythrocytes, Naylor et al. (1980) found lower sodium pump numbers in erythrocytes from normal females over 50 years of age, than in a comparable group under 40 years old. Whether this is a menopausal effect or related to ageing is not clear. Such a correlation can not be seen in the female bipolar cell lines used in this study, but it is conceivable that the lack of age and sex matched controls in this study may influence the results and this possibility must be borne in mind in their interpretation.

The other possible major external influence on this study is the transformation of cell lines in different laboratories, with all but one of the control cell lines being provided by a different laboratory to the bipolar cell lines. Similar protocols were used as discussed in the previous chapter and so no difference would be expected. The majority of the cell lines were established within a year of each other and so the time in culture should be similar. It is notable that the cell lines from the Dundee laboratory possess greater volumes than the majority of the other cell lines and lower bumetanide-sensitive K^+ fluxes. The reason for this is not clear. As demonstrated in the previous chapter there are slight differences in the rate of growth between the various cell lines. This did not seem to depend upon the clinical status of the cell line though and was thought to have little influence over any differences seen between bipolar and control cell lines.

In agreement with the results of Dagher et al. (1984) using erythrocytes, no difference was seen between bipolar and control subjects in the diuretic-sensitive cotransport or passive leak K^+ fluxes of the lymphoblastoid cell lines used in this study. In contrast to the results of Dagher et al. (1984), Naylor et al. (1973), Rybakowski et al. (1981, 1983), and Hokin-Neaverson et al. (1974) using erythrocytes, no difference was found between bipolar and control subjects as regards ouabain-sensitive flux per cell. Rybakowski et al. (1981) and Thakar et al. (1985) did not however find any difference between the Na^+/K^+ -ATPase activity of control subjects and bipolar patients in remission. It is possible therefore that during depression and/or mania, the

apparently normal level of activity of the sodium pump is altered by either a change in susceptibility to some endogenous modifying factor, or that an alteration in the level of a modifying factor occurs. Whereas in the patient, parameters can be compared during the depressed phase, the manic phase, and remission, and can also be compared with non-psychiatric controls, the lymphoblastoid cell lines used here are presumably outwith the phasic nature of the illness if determined by some endogenous factor, and correspond to the remission state of the patient. If this is the case, a difference may not be seen in the lymphoblastoid cell lines used here unless the cation transport systems are experimentally stressed. The results of this part of the study are discussed later.

The higher mean number of sodium pumps per cell found in this study in the bipolar lymphoblastoid cell lines compared with control cell lines has not been reported before. Whether this correlates with altered ion contents of the cells could not be determined due to the difficulty in measuring ions. The finding by Sengupta et al. (1980) of higher total Na^+/K^+ -ATPase activity in erythrocytes and platelets of manic and depressed patients may have been due to a similarly increased number of sodium pumps per cell but alternatively may have been due to an increased activity per pump or to poor experimental technique. Although it is not possible to determine the exact flux per pump in this study due to the variability from day to day in the parameters measured, if the overall mean values for flux and sodium pump number in each group are used, it would appear that the activity per pump is reduced in bipolar cell lines compared with controls. The

combination of higher pump numbers with reduced activity per pump produces no overall difference in total ouabain-sensitive K^+ influx per cell between bipolar and control cell lines. If this is the case, whether the increased pump number is a homeostatic mechanism to compensate for the reduced activity or vice versa is not clear. By stressing the transport systems of the cells using, for example, ouabain, and establishing whether or not bipolar cell lines compensate by increasing their flux per pump, it may be possible to differentiate between these possibilities. This is purely speculative however, as it is impossible to say whether this is a real difference in pump activity unless the sodium pump numbers are determined on the cell lines at the same time as the ouabain-sensitive flux. The report by Naylor et al. (1980) of a lower activity per pump site in erythrocytes from female manic and depressed patients compared with a recovered group would be in agreement with such a finding although they found no difference in sodium pump numbers.

The use of cell lines from family members of the proband reduces the effects of environmental and genetic heterogeneity present within the general population, on the results. The day to day variability in the results complicates the analysis but if the higher specific ouabain binding is used as a marker for the illness, it would appear that 08-04 may be affected whereas 08-02 would be normal. The predictions in the family of 10-01 would be more difficult due to the greater variation and may appear to all be normal. Unfortunately the clinical status of the family members have not yet been obtained from the double-blind study instigated in the USA so these predictions can not be checked.

As already reviewed in Chapter 1, whether the transmission of bipolar manic depressive psychosis is polygenic or due to a single gene is not clear. In addition the possibility of genetic heterogeneity and/or reduced penetrance are complicating factors and if this is the case a "marker" would not be expected to be exclusively present in affected subjects but would occur with greater frequency in this group compared with normal individuals (Gershon et al. 1977). With the considerable overlap of ouabain binding values for bipolar and control subjects however, even if the day to day variation was not present this parameter would probably be of little use as a predictive aid but its identification as a "marker" would be of considerable help in determining the precise aetiology of the illness and may be useful in predicting relative susceptibility to the illness.

The source of the day to day variability in each cell line during this study is not clear. In addition to the variation seen in the basal values of the parameters measured, inconsistent treatment effects were seen in many cell lines, with the exception of the effect of ouabain on sodium pump number. The effects of the various treatments are discussed further later in this section. Such variation has also been reported in the beta-adrenergic receptor numbers of these cell lines (Wright - personal communication). The reason for the variation could not be determined. Experiments were performed using the same protocol each time and all medium changes were kept to the same routine with the cell lines being exposed to identical conditions in culture. The slight variations between the growth rates of

the various cell lines would not explain the day to day variations seen within the same cell line, and all medium changes were kept to the same routine relative to the time of the experiment in order to minimise possible differential effects of the phase of growth of the cell lines. There did not appear to be any relationship between the variation in flux measurements and the variable seeding density of the cell lines. One obvious candidate for the source of variation was the foetal calf serum. However variation in results was seen even within the same batch of serum and this was discounted. Retrospectively, in view of such variation, it would be advisable to carry out the ouabain binding studies on the same day as the flux measurements.

The effects of viral transformation on the parameters measured here have not been documented in the literature. Studies by Boon et al. (1984) and Pedersen and Klitgaard (1983) using non-transformed lymphocytes show similar time courses of ouabain binding to those found for the transformed lymphocytes in this study. The number of sodium pumps per cell (37000 - 43000) however is approximately one tenth of that found in transformed lymphocytes and represents a density of approximately 200 sites/ μ^2 of membrane, assuming the cells are perfect spheres. The volume of the non-transformed cells is also considerably smaller than that of the transformed cells i.e. $314 \mu^3$ compared with $1582 \mu^3$. This is probably due to the reversion of the lymphocytes to "blast-like" forms upon transformation and would largely account for the differences in specific ouabain binding per cell. However, assuming the cells are perfect spheres, the density of the pump sites in the transformed cells would still be

greater than that of the non-transformed i.e. 590 or 715 sites/ μ^2 (control or bipolar respectively) versus 200 sites/ μ^2 . The limitations of making such an approximation for cell surface area have already been discussed in Chapter 3 and apply equally to lymphocytes (see Figure 5.5 in Chapter 5). Whether this difference in sodium pump density (within the limitations of the approximation) is due solely to the viral transformation is not clear. A 50% increase in sodium pump numbers has been found for example in non-transformed lymphocytes cultured for 24 hours in RPMI containing 10% foetal calf serum (Oh et al. 1983).

It must also be appreciated that the non-transformed populations of cells used in these studies were a mixture of B and T lymphocytes (probably mainly T) compared with the transformed populations which are thought to be B in origin but may represent an earlier stage in the development with the potential to be either B or T cells (see Chapter 5). Whether any differences exist between B and T lymphocytes with regard to sodium pump number is not known. The change in volume seen with transformation seems to be more pronounced in some cell lines than others, particularly the cells supplied by Dundee. The only obvious difference between these cell lines and the remainder is their slightly longer period in culture.

The ouabain-sensitive ^{86}Rb influx reported by Oh and Taylor (1985) is considerably smaller per cell for non-transformed lymphocytes (9.18 pmol/hr/ 10^6 cells) and can not be accounted for solely by the differences discussed above. If the K^+ influx per pump site is calculated using the results of the ^{86}Rb influx and ouabain binding studies of Oh and Taylor (1985) for the non-

transformed lymphocytes (i.e. $9.18 \text{ pmol}/10^6 \text{ cells/hr}$ and 45,000 sites/cell) and the mean results for the control transformed cell lines in this study bearing in mind the variability, there appears to be a large difference in the pump turnover rate with approximately 2 K^+ ions transported per site per minute in the non-transformed lymphocytes compared with approximately 1950 in the transformed lines. The flux values reported by Oh and Taylor (1985) appear to be much lower than those reported in other studies. For example Bui and Wiley (1981) report a ouabain-sensitive K^+ influx of $3.3 \text{ nmol}/10^6 \text{ cells/hour}$ in a mixed lymphocyte population with an average cell volume of 211 u^3 . The ouabain-sensitive K^+ influx was found to represent approximately 70% of the total K^+ influx, in good agreement with the figure of 68% found for transformed lymphocytes in this study. Unfortunately sodium pump site numbers were not determined in the study by Bui and Wiley (1981), but if the mean figure of 40,000 sites per cell is used from the above studies by Pedersen and Klitgaard (1983) and Boon et al. (1984), a pump turnover rate of approximately 900 K^+ ions transported per site per minute is found. This may be an underestimate since assuming the same sodium pump density per area of membrane in the various studies, there are probably less pump sites per lymphocyte in the study by Bui and Wiley (1981) due to their smaller volume. It is possible that the units of measurement quoted by Oh and Taylor (1985) should actually be $\text{nmol}/10^6 \text{ cells/hour}$ in which case there is no difference between the flux per pump in transformed and non-transformed cells. This

uncertainty coupled with the variability of the flux results in the transformed lymphocytes does not allow firm conclusions to be drawn regarding the activity per sodium pump but it would appear that if there are differences in activity per pump between transformed and non-transformed lymphocytes, the transformed lymphocytes have a greater flux per pump per minute by a factor of 2 at the most.

The diuretic-sensitive and passive leak K^+ pathways of the lymphoblastoid cell lines used in this study are relatively small and in some cases nearly zero. As already discussed in Chapter 3, the role of the diuretic-sensitive pathway is not entirely clear and depends on the cell type e.g. it has been implicated in volume regulation in duck erythrocytes under certain conditions (Kregenow - from Saier Jr. and Boyden 1984). In non-transformed lymphocytes however, it would appear that the RVD associated with suspension in hypotonic medium is associated with a loss of KCl, probably by independent pathways and in the case of K^+ , dependent on Ca^{2+} (Grinstein et al. 1984). This occurs much more rapidly in T lymphocytes compared with B lymphocytes. RVI is only seen in lymphocytes if hypotonically pre-equilibrated and then returned to isotonic medium (Grinstein et al. 1984). This is due to an exchange of Na^+ for H^+ through an amiloride-sensitive pathway, and an exchange of Cl^- for HCO_3^- similar to that seen in Amphiuma red blood cells (Cala 1980). The diuretic-sensitive pathway does not appear to be physiologically involved in volume regulation in lymphocytes and judging by its magnitude relative to the ouabain-sensitive pathway in lymphocytes its function may be not only less apparent but also less important under control

conditions.

The lack of effect of lithium treatment on either K^+ influx or ouabain binding may be due to the relatively short incubation (24 hours) compared with the time taken before therapeutic effects are seen in patients. The delay between treatment onset and therapeutic effects (2 - 3 weeks) is thought to be the time taken for the blood lithium to reach therapeutic levels. If the therapeutic effect of lithium is due to an induction of increased Na^+/K^+ -ATPase activity such as that reported in erythrocyte membranes from lithium-treated bipolar patients (Naylor et al. 1974, Hesketh 1976), this may be due to either an increased activity per pump or an increased number of pumps. Lithium has been reported to produce an increased number of sodium pumps in HeLa cells albeit at higher than therapeutic levels (Boardman et al. 1975) within 24 hours. It is possible that at therapeutic levels, longer than 24 hours is necessary before such changes are seen. Future studies with the lymphocyte cell lines involving longer treatment periods may reveal changes in sodium pump numbers with lithium treatment and differences in response between the control and bipolar groups.

Vanadate also produced no consistent effect on the lymphoblastoid cell lines, despite using higher levels than normally found in the blood. Based on the results of the vanadate experiments using HeLa cells, no effect on fluxes would have been expected, probably due to the intracellular reduction of vanadate to vanadyl. Changes could have been noted if the lymphoblastoid cell lines were less capable of reduction, or some defect was present for example in the bipolar group conferring

upon them a susceptibility to vanadium as suggested by Dick et al. (1980). Based on the results in this study this does not appear to be the case.

The effects of ouabain on sodium pump numbers were very consistent and revealed no difference between the two groups of cell lines. Only approximately half the cell lines which showed this decrease in pump numbers showed a concomitant decrease in the ouabain-sensitive flux. The cell lines showing the decrease in flux were not confined to either of the two groups however and the magnitude of the mean decrease was not significantly different in either group. It is possible that if the ouabain treatment had been continued over a longer time period, a compensatory up-regulation of sodium pump numbers may have occurred which may vary depending on whether control or bipolar.

There are a number of possible ways in which cation transport may be involved in the aetiology of bipolar manic-depressive psychosis:- i). differences may be present in basal values and may account for the illness, ii). no difference may be present in basal values but in bipolars and controls, susceptibility to modifying factors may be different, iii). no difference in either basal values or susceptibility may be present but levels of endogenous modifying factors may be different in the two groups. Alternatively any changes in ion transport in the illness may be secondary to another change e.g. in the endocrine system, which only occurs in the affected group. The reason for the phasic nature of the illness is not clear. Based on the results in this study, there seems to be a difference in basal sodium pump site number but no difference in total ouabain-

sensitive flux, hence it is unlikely that this single difference is totally responsible for the disorder. Unless any possible modifying factor has a specific action which is not seen with any of the treatments used here, the second possibility is unlikely. The third possibility has not been investigated in this study with the exception of the possibility of vanadium as the endogenous modifying factor. The involvement of vanadium is not supported by this study but other modifying factors can not be ruled out and changes in ion transport occurring secondarily to some other change but being responsible for some of the symptoms can also not be ruled out. In addition if the ouabain binding and flux measurements were carried out on the same day, a clearer picture may have emerged.

In summary therefore, no difference has been detected between lymphoblastoid cell lines from control subjects and bipolar patients as regards the K^+ influx pathways although the investigation was complicated by the considerable variation which was seen from day to day in each cell line. The source of this variation is not known. The bipolar group however have a higher mean specific ouabain binding value which is statistically significant. This may be due in part to the lack of age and sex-matched controls and needs to be investigated further. Transformation of the cells may also have influenced the sodium pump number and differences can be found between transformed and non-transformed cells, most notably in the cell volume. The flux per pump site could not be determined due to the fact that the flux experiments and ouabain binding measurements were carried out on different days and the day to day variability made

the use of mean values questionable. Due to experimental difficulties, the measurement of intracellular ions was not possible. No consistent effect of vanadate or lithium (24 hours) was seen on ouabain binding or K^+ influx, in either group. The decrease in sodium pump numbers with ouabain treatment was similar in both groups, both with respect to the number of cell lines affected and the magnitude of the change. Overall no difference was seen between the bipolar and control groups, in the susceptibility of the cation transport systems to either lithium, ouabain or vanadate, although significant differences in basal pump numbers were found. What we can not conclude directly from this study is whether or not transformation has altered any possible existing differences between bipolar and control subjects with regard to cation transport, and whether the results can be extrapolated to the brain.

CHAPTER 7. PHOSPHATIDYLINOSITOL TURNOVER IN LYMPHOBLASTOID CELL
LINES FROM BIPOLAR MANIC-DEPRESSIVE PATIENTS AND CONTROL SUBJECTS

7.I. INTRODUCTION

7.I.1. Receptor signalling mechanisms

Cells employ various intracellular transduction or "2nd messenger" systems which translate receptor-mediated information such as the presence of hormones, growth factors, neurotransmitters etc., into cellular responses. Probably the best characterised of these systems is that involving cyclic AMP in which binding of, for example, beta-adrenergic agonists to receptors, activates the enzyme adenylate cyclase and results in increased intracellular levels of the 2nd messenger, cyclic AMP. This then mediates the cellular response.

Less well characterised, at least until fairly recently, is the phosphatidylinositol (PtdIns or monophosphoinositide) system whereby stimulation of, for example, α_1 -adrenergic receptors, provokes hydrolysis of membrane-bound inositol phospholipids and a consequent increase in the level of the 2nd messenger, inositol-1,4,5-trisphosphate (IP_3). This results in a mobilisation of Ca^{2+} and produces the appropriate cellular response. A brief overview of this very complex system is presented in the following sections, together with its possible involvement in the aetiology of manic-depressive psychosis. Further details can be found in the cited reviews and references.

7.I.ii. Receptor-stimulated inositol phospholipid metabolism

PtdIns consists of phosphatidate (diacylglycerol-3-phosphate; i.e. a glycerol backbone with 2 fatty acid chains, usually predominantly arachidonic and stearic acid in animal cells, and

esterified to phosphoric acid) attached to the 1-hydroxyl of myo-inositol, a cyclic 6 carbon alcohol. PtdIns is formed together with CMP, by the enzymatically-catalysed combination of CDP-diacylglycerol with myo-inositol (Hawthorne 1982, Hokin 1985). Of the possible stereoisomers, only the optically inactive myo-inositol has been found in naturally-occurring PtdIns and its phosphorylated derivatives, phosphatidylinositol-4-phosphate (PtdIns(4)P or diphosphoinositide) and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂ or triphosphoinositide; Hawthorne 1982). The structures and metabolism of these compounds are shown in Figure 7.1.. The kinases responsible for the sequential phosphorylation of the inositol ring both require Mg²⁺, and the presence of the phosphate groups confers on the polyphosphoinositides the property of being the most polar phospholipids (Downes 1983). Phosphomonoesterases reverse this phosphorylation and there is a constant futile cycling between the 3 pools with the turnover of the whole pools occurring within minutes in many cell types (Downes 1983, Berridge and Irvine 1984). Whether these enzymes are membrane-bound or soluble appears to depend on the tissue (Michell 1975, Hokin 1985).

The inositol phospholipids usually account for less than 10% of the total phospholipid in animal cells with the actual amount varying depending on the tissue, and with PtdIns representing over 90% of this (Michell 1975, Hawthorne 1982, Hokin 1985, Williamson 1986). Most of the di- and triphosphoinositides are thought to be located within the plasma membrane, probably near the inner face, together with a small amount of the PtdIns; the majority of the PtdIns is in the endoplasmic reticulum (E.R.)

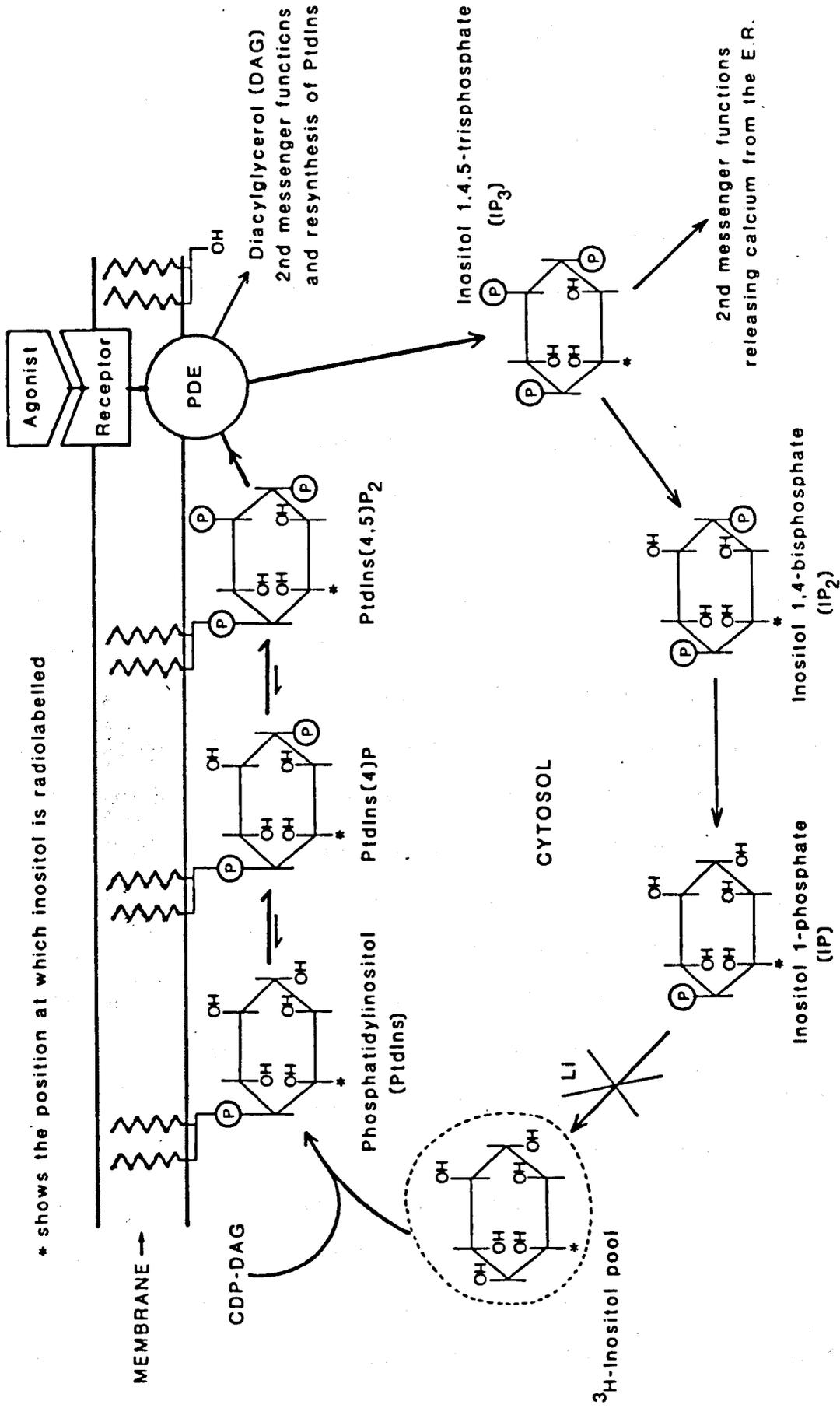


Figure 7.1. Cellular metabolism of phosphoinositides and inositol phosphates

where it is thought to be synthesised (Hawthorne 1982, Hokin 1985). Whether a closed cycle of PtdIns transport exists between the membrane and E.R. is not known (Hokin 1985).

The stimulation of phosphoinositide metabolism in response to external stimuli was first identified by Hokin and Hokin in the early 1950's using avian pancreas (reviewed by Hokin 1985) when they demonstrated an increased incorporation of ^{32}P into PtdIns, following stimulation with acetylcholine. Subsequently, stimulation of the hydrolysis of PtdIns(4,5) P_2 has been demonstrated in many tissues e.g. brain, parotid gland, liver, platelets, adrenal cortex, and T-lymphoblastoid cells, involving many receptor types e.g. muscarinic-cholinergic, serotonergic, alpha-adrenergic, histaminergic, phytohaemagglutinin (PHA), and platelet derived growth factor (PDGF), to name but a few (reviewed by Berridge and Irvine 1984, Hokin 1985). In 1975 Michell proposed that the stimulation of inositide metabolism was a feature of those agonists which produced raised intracellular $[\text{Ca}^{2+}]$ levels. This is true in the majority of cases, but mobilisation of Ca^{2+} has been shown to occur without changes in phosphoinositide metabolism (reviewed by Berridge and Irvine 1984). Michell's hypothesis accounted for the raised Ca^{2+} by proposing a "gating" of Ca^{2+} through channels in the membrane as a result of PtdIns hydrolysis (Michell 1975, Michell et al. 1981). Subsequently however it has been shown that the Ca^{2+} release appears to occur from intracellular stores and that hydrolysis of PtdIns(4,5) P_2 is the initial event, as discussed below.

The proposed metabolism of the inositol phospholipids is shown in Figure 7.1.. The agonist binding to the external receptor

stimulates the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by a phosphodiesterase (PDE:- a phospholipase C) forming diacylglycerol (DAG) and the water-soluble IP_3 (Berridge and Irvine 1984, Williamson 1986). Controversy exists as to whether this PDE is cytosolic or membrane-bound (Hokin 1982, Hokin 1985, Plantavid et al. 1986), and the mechanism of coupling of receptors to the PDE with the resulting hydrolysis of the phosphoinositides is unclear. It is possible that the agonist binding to the receptor induces a conformational change in the receptor and perturbs the membrane so that the PDE, whether membrane-bound or cytosolic, has access to the substrate (Berridge and Irvine 1984, Plantavid et al. 1986). However it has also been proposed that the PDE activity may be modulated by a GTP-binding protein in a similar fashion to the coupling of adenylate cyclase activity with receptor occupation (reviewed by Berridge and Irvine 1984, Williamson 1986).

The IP_3 produced is then thought to bind to a specific receptor on the E.R. and cause the release of Ca^{2+} (Berridge and Irvine 1984). The Ca^{2+} released is thought to mediate the cell response through changes in the activity of various proteins including protein kinases, either directly or after binding to calmodulin or other Ca^{2+} -binding proteins (Downes 1983, Williamson 1986). Spat et al. (1986) have demonstrated the presence of a specific intracellular receptor for IP_3 in permeabilised guinea-pig hepatocytes and rabbit neutrophils. Binding to the receptor is saturable, reversible and restricted to those inositol phosphates such as inositol 1,4,5-trisphosphate which had previously been shown to invoke Ca^{2+} mobilisation.

The removal of IP_3 is accomplished by an inositol trisphosphatase which results in the formation of inositol 1,4-bisphosphate (IP_2). This is also hydrolysed forming inositol-1-phosphate (IP), or in some cases inositol-4-phosphate, and subsequently free inositol. The inositol then combines with the CDP-DAG formed from the DAG produced by the initial receptor-stimulated PDE activity, to form PtdIns. Thus the intracellular levels of IP_3 and DAG are determined by a balance between their rate of formation and rate of removal (Berridge and Irvine 1984).

In many cases, a dissociation between the physiological response and the phosphoinositide effects is seen at increasing agonist concentrations (Hokin 1985), e.g. 5-HT stimulation of salivary gland in Calliphora (Berridge et al. 1982). This is thought to be due to the existence of spare receptors for the agonist-induced response whereas the phospholipid effect corresponds much more closely to receptor occupancy.

Initially, it was unclear whether the increased inositol phospholipid metabolism following agonist stimulation occurred as a consequence of the increased intracellular $[Ca^{2+}]$ or vice versa. However, following the discovery by Michell et al. (1981) that in rat hepatocytes stimulated by antidiuretic hormone (ADH or vasopressin), the decrease in PtdIns(4,5) P_2 preceded the change in PtdIns and was largely independent of extracellular Ca^{2+} , it appeared that the triphosphoinositide hydrolysis was the initial event and preceded any changes in Ca^{2+} . This has since been confirmed in many other tissues (reviewed by Berridge and Irvine 1984), although partial dependence on Ca^{2+} has also been reported in some tissues (Berridge and Irvine 1984, Michell 1975,

Michell et al. 1981, Michell 1982), together with an induction of phosphoinositide hydrolysis by the ionophore A23187. However it has recently been suggested that this latter effect in smooth muscle is not due to increased Ca^{2+} but is due to increased acetylcholine release (Hokin 1985). Such partial dependence on Ca^{2+} is not irreconcilable with the model as it does not constitute proof that a change in cytosol Ca^{2+} causes the change in phosphoinositide metabolism (Michell et al. 1981, Michell 1982). For example, the phosphoinositide phosphodiesterases are Ca^{2+} -dependent but do not require elevated Ca^{2+} levels and are therefore not normally ratelimiting (Hokin 1985).

IP_3 formation in Calliphora salivary glands has also been shown to occur at least 1 second before the onset of the calcium-dependent cellular response and led to the initial suggestion that IP_3 was the 2nd messenger (Berridge 1983, Berridge and Irvine 1984). In addition IP_3 has been demonstrated to form before IP (the expected product of PtdIns hydrolysis) in for example, Calliphora salivary glands (Berridge 1983), rat parotid glands (Downes and Wusteman 1983), and human platelets (Siess and Binder 1985), providing more evidence that PtdIns(4,5) P_2 is the primary substrate for the receptor. Other evidence which indicates that Ca^{2+} mobilisation is a consequence of phosphoinositide metabolism includes the fact that in human platelet membranes, the phospholipase C responsible for the hydrolysis of PtdIns(4,5) P_2 to IP_3 does not require increased Ca^{2+} levels for activation (Plantavid et al. 1986). Similarly in human neutrophils, IP_3 production has been shown to occur at basal intracellular [Ca^{2+}] (Di Virgilio et al. 1985). Results

using carbachol-stimulated PC12 cells support phosphoinositide hydrolysis and Ca^{2+} mobilisation as being successive events with Ca^{2+} mobilisation occurring last, as the Ca^{2+} signal can be abolished without any effect on the inositol phosphate generation (Vicentini et al. 1986). Lew et al. (1986) have shown that in the human promyelocytic cell line HL-60, the receptor-triggered PtdIns(4,5)bisphosphate phosphodiesterase activation does not require elevated Ca^{2+} levels, and elevated Ca^{2+} levels above micromolar decrease the agonist induced rise in inositol 1,4,5-trisphosphate, thus acting as a negative feedback mechanism.

The release of intracellularly sequestered Ca^{2+} is thought to occur from the E.R. rather than the mitochondria and experiments have indicated that it is due to a stimulation of Ca^{2+} efflux from the stores rather than an inhibition of uptake. Using permeabilised cell models, IP_3 has been shown to be a very potent stimulator of Ca^{2+} release, producing half-maximal effects at concentrations less than 1 μM in many cases. IP_2 and IP are however, ineffective (reviewed by Berridge and Irvine 1984). The mobilisation of Ca^{2+} is thought to be an amplification step and it has been calculated that in permeabilised liver cells, each IP_3 molecule releases at least 20 Ca^{2+} ions (Berridge and Irvine 1984). In order to maintain these raised intracellular Ca^{2+} levels long enough for the cellular response to be produced, some inhibition of the Ca^{2+} -ATPase on the cell membrane is likely as intracellular stores would not be sufficient to maintain these levels for very long and therefore extracellular Ca^{2+} is needed to maintain the response (Hokin 1985, Williamson 1986). It has been hypothesised that since PtdIns(4,5) P_2 regulates membrane

fluidity in erythrocytes (Sheetz et al. 1982), as it is consumed during hydrolysis, this may perturb the membrane in such a way as to alter Ca^{2+} removal across the membrane (Berridge and Irvine 1984). IP_3 has also been shown to cause Ca^{2+} release from the sarcoplasmic reticulum of rabbit skeletal muscle (Volpe et al. 1985) but the physiological implications of this are not yet clear.

The situation has been further complicated by the discovery of two naturally-occurring isomers of IP_3 in carbachol-stimulated rat parotid glands (Irvine et al. 1984), D-myo-inositol 1,4,5-trisphosphate and D- or L-myo-inositol 1,3,4-trisphosphate. The kinetics of the production of these two isomers has been investigated in carbachol-stimulated (15 minutes), ^3H -inositol-loaded rat parotid gland, using HPLC to separate the isomers (Irvine et al. 1985). The most marked increase in levels of the 1,4,5, isomer of IP_3 occurs in the first 5 seconds with levels then declining to control values and gradually increasing over the following 15 minutes. The increased levels after 15 minutes were abolished if an antagonist was introduced. In contrast, the increase in the level of the 1,3,4 isomer was delayed, being greater than that of the 1,4,5 isomer by 15 seconds and continuing to rise at a faster rate over the following 15 minutes. If an antagonist was introduced, the levels did not reach control values for at least 15 minutes. The specific activities of these pools relative to each other were not known however so caution must be exerted and exact magnitudes of increases not known, but it appears that the 1,4,5 isomer appears to behave more like a 2nd messenger. It is obvious though that

the differing kinetics of the production of the two isomers will complicate the analysis of IP₃ production unless both are examined as separated entities. For example, Downes and Wusteman (1983) calculated that on the basis of the differing rates of hydrolysis of IP₃ and IP₂, not all the IP₂ could be produced by IP₃ and proposed an additional phosphodiesteratic cleavage of PtdIns(4)P forming IP₂ to account for this. However if the differing kinetics of the two IP₃ isomers are taken into account, this may not be the case. Most studies have failed to differentiate between the two and the term IP₃ will be used to include both isomers unless otherwise indicated.

Batty et al. (1985) have since found inositol 1,3,4,5-tetrakisphosphate (IP₄) in rat cerebral cortex after muscarinic stimulation and this has been confirmed in GH₄ cells by Heslop et al. (1985), together with the presence of inositol pentakisphosphate (IP₅) and inositol hexakisphosphate (IP₆). It was postulated that these compounds were formed from the successive phosphorylation of IP₃ although formation as a result of phosphodiesteratic attack on possible corresponding polyphosphoinositides can not be ruled out. The possibility of these compounds also having a role as 2nd messengers can not be confirmed without further investigation. A specific inositol 1,4,5-trisphosphate kinase which produces inositol 1,3,4,5-tetrakisphosphate (IP₄) has been found in rat liver, brain and pancreas (Irvine et al. 1986). This kinase is ATP and Mg²⁺-dependent and is thought to play a possible role in aiding the curtailment of IP₃ activity by forming first IP₄, then the 1,3,4 isomer of IP₃ and then IP₂ although this last step has not yet

been established. In support of this hypothesis, it has been shown using the human promyelocytic cell line HL-60, that inositol 1,3,4-trisphosphate generation appears to be favoured both by increased Ca^{2+} levels, and by increased inositol 1,4,5-trisphosphate levels (Lew et al. 1986).

DAG is also thought to function as a 2nd messenger as outlined below. In many tissues an increase in cyclic GMP is seen with the phosphoinositide response and this is thought to occur as a consequence of the arachidonate released upon DAG breakdown (Hokin 1985). The possibility of synergism between DAG, IP_3 and Ca^{2+} has been reviewed by Berridge and Irvine (1984), Nishizuka (1984) and Hokin (1985).

Both IP_3 and DAG, which is transiently produced as a result of the inositol phospholipid metabolism before being presumably recycled to form PtdIns, have been implicated in normal cellular proliferation and proliferation occurring as a result of oncogenic stimulation. For example, PDGF has been shown to increase the formation of DAG and IP_3 in Swiss 3T3 cells (Hasegawa-Sasaki 1985). Both intracellular pH and $[\text{Ca}^{2+}]$ are thought to be important in the regulation of cell growth. DAG stimulates the Ca^{2+} -activated membrane-bound enzyme, protein kinase C by increasing its affinity for Ca^{2+} thereby activating it without any change in intracellular Ca^{2+} levels (reviewed by Nishizuka 1984, Hokin 1985). This enzyme has multifunctional catalytic activity and one of its actions, either directly or indirectly, is to produce an activation of the amiloride-sensitive Na^+/H^+ exchanger, increasing the intracellular pH. IP_3 of course increases the intracellular $[\text{Ca}^{2+}]$. Thus

stimulation of the receptor by PDGF activates two signal pathways which are thought to be involved in cellular proliferation (Berridge and Irvine 1984). In addition, PDGF has been shown to activate a PDGF-dependent tyrosine-specific protein kinase but whether these actions are connected or act synergistically in inducing cellular proliferation has not been determined (Hasegawa-Sasaki 1985). Oncogenes may also produce similar effects as it is known that the *sis*-oncogene codes for a protein almost identical to PDGF, and the *erb-B* gene codes for a receptor similar to that for EGF (Berridge and Irvine 1984, Hokin 1985).

In addition, the tumour promoting phorbol esters act on the protein kinase C in a similar fashion to DAG and it has been proposed that protein kinase C may be the receptor for tumour promoters (Nishizuka 1984). The phorbol esters have also been shown to phosphorylate proteins at tyrosine residues in fibroblasts (Gilmore and Martin 1983). Protein kinase C is a serine/threonine specific kinase and therefore it appears that either other kinases are acted upon by DAG and TPA in addition to kinase C, or that the activation of kinase C may result in the activation, either directly or indirectly, of tyrosine-specific kinases. The function of the phosphorylated protein is not known. The reason why such effects on cell growth are not seen with all the agents which stimulate hydrolysis of phosphoinositides is not clear unless for example, the magnitude of the effect is different due to a control defect, and/or additional effects of these agents are also important e.g. the tyrosine-specific kinase activity may be needed in addition to the stimulation of inositol phospholipid metabolism. Nishizuka

(1984) has speculated that in carcinogenic cells, the protein kinase C may be a mutant uncontrolled form.

7.I.iii. Possible role of phosphoinositides and inositol phosphates in the aetiology of bipolar manic-depressive psychosis

Many of the initial studies into inositol phospholipid metabolism have measured incorporation of either ^{32}P or ^3H -inositol into the various pools before and after agonist stimulation. Initially, because of the difficulties of knowing whether all the pools were in isotopic equilibrium and hence whether changes in activity could be regarded as changes in pool size, together with the simultaneous occurrence of both synthesis and breakdown of the various pools, the results were difficult to interpret. Following the discovery that lithium decreased the concentration of myo-inositol in the cerebral cortex of rats (Allison and Stewart 1971, Allison et al. 1976, Allison and Blisner 1976), further studies revealed that this was most likely due to an inhibition of the enzyme myo-inositol 1-phosphatase (Hallcher and Sherman 1980, Sherman et al. 1981). Lithium has since been used as a selective inhibitor of this enzyme in many studies of phosphoinositide metabolism as it allows the effects of agonists to be visualised much more easily due to the accumulation of IP and depletion of the inositol pool.

This action of lithium has obvious implications for the aetiology of manic-depressive psychosis. As discussed in Chapter 1, the actions of lithium which are responsible for its therapeutic effects in this illness are not entirely clear and it is possible that lithium may modify neurotransmitter-mediated

responses in the brain via the PtdIns system. In several tissues it has been shown that half-maximal effects of lithium on IP accumulation and inositol depletion occur at approximately 1mM lithium (Berridge et al. 1982, Hokin 1985), i.e. the therapeutic concentration in man. In quiescent cells, little effect of lithium is seen, and this has been ascribed to the low basal turnover of the inositol phosphates. With increasing receptor occupancy by 5-HT in the blowfly salivary gland (Berridge et al. 1982), increasing effects of lithium were seen, presumably due to the increased inositol phospholipid metabolism with increasing receptor occupancy as shown in ADH-stimulated rat hepatocytes for example (Michell et al. 1981). This may imply that lithium preferentially affects those receptor pathways that are abnormally active and may account for its effectiveness in both mania and depression. The time lag before the onset of therapeutic actions of lithium in man may be due largely to the time taken for serum concentrations of lithium to reach therapeutic levels but may also be due in part to the time taken to deplete the intracellular inositol stores. Thus if depression was due to e.g. a supersensitivity of receptors which involve phosphoinositide metabolism, e.g. 5-HT or alpha-adrenergic, as discussed in Chapter 1, continual agonist stimulation in the presence of lithium may produce a depletion of inositol and hence phosphoinositides, and eventually reduce the receptor-mediated response (Berridge et al. 1982).

Many peripheral receptors where agonist stimulation promotes phosphoinositide hydrolysis e.g. muscarinic in pancreas and smooth muscle, are not susceptible to the actions of lithium

(Berridge et al. 1982). This may be due to the fact that they can readily replenish their inositol pool from extracellular sources and it has been shown for example that the serum [inositol] of rats increases with lithium treatment (Allison and Stewart 1971, Allison and Blisner 1976). This may be a protective effect for peripheral tissues. Inositol can not however cross the blood-brain barrier (Margolis et al. 1971, from Berridge et al. 1982) and brain cells must synthesise some of their inositol from glucose via the L-isomer of inositol-1-phosphate. The remainder is thought to be taken up across the choroid plexus into the CSF (Spector 1976). Thus brain tissue will be more susceptible to the actions of lithium, especially any hyperactive pathways.

Using fluorescence spectroscopy, with fluorescent probes of known affinity for specific regions of cell membranes, Pettegrew et al. (1982) have demonstrated abnormalities of the hydrocarbon region of the plasma membrane of erythrocytes and the surface of lymphocyte plasma membranes, in manic-depressive patients. This would also be in keeping with a possible role for phospholipids in the aetiology of manic-depressive psychosis.

This part of the study is aimed at using the lymphoblastoid cell lines from manic-depressive patients and control subjects and examining them for any differences in the metabolism of phosphoinositides and inositol phosphates which may possibly underlie the illness. Speculation as to the possible defect in the PtdIns system, if indeed there is one, is discussed at the end of the chapter.

7.II. MATERIALS AND METHODS

7.II.1. Reagents

Reagents used were purchased from the following:-

Alfa Chemicals Ltd. (Wokingham, Beds.):- Versilube F50.

Amersham International (Amersham, Bucks.):- D-myo-[2-³H]Inositol (16.3 Ci/mmol), D-myo-[2-³H]Inositol 1-phosphate (IP; 1.0 Ci/mmol), D-myo-[2-³H]Inositol 1,4-bisphosphate (IP₂; 1.0 Ci/mmol), and D-myo-[2-³H]Inositol 1,4,5-trisphosphate (IP₃; 1.0 Ci/mmol).

BDH Chemicals (Poole, Dorset):- Sucrose, EDTA, (COOK)₂.H₂O, H.COONH₄, concentrated acetic and hydrochloric acids (all Analar grade), methanol, acetone and chloroform (all chromatographic grade), "Cocktail T" scintillant, and Merck Kieselgel 60 HPTLC (10 x 20 cm) and TLC (20 x 20 cm) plates.

Capital HPLC (Edinburgh, Scotland):- 250 x 4 mm i.d. SAX-Partisil 10 HPLC column.

Coulter Electronics Ltd. (Luton, Beds.):- Isoton.

Gibco-Biocult (Paisley, Scotland):- all tissue culture supplies as detailed in Section 5.II.1., and inositol-free RPMI-1640 (made to order).

New England Nuclear (W. Germany):- D-myo-[2,3-³H]Inositol (54.5 Ci/mmol).

Sigma (Poole, Dorset):- 2-p-toluidinylnaphthylene 6-sulfonate (TNS), AMP, ADP, ATP, PtdIns, PtdIns(4)P, PtdIns(4,5)P₂, myo-inositol, carbachol, noradrenaline, 5-HT, phytohemagglutinin (PHA), pokeweed mitogen (PWM), concanavalin A (Con A), Phaseolus-

coccineus lectin (PCo), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidic acid (PA), sphingomyelin (S), cholesterol (C), lysophosphatidylserine (LPS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylglycerol (LPG).

Water used for solutions was produced using a Milli-Q water system (Millipore S.A., France). All solutions were dispensed using Gilson adjustable pipettes during experiments.

7.II.ii. Measurement of inositol uptake by lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

In order to radiolabel all the intracellular inositol-containing pools to such an extent that they could subsequently be detected either by HPLC or TLC as appropriate, considerable time was spent investigating methods of incorporating the maximum amount of radiolabel into the cells. For expense reasons it was not possible to solely use ^3H -inositol but rather to use a mixture of labelled and unlabelled inositol. For similar reasons it was also not possible to use the concentrations of inositol normally present in the medium ($1.94 \times 10^{-4}\text{M}$), as the specific activity of such solutions would have been too low for adequate detection. Various preliminary experiments and their results are outlined below, followed by the detailed experimental protocol which was finally adopted.

In order to vary the inositol concentration of the medium, inositol-free RPMI 1640 was purchased and the appropriate amount

of inositol (either radiolabelled or unlabelled) added. The composition of the complete RPMI 1640 medium (inositol-free) was the same as standard RPMI 1640 (see Table 5.2.), with the exception that the foetal calf serum used was dialysed before being added. Initial incubations (15 minutes to 24 hours) using RPMI containing $5 \times 10^{-8} \text{M}$ ^3H -inositol (final concn. 5.1 mCi/mmol) produced very low labelling of the cells (approx. 2500 cpm/ 10^6 cells). This was thought to be due to a decrease in the specific activity of the extracellular inositol due to the free unlabelled inositol diffusing out of the cell down a concentration gradient. For this reason, it was decided to pre-incubate the cells in inositol-free RPMI 1640 before the incubation in ^3H -inositol, in an attempt to deplete the intracellular "free" inositol pool. Long periods of inositol-free pre-incubation (24 hours) resulted in a substantial loss of cellular viability as assessed using ethidium bromide/acridine orange (see Chapter 5). However 2 x 30 minute pre-incubations in inositol-free RPMI were found to substantially increase the radiolabelling of the cells, without affecting the cellular viability.

Four cell lines were selected at random, preincubated in inositol-free RPMI for 2 x 30 minutes and then incubated in RPMI containing various concentrations of unlabelled inositol (10^{-8}M - $2 \times 10^{-4} \text{M}$) for 24 hours, at which point viability was assessed. Satisfactory viability was maintained at extracellular inositol concentrations of $3 \times 10^{-7} \text{M}$, 10^{-6}M and $2 \times 10^{-4} \text{M}$ inositol during this period, and as a result it was decided to label the cells using 10^{-6}M inositol over a 6 hour period, following the

inositol-free pre-incubation. During the preliminary experiments this consisted of RPMI 1640 containing $8.44 \times 10^{-7}M$ unlabelled inositol and 2.5 uCi/ml of 3H -inositol (final inositol concentration = $10^{-6}M$). During the later experiments, the double-labelled inositol (N.E.N.) was used as detailed under "Reagents". In this case, unlabelled inositol accounted for $9.53 \times 10^{-7}M$ inositol and 2.5 uCi/ml 3H -inositol accounted for $0.47 \times 10^{-7}M$ of the final $10^{-6}M$ inositol medium. Both these conditions were found to produce adequate labelling of the cells without any possible compromise in viability.

The final protocol for measuring inositol uptake is described below for one bottle of cells. Lymphoblastoid cell lines were routinely cultured as described in Chapter 5. Twenty-four hours prior to the experiment, the medium was changed as normal. On the day of the experiment, 25×10^6 cells were placed in a 50 ml disposable plastic centrifuge tube, which was capped and centrifuged at 1200 rpm for 3 minutes ($37^{\circ}C$; Fisons MSE Coolspin). The supernatant was removed and the cells resuspended in 25 mls of inositol-free RPMI 1640 ($37^{\circ}C$). The cells were washed in inositol-free medium a further 2x, finally being resuspended in 25 mls of inositol-free medium and left to incubate for 30 minutes at $37^{\circ}C$. At the end of this period, the cells were washed once more in inositol-free medium and left at $37^{\circ}C$ in inositol-free medium for a further 30 minutes. The cells were then centrifuged again, the supernatant removed and the cells resuspended in 3 mls of RPMI containing $9.53 \times 10^{-7}M$ unlabelled inositol. Immediately, 3 mls of 2x radioactive standard solution (inositol-free RPMI containing $9.53 \times 10^{-7}M$

unlabelled inositol and 5.08 μCi ^3H -inositol/ml) was added, resulting in a final cell suspension of $3.5 - 4 \times 10^6$ cells/ml in 10^{-6}M inositol.

Immediately following the addition of the radioactivity to the cell suspension, 3 x 100 μl aliquots were layered onto 50 μl of Versilube F50 silicone oil in 450 μl microfuge tubes. The cells were then separated from the extracellular radioactivity by microfuging them through the oil (15 seconds; Beckman Microfuge B) into 50 μl of 0.25M sucrose, as described in the previous chapter. The microfuge tubes were then placed in liquid nitrogen until the end of the experiment. These served as blanks, measuring the radioactivity trapped between the cells when separated from the radioactive incubation medium by spinning through oil. The cell suspension was then incubated at 37°C . Every hour following the start of the experiment, upto and including 6 hours, 3 x 100 μl aliquots of cell suspension were taken for the measurement of inositol uptake and spun through oil as described above and the cell pellets frozen. At a convenient time point during the experiment, 3 x 100 μl aliquots of cell suspension were taken and each added to 19.9 mls of Isoton for the determination of cell numbers, as previously described in Chapter 2.

At the end of the uptake experiment, the tips were cut off the frozen 450 μl microfuge tubes and placed in scintillation vials containing 0.5 mls of water. When the frozen pellet had melted into the water, 5 mls of scintillant was added to each vial, the vials capped, shaken and the radioactive content determined as previously described. 3 x 50 μl aliquots of the 2x radioactive

standard were also taken and counted in a similar way for the determination of specific activity (each aliquot is equivalent to 100 ul of final incubation medium). Inositol uptake was calculated using the equation in Appendix 6 and expressed as either $\mu\text{mol}/\text{lcw}$ or $\text{pmol}/10^6$ cells, per unit time. The experiment was repeated only twice for each cell line due to the cost and time involved, with each measurement being made in triplicate within an experiment. Results were analysed by Student's t-test using the "Minitab" statistical package on the VAX mainframe computer.

7.II.iii. Extraction and partitioning of the intracellular water-soluble and lipid-soluble inositol-containing pools of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Cells were loaded with ^3H -inositol as described in the previous section, with aliquots being taken for blanks at zero time and aliquots for extraction at 6 hours. In contrast to the uptake experiment however, the aliquots were 500 ul in size and were microfuged through 200 ul of Versilube F50 into 50 ul of 0.25M sucrose in 1.5 ml microfuge tubes (Sarstedt) before being frozen in liquid nitrogen. The frozen samples were then extracted using a a modification of the method of Shukla and Hanahan (1982) and Shukla et al. (1985), as follows.

The tips of the microfuge tubes containing the cell pellets were cut off and added to 1 ml of inositol-free RPMI (serum-free) in 20 ml Quickfit tubes with ground glass stoppers. Immediately 3.75 mls of chloroform/methanol/11.3M HCl (200:400:1.6 v/v)

containing 1.33mM EDTA was added to each tube in turn which was then stoppered and shaken thoroughly, releasing the pressure at intervals. The tubes were then left at room temperature for 30 minutes and shaken periodically. At the end of this period, 1.25 mls of chloroform followed by 1.25 mls of water were added to each tube which was then shaken thoroughly before being centrifuged at 3000 rpm for 15 minutes (4°C; Fisons MSE Coolspin).

From each tube, 3 x 100 ul aliquots were taken from the resultant upper aqueous phase (4.75 mls) and added to scintillation vials containing 5 mls of scintillant. These were capped, shaken and the radioactivity determined as described in the previous section. The total radioactivity per upper phase was calculated and expressed as cpm/10⁶ cells/6 hours or as cpm/lcw/6 hours for each sample. The remainder of the upper phase was removed using Pasteur pipettes, taking care not disturb the interface, and stored at -20°C until analysed using HPLC, as described later. The interface was discarded and 3 x 100 ul aliquots of the lower lipid-soluble phase (2.5 mls) were added to scintillation vials and allowed to evaporate (removes the quenching effects of chloroform). 5 mls of scintillant was then added to each vial, the vials capped, shaken, and the radioactivity determined and expressed as described above. The remainder of the lower phase was removed into clean glass test tubes using Pasteur pipettes and evaporated to dryness in a Techne Dri-block at 60°C, under a stream of nitrogen gas. The lower phase was concentrated in the base of the tube by repeated evaporation and resuspension in chloroform, using progressively

smaller volumes of chloroform and washing the walls of the tube in the process. The evaporated lower phase samples were then stored at -20°C until analysed by TLC as described in the following section.

Contamination of the lower phase by inositol from the upper phase was assessed by extracting an aqueous solution of ^3H -inositol and taking aliquots of the lower phase for the determination of radioactivity as described above. It was not possible to assess the contamination of the upper phase by the lower phase in a similar manner as the labelled inositol compounds contained in the lower phase were either not available commercially or were too expensive to purchase. For similar reasons recovery studies were not carried out. This is discussed further in the "Results" section of this chapter.

7.II.iv. Thin layer chromatographic (TLC) analysis of the lipid-soluble phosphoinositide pools in lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Preliminary experiments failed to find a TLC method which would separate the phosphoinositides from each other and from other phospholipids. Hence the possibility of measuring the size of the phosphoinositide pools by running the samples on TLC plates, scraping off the relevant spots and analysing them for phosphorus content was not feasible. Therefore it was decided to use a method which would adequately separate PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ from each other (although possibly still contaminated with other phosphorus-containing compounds), scrape the spots off the TLC plate and determine their

radioactive content. The limitations of using radioactive incorporation to examine the relative pool sizes are discussed later in the chapter. The method finally used was based on that of Shaikh and Palmer (1977) and Jolles et al. (1981) as described below. Initial attempts at separating various lipid standards as listed in the "Reagents" section (5 mg/ml in 1/2 (v/v) chloroform/methanol), from the phosphoinositides (4 mg/ml in 90/9/1 (v/v) chloroform/methanol/1M HCl), were unsuccessful as mentioned above, but separation of the phosphoinositides relative to each other was achieved. The plate was prepared and samples applied as follows, both for standards and biological samples. Initially Kieselgel 60 TLC plates (20 x 20 cm; Merck) were used but in the final analyses the plates below were used. Similar separations were achieved in both cases.

Kieselgel 60 HPTLC plates (10 x 20 cm; Merck) were impregnated with a solution of potassium oxalate (1% w/v) in methanol/water (2:3 v/v) and dried at room temperature. Before sample application they were activated at 110°C for 15 minutes. The lower phase samples for TLC, prepared as described in the previous section, were then resuspended in 5 ul of chloroform/methanol/1M HCl (90/9/1, v/v), and spotted onto the TLC plate at 1.5 cm intervals using a Hamilton spiking syringe. Each sample tube was washed out twice more with 5 ul of the above solvent, and the washings applied to the appropriate sample spot on the plate. Drying of the spots to minimise spread during their application was accomplished by situating the TLC plate on a bench-top incubator at 50°C and air-drying with a stream of N₂. Some samples were spiked with a mixture of standard PtdIns,

PtdIns(4)P, and PtdIns(4,5)P₂ (4 mg/ml) to aid the later visualisation of the separated phosphoinositide spots when present at very low concentrations. The plate was then run using a chloroform/acetone/methanol/glacial acetic acid/ water mixture (100:37.5:34.5:30:20 v/v) in a glass chamber which had been pre-equilibrated with the solvent.

When the solvent front was approximately 2 cm from the top of the plate, the plate was removed, air dried, and sprayed with an aqueous solution of 1mM TNS (Shukla and Hanahan 1982). After drying the plate at room temperature, the spots were visualised under UV light, identified on the basis of the spiked samples and the R_f values of the previously run standards, the relevant spots marked and scraped off. Using a suction device attached to the water tap, the silica from each lipid spot was sucked into scintillation vials containing 0.5 mls of water. 5 mls of scintillant was added to each and the radioactivity determined as described in the previous section. The origin spots were also scraped off and counted and used as another indicator of the contamination of the lower phase by inositol. No quenching effect of the silica was found.

Due to the difficulties of collecting all the lower phase, together with the problems presented when attempting to wash the tubes thoroughly with very low volumes of solvent after evaporation of the samples to dryness, it was decided to use the ratios of the radioactive counts (corrected for blanks) contained in the mono-, di- and triphosphoinositide spots for each sample as a measure of the relative radioactive proportions present in the cells. By using the ³H-inositol content of the

cells which was attributable to the lower phase as calculated previously for each sample, together with these ratios, the radioactive content of each phosphoinositide pool was determined for each sample and expressed as either cpm/ 10^6 cells/6 hours or as cpm/lcw/6 hours. The assumptions inherent in such a calculation are discussed later in the chapter. This procedure was carried out for all the triplicate samples of each cell line within each experiment and was performed on only two separate occasions for the majority of cell lines due to the time and expense involved. Results were analysed by Student's t-test using the "Minitab" statistical package on the VAX mainframe computer.

7.II.v. High performance liquid chromatographic (HPLC) analysis of the intracellular inositol phosphates of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The method used to determine the basal levels of IP, IP₂ and IP₃ is a modification of that of Irvine et al. (1985) which is an ion-exchange method. HPLC equipment is as detailed in Chapter 4 with the exceptions of the column which was a 250 x 4 mm i.d. SAX-Partisil 10 column, and the sample loop which was 2 mls in capacity. In addition the eluent containing the separated inositol phosphates was collected using a Gilson Model 202 fraction collector with controller. Because the inositol phosphates have no appreciable UV absorbance, the nucleotides ATP, ADP and AMP which elute with a similar profile, were used as intracellular markers for the separation of the inositol phosphates during each run. During preliminary experiments to

establish the method, which had to be modified considerably, radioactive standard inositol phosphates and inositol were used and all the fractions were collected into insert vials and counted for radioactivity. Once a satisfactory and reproducible separation was achieved, a programme was devised so that only the fractions of interest were collected. The separation was checked for each sample by monitoring the elution profile of the above nucleotides using the UV detector ($\lambda = 254 \text{ nm}$). The resultant gradient profile and fraction collector programme were as below.

Eluents:- A: Water

B: 1.5 M Ammonium formate adjusted to pH 3.7 with orthophosphoric acid.

Flow Rate:- 1.25 mls/min.

Gradient Profile:-

<u>Time (mins)</u>	<u>%B</u>
0	0
1	0
26	100
31	100
33	0
40	0

Fraction Collector Programme:-

Fraction size: 0.25 mins (0.31 mls)

Drain:-	2.0 mins.
Collect:-	4.0 mins.
Drain:-	4.5 mins.
Collect:-	5.5 mins.
Drain:-	0.5 mins.
Collect:-	4.0 mins.
Drain:-	4.5 mins.
Collect:-	5.0 mins.

The modifications to the initial method are discussed in the "Results" section. The stored upper phase samples, prepared as described in the previous sections, were filtered through 0.2 um cellulose acetate Millipore filters before injection. The fractions (0.31 mls) were collected in insert vials and 0.2 mls of water added to each followed by 4 mls of scintillant. The vials were then capped, shaken, inserted into scintillation vials and the radioactivity determined as previously described. No significant quenching of the count rates was found with increasing concentrations of Eluent B.

7.II.VI. Determination of agonist-stimulated inositol phosphate turnover in lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Potential agonists were assessed by preparing and loading the cells with ^3H -inositol as described before. Lithium chloride was then added to the incubation medium to produce a final concentration of 10 mM Li^+ (to inhibit the hydrolysis of IP to inositol) and the suspension was incubated at 37°C for 15 minutes. Potential agonists were then added to the cell suspension and incubated for a further 15 or 30 minutes. At the end of this incubation period, 500 ul aliquots of the cells were microfuged as described previously, frozen and extracted. The upper phase samples were analysed by HPLC to determine whether any accumulation of IP had occurred. Agonists investigated were carbachol (10^{-3}M), 5-HT (10^{-4}M), noradrenaline (10^{-4}M), PHA (50 ug/ml), PWM (50 ug/ml), PCo (50 ug/ml) and Con A (50 ug/ml). Unfortunately, as described in the following "Results" section,

none of these potential agonists produced any effect on the inositol phosphates of the lymphoblastoid cell lines. As time was limited, this area was not pursued further and the ^3H -inositol incorporation into the cells was examined under non-stimulated conditions only.

7.III. RESULTS

The separation of phosphoinositide standards by TLC and inositol phosphate standards by HPLC are shown in Figures 7.2. and 7.3. respectively. As can be seen in Figure 7.2. the TLC separation of phosphoinositides relative to each other has been achieved with R_f values of 0.51, 0.40 and 0.34 for PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ respectively. The incomplete separation from other phospholipids can also be seen illustrating the need to analyse for radioactive incorporation rather than measuring pool size by phosphorus determination. The HPLC separation of inositol phosphate standards (Figure 7.3.) illustrates their elution profile relative to the nucleotides AMP, ADP, and ATP. The unknown peak eluting just prior to IP is a contaminant present in the ³H-inositol stocks. The 1,4,5 isomer of IP₃ was used as the radiolabelled 1,3,4 isomer of IP₃ was not available commercially. In the original method of Irvine et al. (1985), both isomers present in the cells were separated. It is assumed that they would also be separated using the modified method in this study which yields a comparable chromatographic profile, although Eluent B had to be adjusted from 1M ammonium formate to 1.5M and the gradient was also adjusted in order to achieve this profile.

When biological samples were examined however, after being loaded with ³H-inositol and extracted, only a radioactive inositol peak was seen, with no detectable IP, IP₂ or IP₃ peaks. Incubation with lithium and several potential agonists as described in the "Methods" section failed to produce any

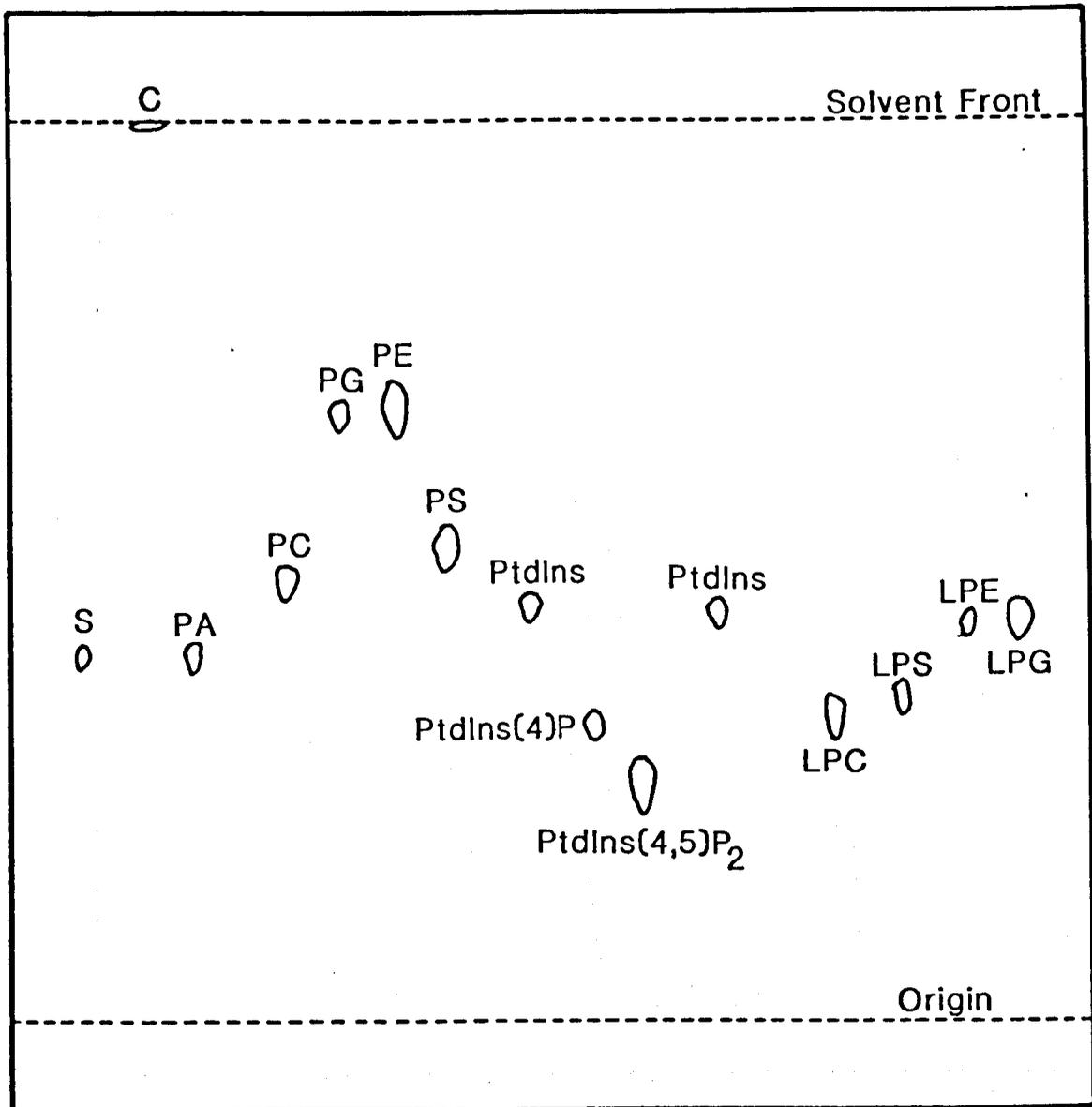


Figure 7.2. TLC separation of lipid standards

Solvent conditions, lipid concentrations and abbreviations are as described in the previous "Methods" section.

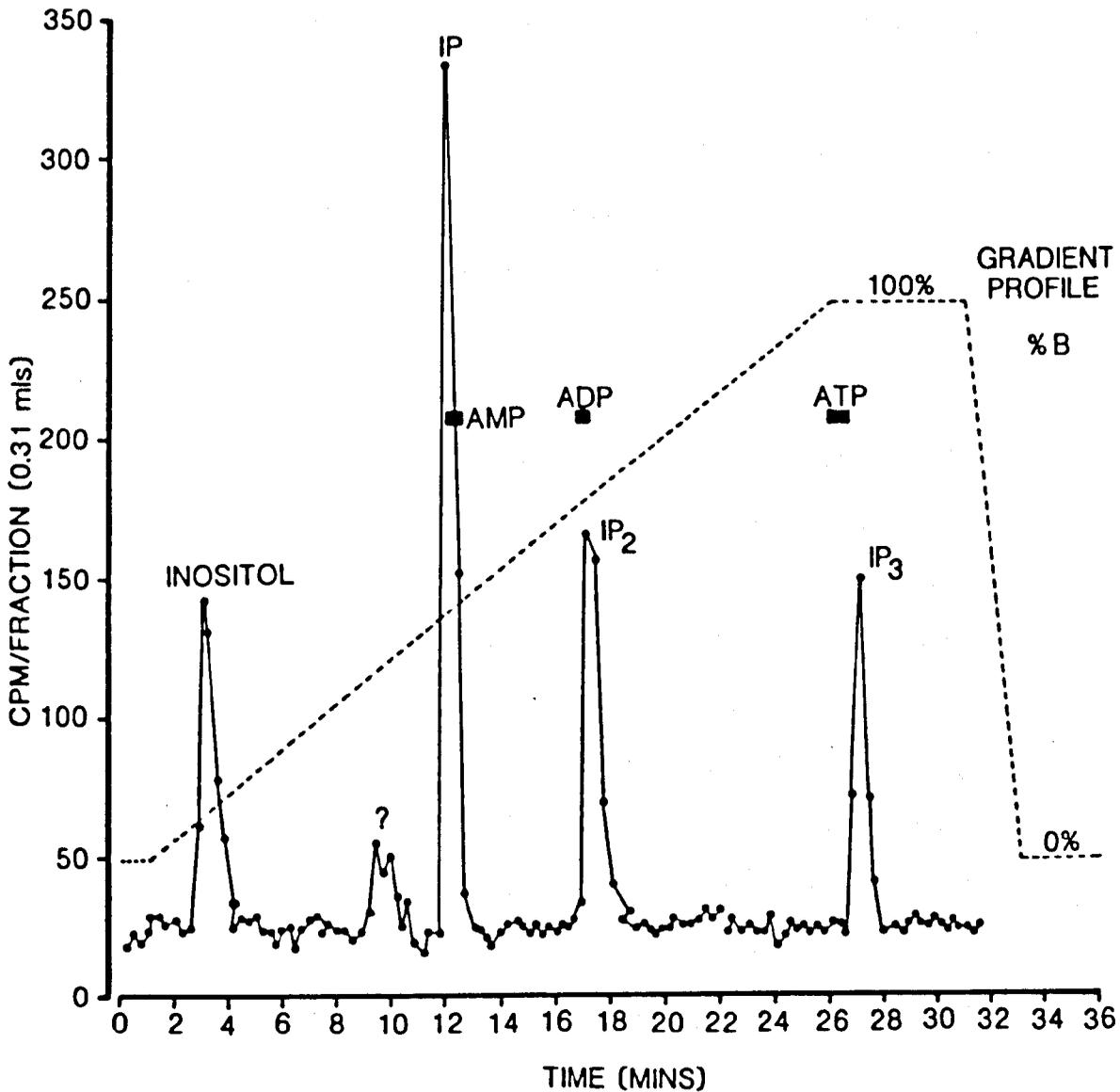


Figure 7.3. HPLC separation of inositol and inositol phosphates standards

Equipment and operating conditions are as described in the previous "Methods" section.

detectable labelling in any of these inositol compounds. The possible reasons for this lack of initial and stimulated labelling of inositol phosphates are discussed in the following "Discussion" section. Because of this, only the uptake of inositol and basal incorporation into the phosphoinositide pools was examined as these could be detected under these conditions.

During the extraction, the contamination of the lower phase by inositol from the upper phase was negligible. As discussed previously it was not possible to assess the contamination of the upper phase by the lower phase due to the unavailability and/or expense of standard radiolabelled phosphoinositides. For this reason recovery studies were also not carried out and therefore actual values may be slightly higher than the results given here. However this does not affect comparisons made between cell lines as recovery should be similar in all cases. The assumption that the relative incorporation of radioisotope into the various phosphoinositide pools is the same as that found in the sample phosphoinositide spots on the TLC plate is justified providing that recoveries of the phosphoinositides are similar. The acidic solvent was used for the resuspensions as it has been shown that lower recoveries of $\text{PtdIns}(4,5)\text{P}_2$ result from tissue if the extractant is neutral (Creba et al. 1983).

The inositol uptake measurements are shown in Figures 7.4. and 7.5., expressed as $\text{pmol}/10^6$ cells and umol/lcw respectively. Error bars have been omitted for clarity but are usually less than 5%. It is possible to quantify the results in this way as the specific activity of the extracellular solution is known, assuming that no dilution is occurring as a result of the

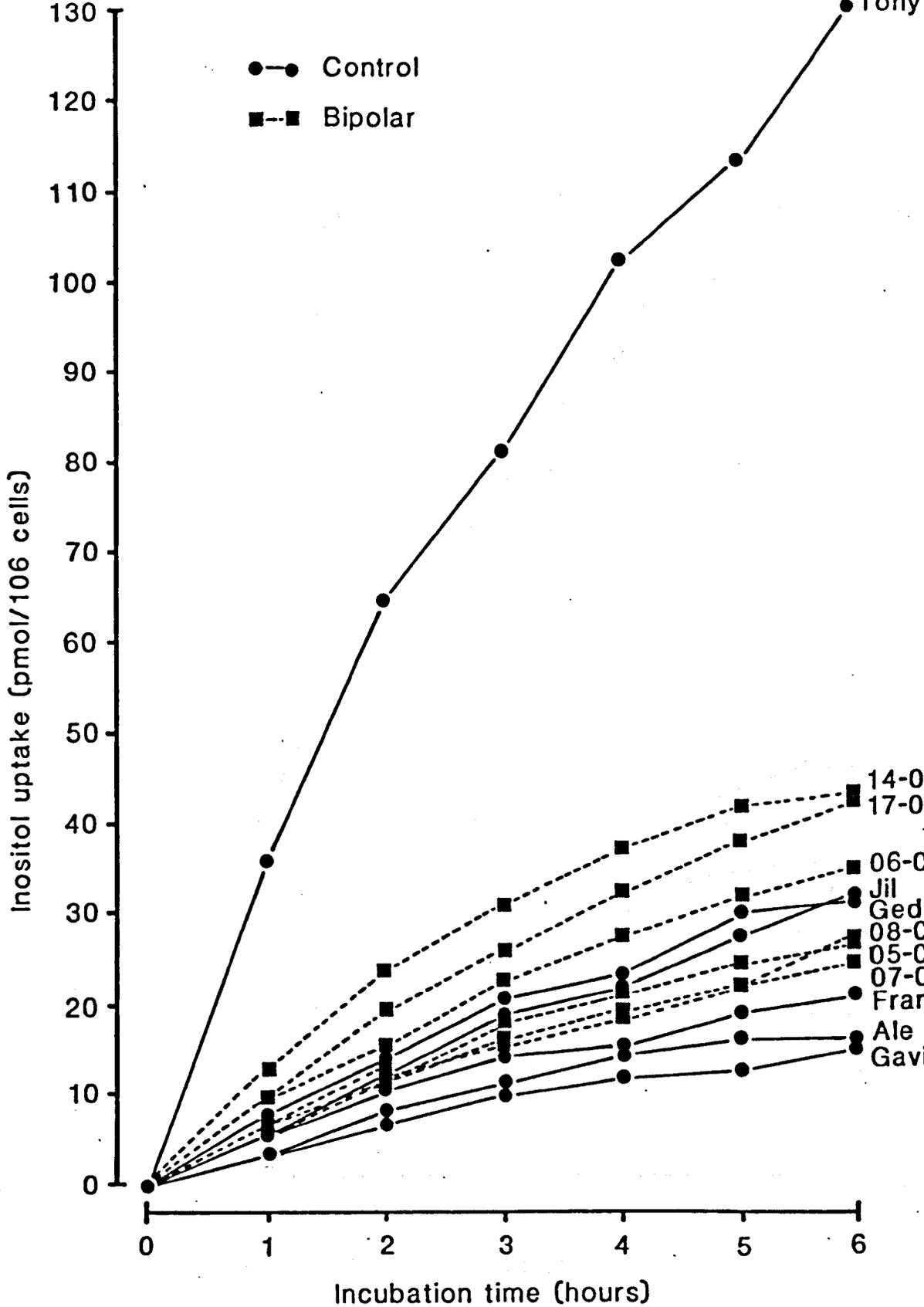


Figure 7.4. Inositol uptake (pmol/10⁶ cells) of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean values of data from 2 experiments per cell line, with each measurement being triplicated within an experiment. Extracellular [inositol] = 1 uM. Inositol-free pre-washes = 2 x 30 minutes.

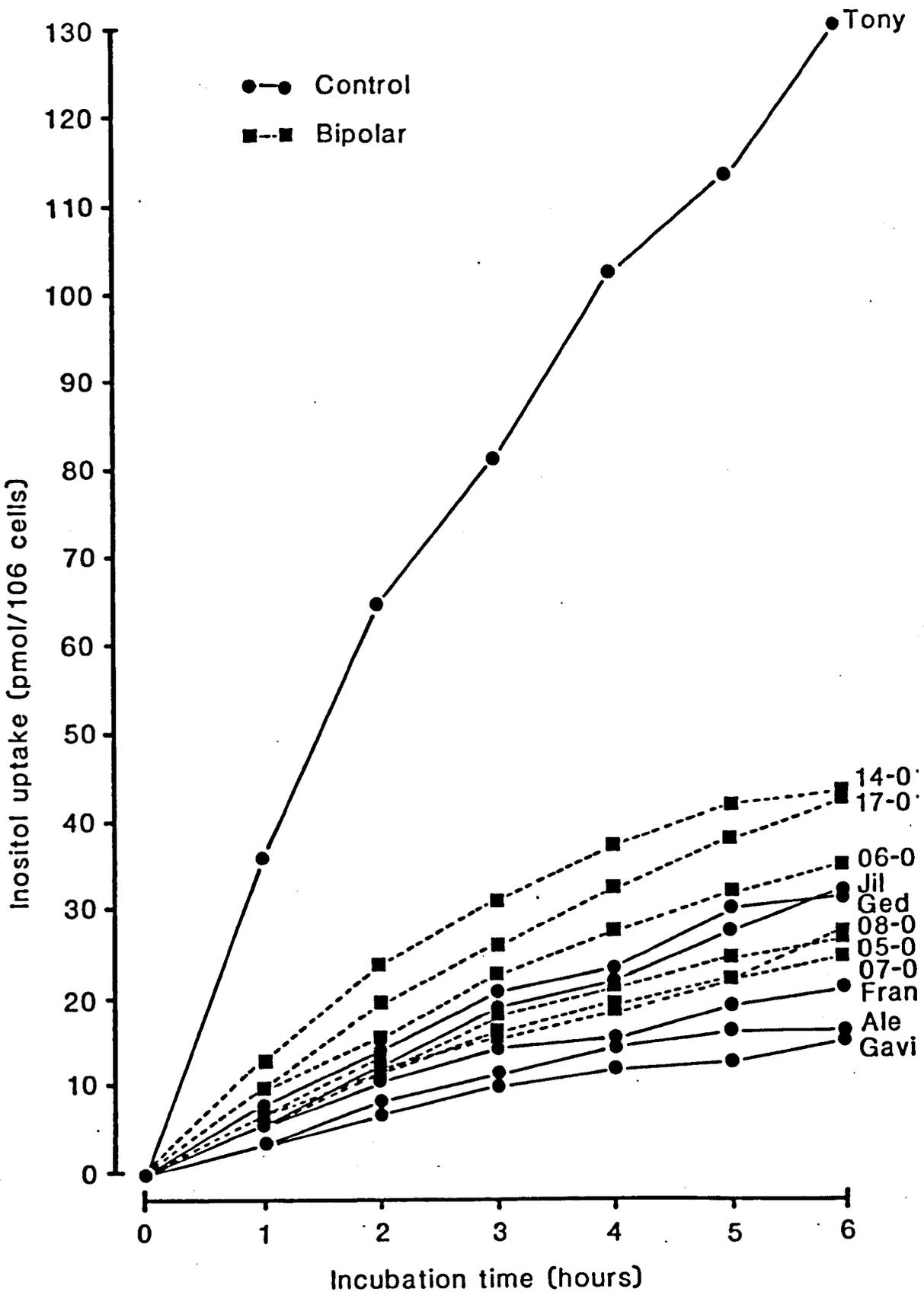


Figure 7.4. Inositol uptake (pmol/10⁶ cells) of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean values of data from 2 experiments per cell line, with each measurement being triplicated within an experiment. Extracellular [inositol] = 1 uM. Inositol-free pre-washes = 2 x 30 minutes.

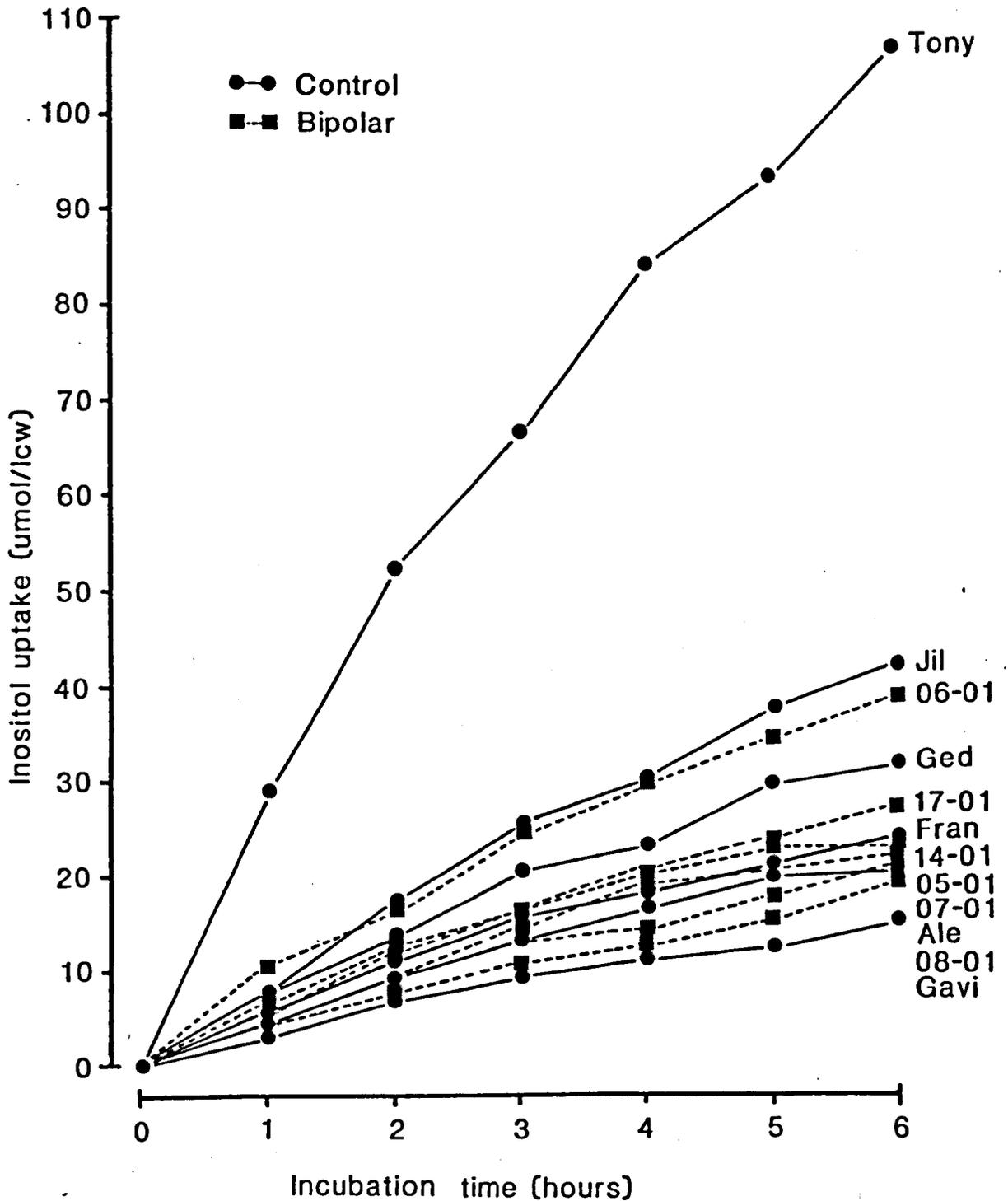


Figure 7.5. Inositol uptake (umol/lcw) of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean values of data from 2 experiments per cell line, with each measurement being triplicated within an experiment. Extracellular [inositol] = 1 uM. Inositol-free pre-washes = 2 x 30 minutes.

unlabelled inositol diffusing out of the cells. Such a diffusion is likely to be negligible, due to the inositol-free pre-incubations. In the majority of cell lines, uptake appears to be linear over the first 2 - 3 hours before appearing to slow down although not reaching a plateau within the 6 hour period. The cell line Tony is a notable exception with an uptake far greater than any of the other cell lines although there is considerable interindividual variation in both groups. Tony was established from a person suffering from myeloma and the possible importance of this is discussed later. For the overall analysis of the results of the control group this cell line has been omitted and is analysed separately.

When the results are expressed as $\text{pmol}/10^6$ cells, greater uptake seems to occur in the bipolar cell lines compared with the controls (with the exception of Tony) but this difference is abolished if the results are expressed as umol/lcw and can be explained in terms of the higher mean cell volumes of the bipolar group compared with the control group (mean + S.E.M. = $1695 + 172 \text{ u}^3$ vs $1104 + 70 \text{ u}^3$ respectively). In view of the significantly greater volume of the bipolar cell lines compared with the control cell lines used in this part of the study, the incorporation of ^3H -inositol into the various intracellular and membrane-bound pools has been expressed solely in terms of per lcw thus negating any effects of the differing volumes. This is the most convenient way of normalising the data with respect to cell size but although expressed in this way, this does not imply that all the pools are freely distributed in the cytoplasm. This is not the case, as discussed in the "Introduction" to this

chapter. Although all the cell lines appear to accumulate inositol to a much greater concentration than that of the extracellular solution, this may not be the case depending upon the distribution within the intracellular pools i.e. whether most of the inositol is in the "free" inositol pool. This is examined below.

The distribution of radiolabelled inositol within the cells is expressed as cpm/lcw rather than molar concentrations. This is due to the limitations imposed by the presence of several inositol-containing pools with interconversions as shown previously in Figure 7.1.. Whether the specific activity of each is equal to that of the extracellular solution and hence whether the distribution of radiolabelled inositol reflects their relative magnitudes, is determined by the rates of equilibration between the various pools. This is discussed further later in the chapter.

A summary of the incorporation of ^3H -inositol into the various intracellular pools of bipolar and control cell lines is shown in Table 7.1.. The values for Tony are not included in this analysis. The results have all been normalised for the same standard counts so that they can be directly compared. If the values for the individual cell lines are examined in Table 7.2. with the distributions of each inositol-containing pool for each cell line shown in Figures 7.6. to 7.10. inclusive, considerable interindividual variation can be seen although the results from experiment to experiment for each cell line are very consistent in the majority of cases. There appears to be a much wider range of inositol uptake values in the control group of cell

Table 7.1. Incorporation of ^3H -inositol into lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The results below are the mean values \pm S.E.M. from 5 control and 6 bipolar cell lines. The mean value for each individual cell line within these groups is derived from (usually) 2 experiments, with each parameter measured in triplicate within an experiment. The results from the cell line Tony have been omitted from this analysis. Significance testing of the results for the bipolar group relative to the control group is by Student's t-test.

Parameter	Mean value \pm S.E.M.		Level of Significance
	Control	Bipolar	
Total uptake ($\times 10^9$ cpm/lcw/6 hrs)	26.58 \pm 5.03	27.61 \pm 2.47	N.S.
"Free" inositol ($\times 10^9$ cpm/lcw/6 hrs)	19.09 \pm 4.85	22.33 \pm 1.85	N.S.
PtdIns ($\times 10^9$ cpm/lcw/6 hrs)	6.25 \pm 1.92	4.99 \pm 0.92	N.S.
PtdIns(4)P ($\times 10^9$ cpm/lcw/6 hrs)	0.35 \pm 0.06	0.23 \pm 0.04	N.S.
PtdIns(4,5)P ₂ ($\times 10^9$ cpm/lcw/6 hrs)	0.11 \pm 0.03	0.05 \pm 0.01	p < 0.05
"Free" inositol (% of total uptake)	69.5 \pm 5.8	81.4 \pm 2.5	p < 0.05
PtdIns (% of total uptake)	28.6 \pm 5.5	17.7 \pm 2.4	N.S.
PtdIns(4)P (% of total uptake)	1.5 \pm 0.3	0.8 \pm 0.1	p < 0.05
PtdIns(4,5)P ₂ (% of total uptake)	0.4 \pm 0.2	0.2 \pm 0.03	N.S.

Table 7.2. Incorporation of ^3H -inositol ($\times 10^9$ cpm/lcw/6 hrs) into lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The values below represent the mean of data from 2 experiments per cell line (1 in the cases of Fran, 07-01 and 17-01), with measurements triplicated within an experiment. The upper and lower groups represent control and bipolar groups respectively.

Cell line	Mean ^3H -inositol incorporation ($\times 10^9$ cpm/lcw/6 hrs) \pm S.E.M.					
	Total uptake	"Free" inositol	PtdIns	PtdIns(4)P	PtdIns(4,5)P ₂	
Ale	21.70 \pm 3.25	15.28 \pm 2.63	5.94 \pm 0.55	0.35 \pm 0.01	0.13 \pm 0.06	
Fran	20.47 \pm 0	13.92 \pm 1.58	5.94 \pm 0.04	0.40 \pm 0.02	0.22 \pm 0.05	
Gavi	13.88 \pm 0.59	8.30 \pm 0.28	5.22 \pm 0.96	0.31 \pm 0.06	0.06 \pm 0.03	
Ged	36.36 \pm 0.74	21.12 \pm 0.96	14.58 \pm 0.24	0.54 \pm 0.02	0.11 \pm 0.04	
J11	40.49 \pm 1.23	36.85 \pm 1.17	3.45 \pm 0.03	0.14 \pm 0.002	0.05 \pm 0.02	
Tony	117.60 \pm 3.24	115.13 \pm 3.54	2.34 \pm 0.29	0.10 \pm 0.02	0.04 \pm 0.002	
05-01	24.45 \pm 0.98	21.21 \pm 0.87	2.98 \pm 0.03	0.22 \pm 0.06	0.04 \pm 0.02	
06-01	33.80 \pm 1.29	27.06 \pm 3.32	6.36 \pm 1.84	0.28 \pm 0.16	0.07 \pm 0.03	
07-01	30.68 \pm 0	21.93 \pm 0.47	8.33 \pm 0.09	0.38 \pm 0.08	0.04 \pm 0.04	
08-01	17.84 \pm 1.23	15.61 \pm 1.60	2.10 \pm 0.32	0.09 \pm 0.03	0.04 \pm 0.02	
14-01	26.03 \pm 0.61	20.40 \pm 0.29	5.35 \pm 0.35	0.22 \pm 0.03	0.06 \pm 0.01	
17-01	32.87 \pm 0	27.78 \pm 0.02	4.82 \pm 0.05	0.21 \pm 0.04	0.06 \pm 0.01	

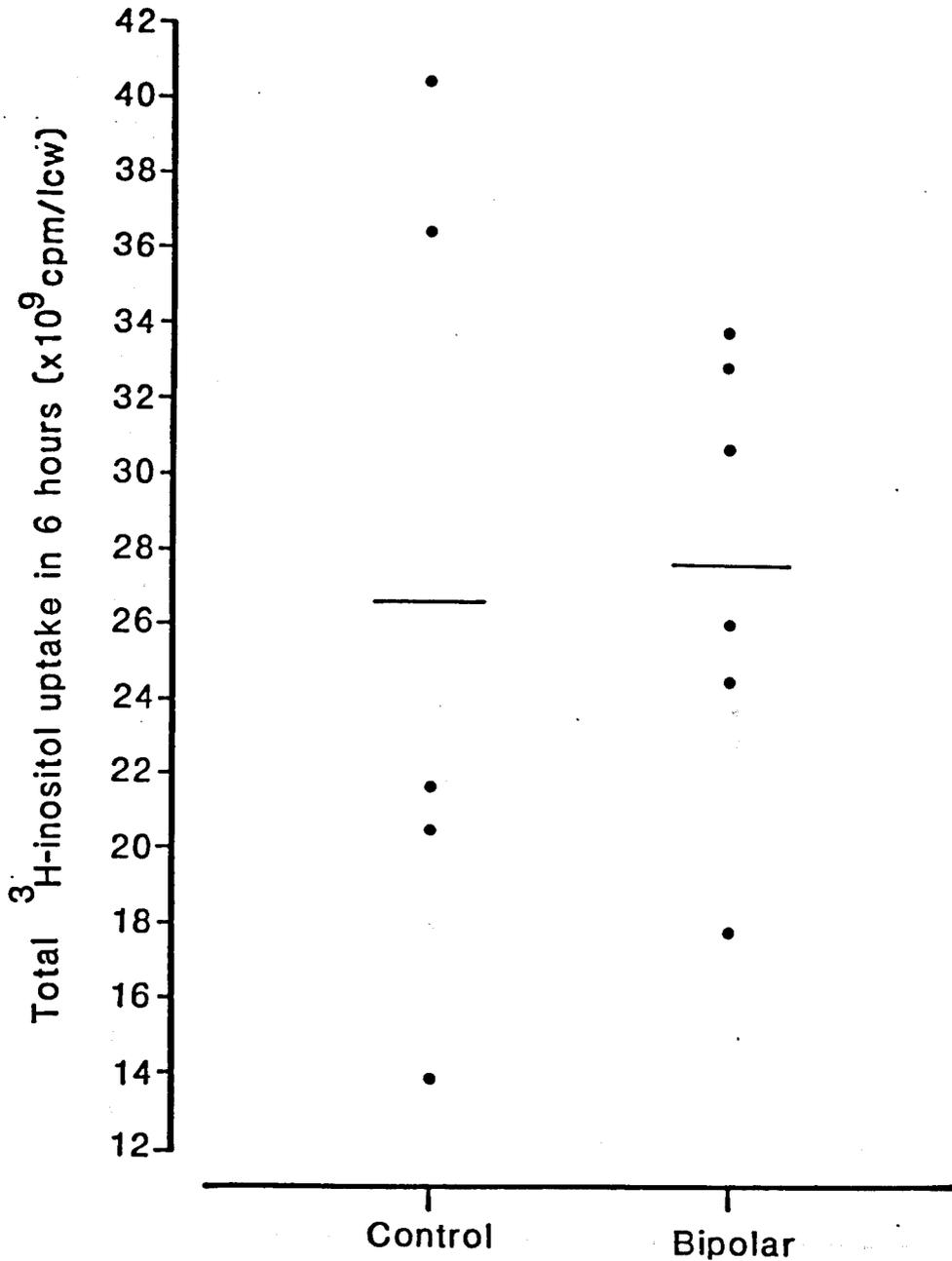


Figure 7.6. Distribution of the values for the ³H-inositol uptake of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

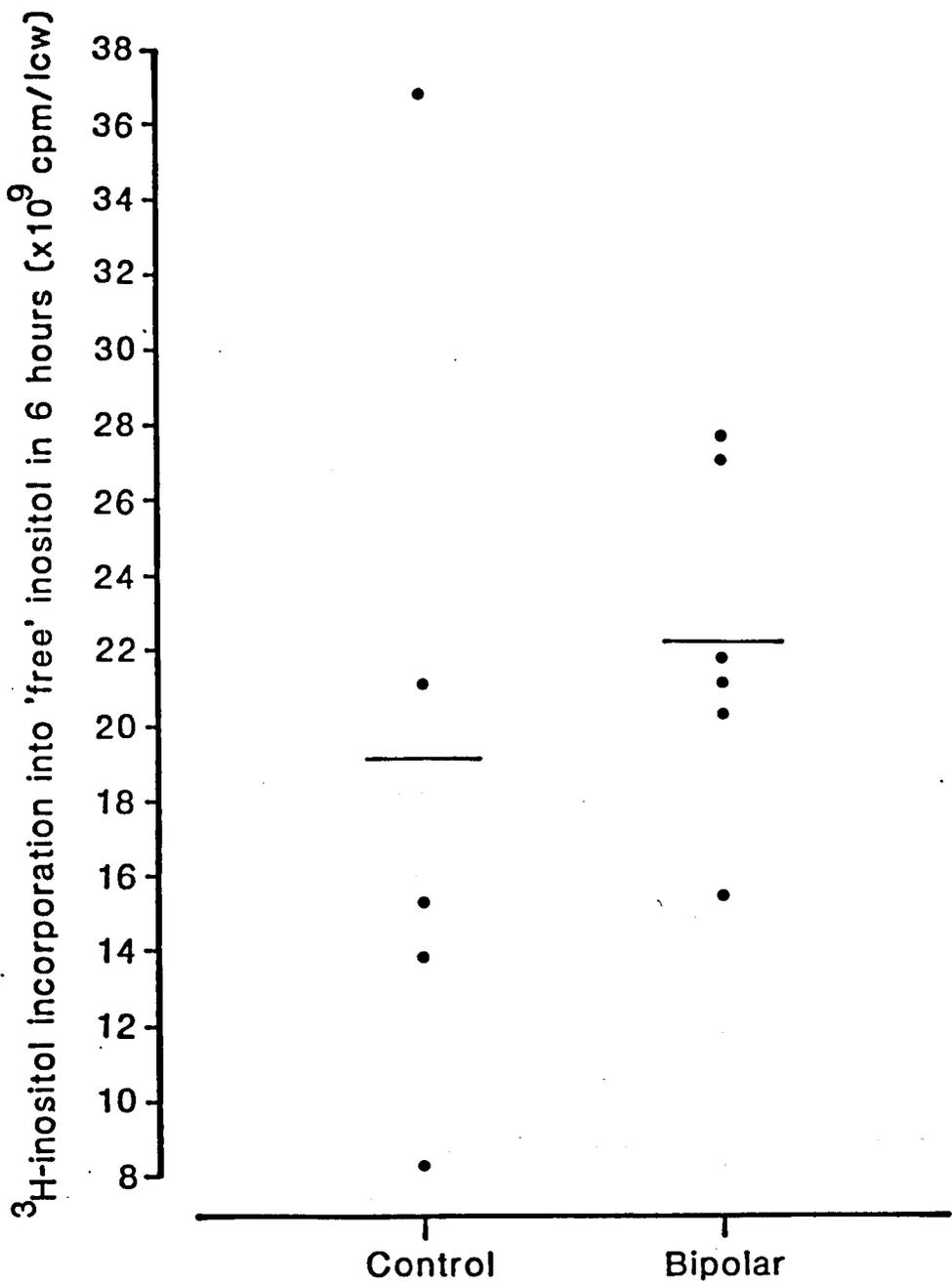


Figure 7.7. Distribution of the values for incorporation of extracellular ³H-inositol into the "free" inositol pool of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

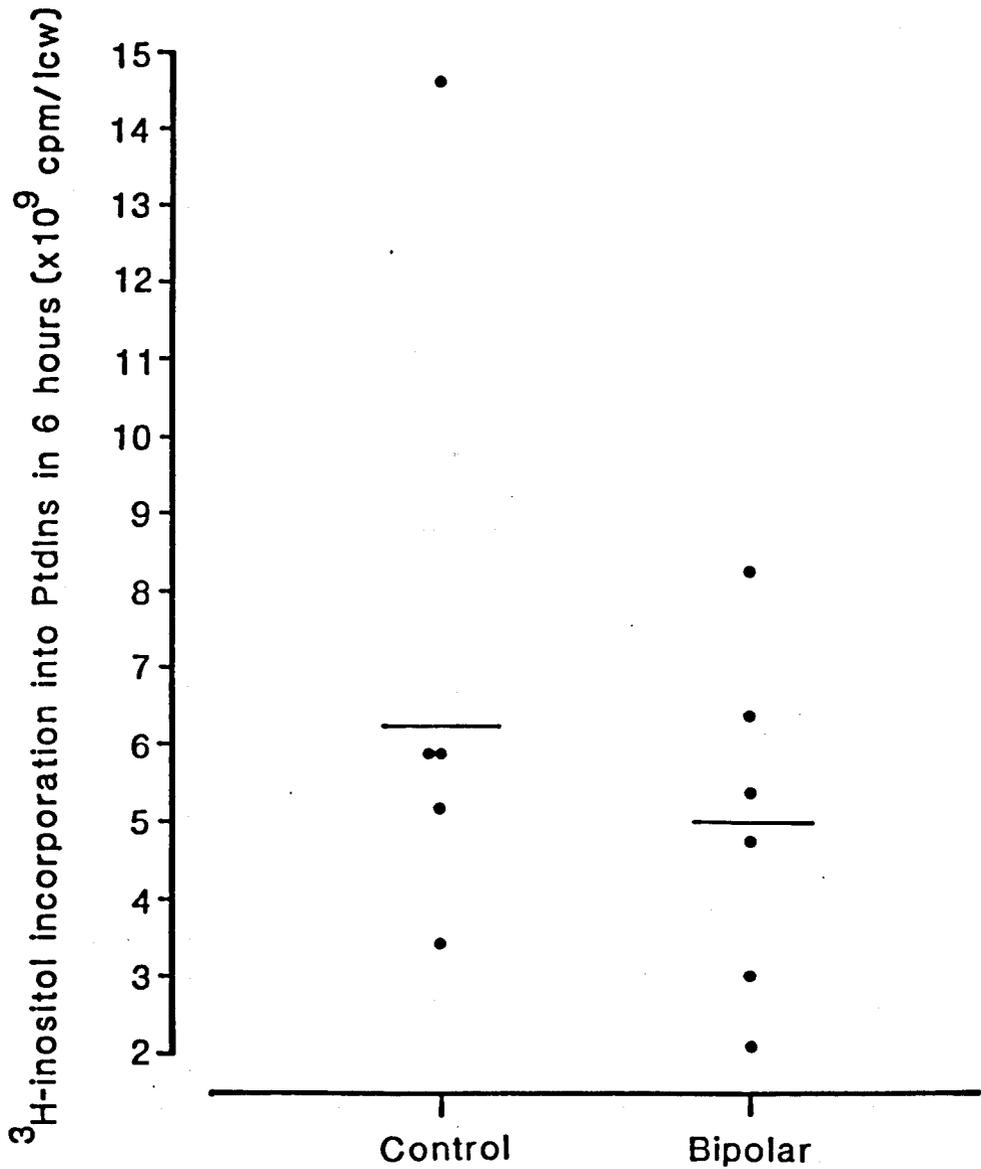


Figure 7.8. Distribution of the values for the incorporation of extracellular ^3H -inositol into PtdIns of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

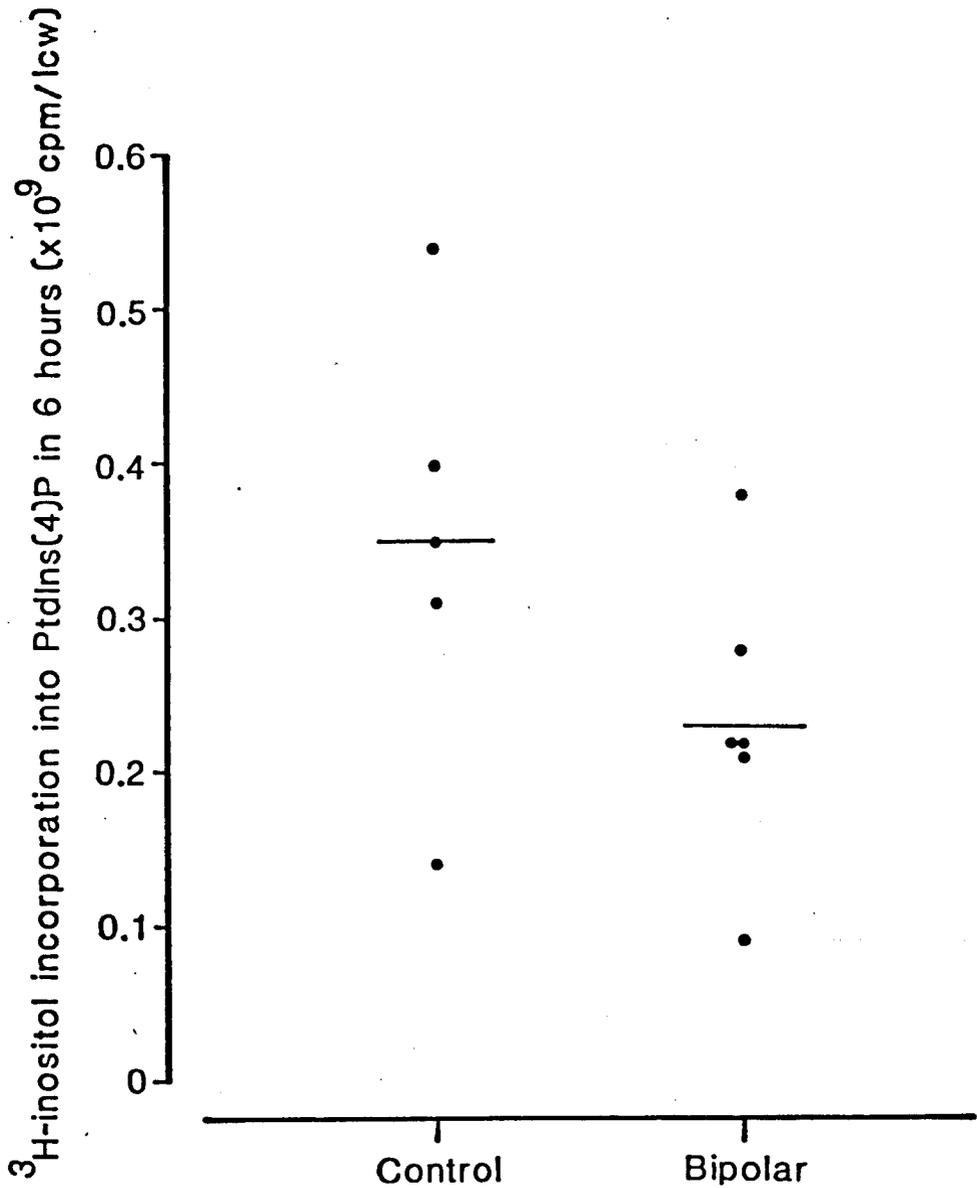


Figure 7.9. Distribution of the values for the incorporation of extracellular ³H-inositol into PtdIns(4)P of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

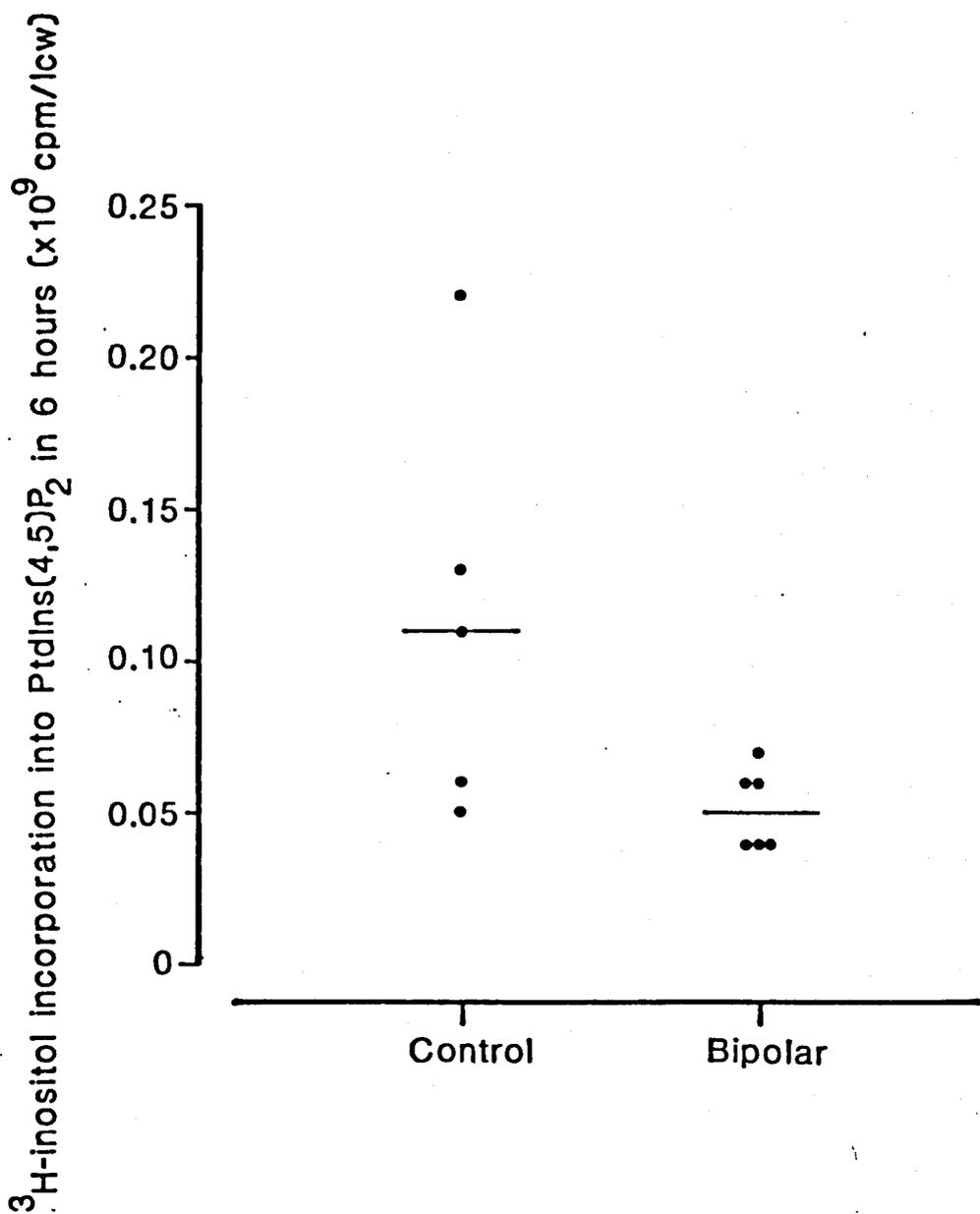


Figure 7.10. Distribution of the values for the incorporation of extracellular ^3H -inositol into PtdIns(4,5)P₂ of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

lines than the bipolars although the average value is similar (Figure 7.6.). The same picture can be seen in the incorporation of ^3H -inositol into the "free" inositol pool within the cells (Figure 7.7), although the majority of the bipolar cell lines appear to have greater incorporation into this pool, compared with the controls. The difference is not significant however. When the incorporation of ^3H -inositol into the phosphoinositide pools is examined, no significant difference is seen between the two groups of cell lines with respect to PtdIns and PtdIns(4)P although in the latter case the average value is 50% higher in the control group but not significantly so due to the relatively large variation in values. An anomalously high value is seen in the case of PtdIns for the cell line Ged, especially when compared with other cell lines which also have higher incorporations into "free" inositol but much smaller values for PtdIns. A significantly lower incorporation into PtdIns(4,5)P₂ is seen for the bipolar group despite their being no difference in any of the other pools. If the ratios of the incorporation of ^3H -inositol into the PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ pools are examined for each of the cell lines in the two groups and averaged they are (\pm S.E.M.), 100 : 5.3 (\pm 0.6) : 1.8 (\pm 0.5) respectively for the control group and 100 : 4.9 (\pm 0.5) : 1.1 (\pm 0.2) respectively for the bipolar group. Thus there is no significant difference between the ratios for each group. It is also interesting to note that although the cell line Tony has a much greater inositol uptake and incorporation into the "free" inositol pool, a proportionately bigger incorporation into the phosphoinositides is not seen; in

fact less radioactivity is incorporated into these pools.

When the incorporation of inositol into the various pools is examined in terms of % of the total ^3H -inositol uptake (summarised in Table 7.1. with individual data in Table 7.3. and Figures 7.11. to 7.14. inclusive), significant differences are seen between the two groups. Significantly more of the ^3H -inositol taken up by the cells is present in the "free" inositol pool of bipolar cell lines compared with controls (81.4% as opposed to 69.5%, $p < 0.05$). Correspondingly there is a smaller % incorporated into the phosphoinositide pools of the bipolar group although this does not reach statistical significance except in the case of $\text{PtdIns}(4)\text{P}$, largely due to the presence of the cell line J11 in the control group which behaves more like the bipolar cell lines. This lower % incorporation of ^3H -inositol into the phosphoinositide pools is made even more significant in view of the fact that the bipolar cell lines tend to have greater incorporation into "free" inositol pools, compared with controls, although non-significantly so. For example, the mean (\pm S.E.M.) values for the incorporation into the "free" inositol pools of the control and bipolar cell lines are 19.09 ± 4.85 ($\times 10^9$ cpm/lcw/6 hours) and 22.33 ± 1.85 ($\times 10^9$ cpm/lcw/6 hours) respectively, whereas the values for the total incorporation into the phosphoinositides as a percentage of the total uptake are 30.6 ± 5.8 and 18.7 ± 2.5 respectively. The former figures just fail to reach statistical significance largely due to the anomalous values for the cell line J11, but the latter figures are significantly different at the 5% level and would have been even more significant but for the values for

Table 7.3. Incorporation of ^3H -inositol (% of total uptake) into lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

The values below represent the mean of data from 2 experiments per cell line (1 in the cases of Fran, 07-01 and 17-01), with measurements triplicated within an experiment. The upper and lower groups represent control and bipolar groups respectively.

Cell line	Mean ^3H -inositol incorporation (% of total uptake) \pm S.E.M.			
	"Free" inositol	PtdIns	PtdIns(4)P	PtdIns(4,5)P ₂
Ale	70.2 \pm 1.6	27.6 \pm 1.6	1.6 \pm 0.2	0.6 \pm 0.2
Fran	68.0 \pm 7.7	29.0 \pm 0.2	1.9 \pm 0.09	1.1 \pm 0.3
Gavi	60.0 \pm 4.6	37.4 \pm 5.3	2.2 \pm 0.4	0.4 \pm 0.2
Ged	58.1 \pm 1.5	40.2 \pm 1.5	1.5 \pm 0.1	0.3 \pm 0.1
JII	91.0 \pm 9.1	8.6 \pm 0.2	0.4 \pm 0.05	0.1 \pm 0.07
Tony	97.9 \pm 0.3	2.0 \pm 0.3	0.1 \pm 0.02	0.03 \pm 0
05-01	86.8 \pm 0	12.3 \pm 0.4	0.9 \pm 0.02	0.1 \pm 0.06
06-01	79.9 \pm 6.8	19.1 \pm 6.2	0.9 \pm 0.6	0.2 \pm 0.1
07-01	71.5 \pm 1.5	27.2 \pm 0.3	1.2 \pm 0.3	0.1 \pm 0.02
08-01	87.3 \pm 3.0	12.0 \pm 2.7	0.5 \pm 0.2	0.2 \pm 0.2
14-01	78.4 \pm 0.7	20.6 \pm 0.9	0.9 \pm 0.2	0.3 \pm 0.05
17-01	84.5 \pm 0.07	14.7 \pm 0.2	0.6 \pm 0.1	0.2 \pm 0.04

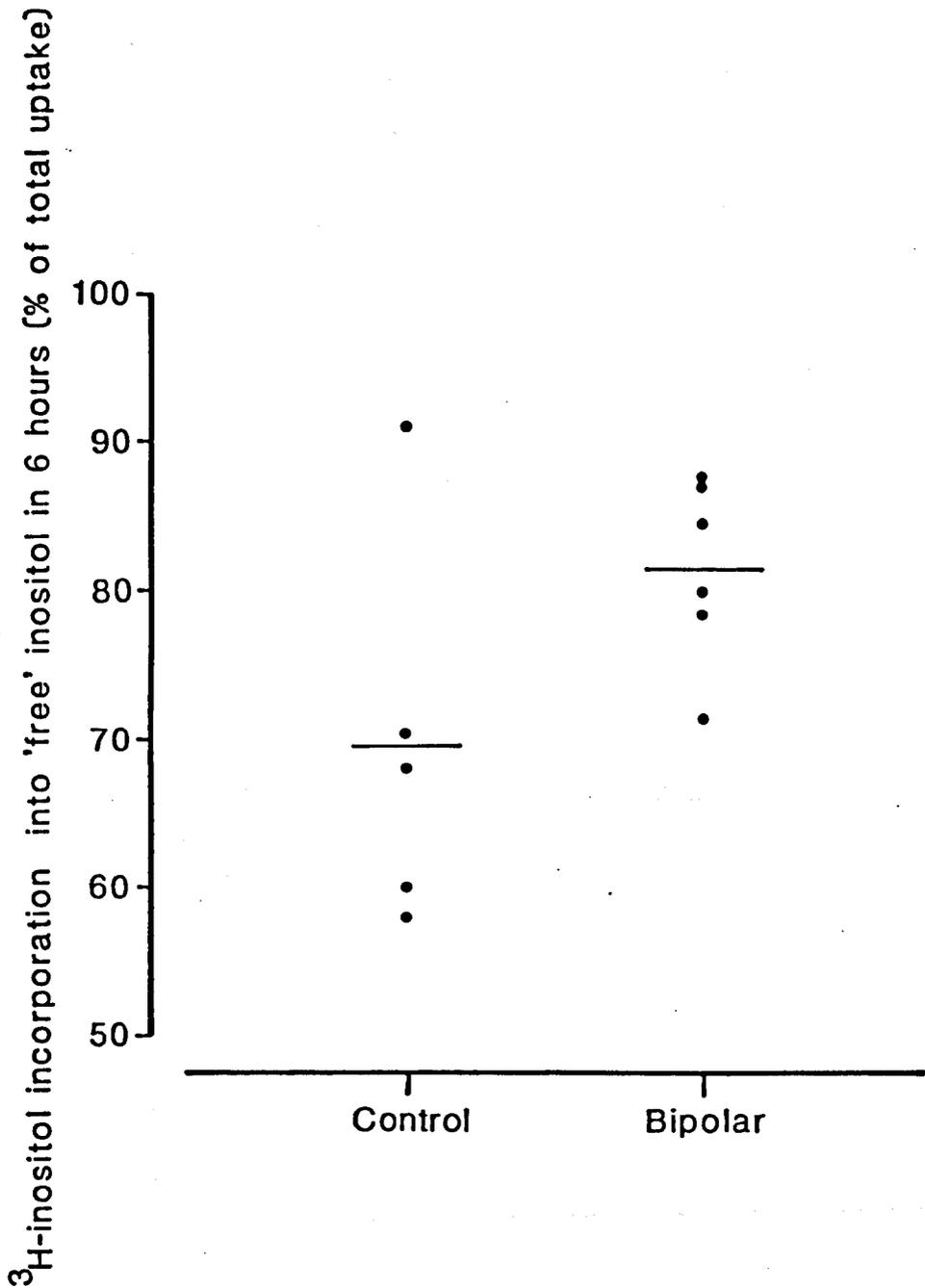


Figure 7.11. Distribution of the values for the percentage of ^3H -inositol uptake incorporated into the "free" inositol pool of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

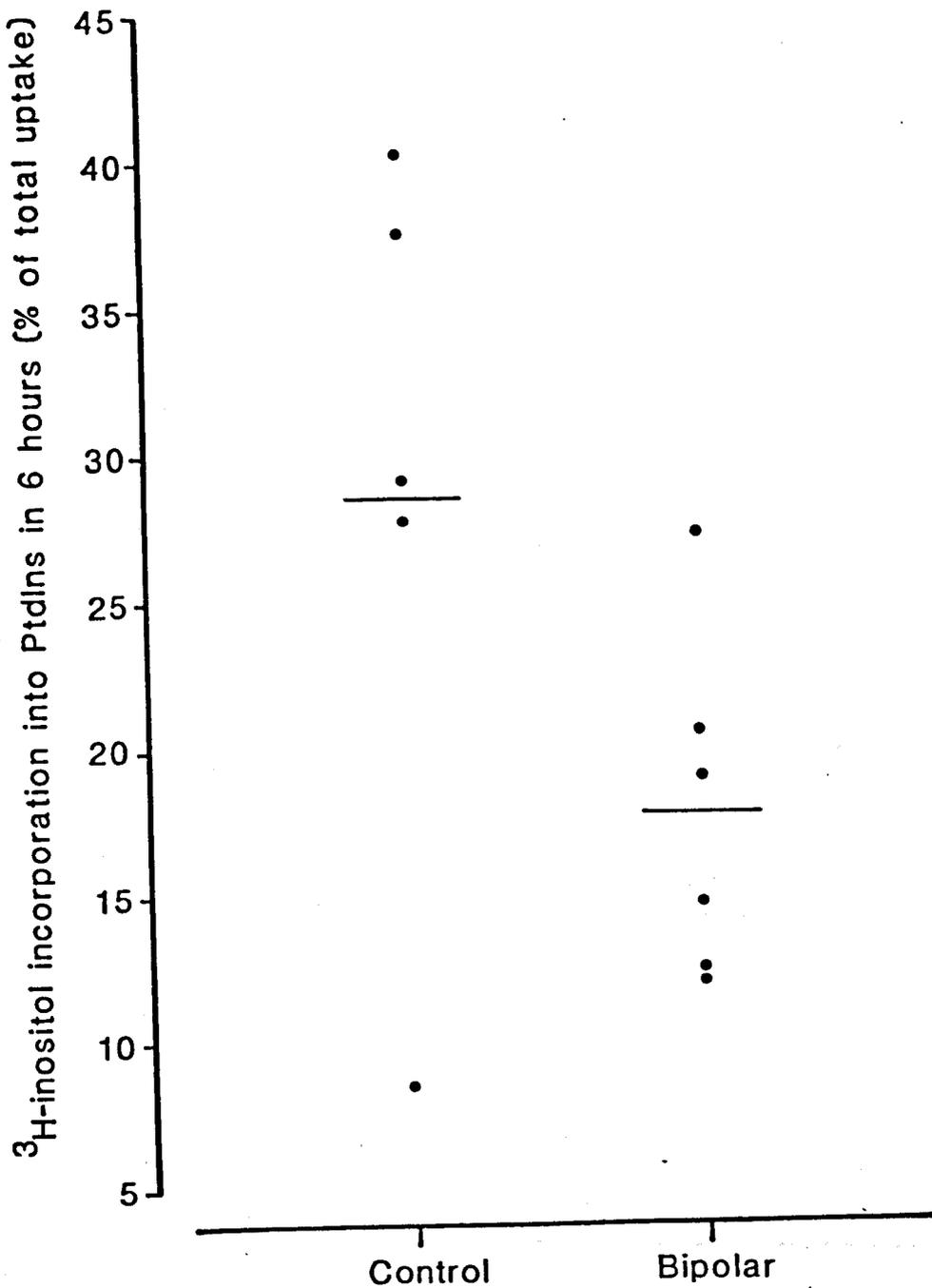


Figure 7.12. Distribution of the values for the percentage of ^3H -inositol uptake incorporated into PtdIns of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

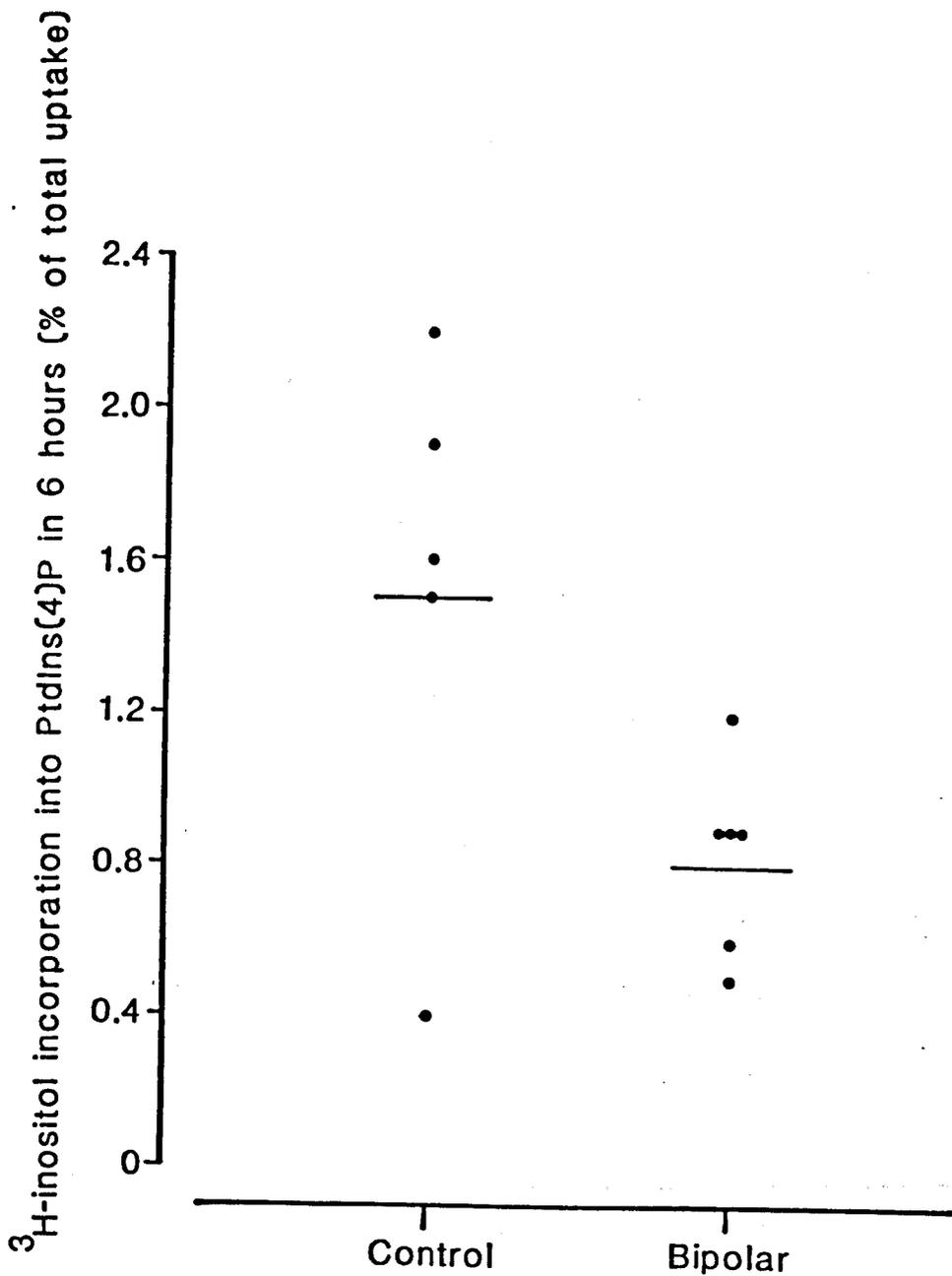


Figure 7.13. Distribution of the values for the percentage of ³H-inositol uptake incorporated into PtdIns(4)P of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

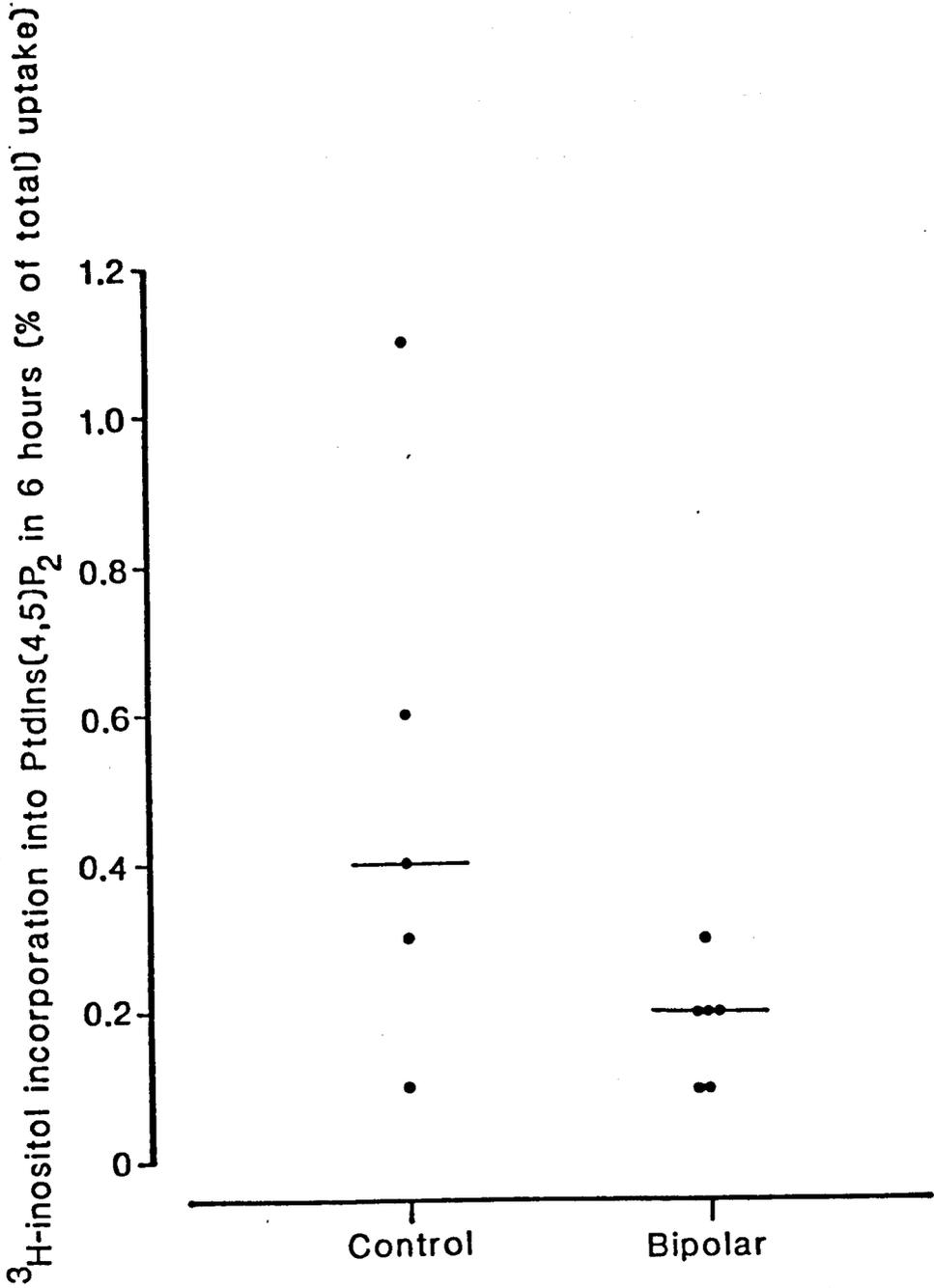


Figure 7.14. Distribution of the values for the percentage of ^3H -inositol uptake incorporated into PtdIns(4,5)P₂ of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

Points represent the mean of triplicate data from 2 experiments (8 cell lines) or 1 experiment (3 cell lines). — indicates the mean value for each group.

J11. Tony was not included in this analysis due to its anomalously high total uptake rendering its value as a control dubious, but exhibits a similar but even more extreme low incorporation of the radiolabel into the phosphoinositide pools.

7.IV. DISCUSSION

Inositol has been shown to accumulate in a variety of mammalian tissues including brain, spleen, kidney, and small intestine, reaching intracellular concentrations in some cases, of greater than 100x that of the extracellular fluid (Hauser 1969a). Normal mammalian plasma levels of "free" inositol have been reported to be approximately 0.1mM (Spector and Lorenzo 1975, Spector 1976). In rabbit brain, CSF levels of "free" inositol have been found to be in the range 0.2 - 0.6mM, with accumulation occurring by a saturable active transport process thought to be located mainly at the blood-CSF barrier i.e. the choroid plexus, with little if any exchange occurring across the blood-brain barrier (Spector and Lorenzo 1975, Spector 1976). From the CSF, inositol appears to diffuse readily into the extracellular space and enters the brain by a similar saturable transport process (Spector and Lorenzo 1975). The turnover of inositol in the brain (0.3%/hr) and the CSF (4%/hr) is much slower than that in the choroid plexus (16%/hr). The transport system responsible for inositol accumulation is half maximally activated at an inositol concentration of 0.1mM i.e. the normal plasma levels (Spector and Lorenzo 1975). Approximately 50% of the "free" inositol in the brain is thought to occur as a result of uptake with the remainder being synthesised from glucose (Spector 1976).

Analogous transport systems have been demonstrated in, for example, rat kidney (Hauser 1969a,b), hamster small intestine (Caspary and Crane 1970) and cultured retinal capillary pericytes

(Li et al. 1986). These transport systems have all been shown to be saturable, Na^+ -dependent, ATP-dependent, ouabain-sensitive, and phlorizin-sensitive. It has been suggested that the transport of inositol is similar to that of sugars i.e. a facilitated diffusion with Na^+ with the energy-dependency being due to the sodium pump activity necessary to maintain the inwardly-directed Na^+ gradient. It is not the same transport system as that for sugars however, with metabolisable sugars such as glucose showing a non-competitive inhibition of inositol uptake and inositol having no effect on the sugar transport (Caspary and Crane 1970, Li et al. 1986).

In the cultured retinal capillary pericytes, two transport systems were found; one similar to that described above and a minor pathway which was Na^+ -independent and non-saturable (Li et al. 1986). The uptake of inositol with time by the pericytes appears very similar in appearance to that found in the majority of lymphoblastoid cell lines in this study, although the time courses are different with initial linearity over 30 minutes for the pericytes and approximately 2-3 hours in the lymphocytes, before the rate of uptake appears to decrease. The extracellular concentrations of inositol were different however i.e. 5 μM for the pericytes versus 1 μM in this study, and the measurements were taken at greater intervals making the assessment of linearity more difficult. Whether two such separate transport systems for inositol are present in lymphocytes can not be confirmed unless the sodium-dependency is examined.

The total uptake is complex, presumably comprising an initial

period where equilibration of the "free" inositol pool with the extracellular solution occurs, superimposed on equilibrations of the various intracellular inositol-containing pools. Obviously with the ^3H -inositol uptake values reached, most of which is "free" inositol, accumulation is occurring. Assuming the "free" inositol is freely distributed in the cytoplasm, inositol accumulations of between 8 and 32x extracellular concentrations are seen over 6 hours for the cell lines with the exception of Tony which is approximately 100x. This anomaly is discussed further below. The actual amounts may be greater than this as some inositol may still have been present in the cells even after the inositol-free washes. In comparison the pericytes were shown to accumulate inositol intracellularly over 2 hours to a concentration approximately 18x that of the extracellular 5uM inositol (Li et al. 1986).

It is not known what effect the use of lower than normal concentrations of inositol in the incubation medium had on the cells in this study (10^{-6}M cf. $1.94 \times 10^{-4}\text{M}$). Viability was not affected over the duration of the experiment and it is therefore assumed that no detrimental effects resulted. As mentioned previously, such a reduction in concentrations was necessary in order to achieve a satisfactory labelling of the cells with the financial restrictions on the amount of ^3H -inositol used per incubation. Similar reductions in inositol concentration have been used in several other studies, for example, using rat cerebral cortical slices (Brown et al. 1984) without any apparent effects on the agonist-induced phosphoinositide hydrolysis.

Until all the radiolabelled inositol-containing pools are

allowed to equilibrate with one another, their specific activities will not be the same and hence the relative radioactive contents would not reflect the pool sizes. Changes in incorporation from one pool to another can thus be examined but not expressed in absolute terms of changes in mass of the inositol-containing pools if equilibrium has not been established. Many studies have been carried out concerning the PtdIns system using many different tissues from various species. Varying incubation protocols measuring either the incorporation of ^{32}P or ^3H -inositol have also been employed and generally the assumption appears to be that equilibrium between the various pools occurs fairly quickly i.e. within 30 - 60 minutes. With a system in equilibrium and the use of lithium to inhibit inositol-1-phosphatase activity, the resulting accumulation of IP in response to agonist-induced production of IP_3 can be used to monitor the production of IP_3 . However although equilibrium of the phosphoinositide pools is usually established and hence changes in radioactive content reflect changes in mass, the inositol phosphates may not be in equilibrium with these and hence changes in incorporation can only be examined qualitatively rather than quantitatively. If the system is not in equilibrium, movement of radioactivity from one pool to another may partly represent a change in mass of the various pools but may also be due to the equilibration process and if specific activities are not equal, the changes can not be related to each other.

In many studies equilibration has been assumed rather than demonstrated. The evidence for equilibration occurring include

the demonstrations that varying the concentration of ^3H -inositol produces a linear incorporation into lipid but a similar percentage accumulation of inositol phosphates, (rat cerebral cortex, 30 minutes; Brown et al. 1984), constant labelling of the inositol-containing pools occurs with further incubation time (rat hepatocytes, 70 minutes; Creba et al. 1983), (this may not apply to the "free" inositol pool which may carry on accumulating inositol from the extracellular medium although the specific activities have reached equilibrium), and turnover times of only a few minutes of the phosphoinositide pools and rapid interconversions between PtdIns and polyphosphoinositides (rat parotid gland and various tissues; Downes and Wusteman 1983). Assuming the lymphoblastoid cell lines behave similarly, the inositol-containing pools should have equilibrated within the 6 hour incubation period used in this study in which case the differences between the cell lines expressed in cpm can actually be interpreted in terms of relative pool size.

If at equilibrium, the lower incorporation of ^3H -inositol into the phosphoinositides of the bipolar cell lines compared with the controls may represent smaller pool sizes. Alternatively, if the system is not at equilibrium, the differences in incorporation of radioactivity may reflect differing levels of enzyme activity or reduced levels of the CDP-DAG needed to combine with the inositol to form PtdIns. In order to investigate which if any of these possibilities are likely, it would be necessary to establish whether the system is at equilibrium by looking at the incorporation of radiolabel into the various pools with time. Depending on the results it may be

necessary to attempt to isolate the enzymes responsible for the synthesis of the phosphoinositides and examine for any differences, and/or measure the levels of CDP-DAG. Less reliability can be placed on the PtdIns(4,5)P₂ results as these were very close to the background levels of radioactivity.

The fact that the ratios of the phosphoinositide pools relative to each other are similar in both the bipolar and control cells would point to similar enzyme activities in the two groups of cell lines with the difference being in pool size, i.e. the system is in equilibrium but further investigations are needed to confirm this, for example, determining the incorporation of ³H-inositol into the various phosphoinositide pools with time.

The reservations expressed in the previous chapter regarding age and sex differences in the control and bipolar groups apply equally to the results in this chapter. The cell lines in this part of the study consist of 5 male controls and 3 male and 3 female probands with mean ages \pm S.E.M. of 32 ± 6.3 and 46 ± 8.7 respectively. Although not age or sex-matched, the differences between the two groups are not likely to be due to this since the results for the mixed-sex bipolar group would then be expected to fall into 2 distinct groups rather than being tightly grouped. It is also notable that the variation seen in this study is much less than that found in the day to day measurements of the fluxes as shown in Chapter 6. Similarly if the differences were due to an artefact of transformation, it is very unlikely that such an effect would divide the cell lines into two groups depending on their clinical status.

The reason for the apparently anomalous behaviour of the cell line Tony is not clear. The control cell lines in this study were all established from non-psychiatrically ill patients and Tony was a myeloma patient. Whether this difference in inositol uptake is related to this clinical condition is a possibility and may be a very important finding if confirmed using cell lines from similar patients. It is also interesting in this context, that the cell line Gavi which was derived from a lymphoma patient, does not show this anomalous uptake and incorporation. One possibility is that it may be age-linked as the subject from whom the cell line Tony was derived was 75 years old at the time that the cell line was established. This is unlikely to be the explanation however, as the proband from whom 17-01 was established was a similar age, and 17-01 does not exhibit this anomalous behaviour. Alternatively it could be a transformation-induced artefact which occurred by chance. The effects of EBV-transformation on the PtdIns system are not known but it has been demonstrated for example in C3H10T1/2 cells that chemical transformation enhances the labelling of polyphosphoinositides and appears to increase the permeability of the membrane, at least to phosphate (Kubota et al. 1986). Whether or not such effects have occurred in the cell lines used in this study is not known and hence any possible differences which may exist in the PtdIns system in vivo between bipolars and controls may or may not be altered upon transformation. Presumably any transformation-induced differences would be unlikely to separate themselves into two groups depending on clinical status unless mirroring the situation in vivo as

mentioned above. If the differences observed here between bipolar and control cell lines reflect differences in vivo, it is still not certain that these differences represent the situation prevailing in the brain.

An alternative explanation for the anomalous behaviour of Tony regarding uptake and to a lesser extent J11 regarding percentage incorporation into the phosphoinositide pools, may be that this is just random variation within the population and that with the small samples used in this study, they appear anomalous. If more samples were used, the anomaly may disappear since more such apparently extreme examples may be seen. An extension to the work done in this study would be to repeat the measurements using more samples and also using the family members to see if any predictive value of these differences is apparent when the between-family heterogeneity within the population is removed. Other improvements may be to use more cells and thereby aid the detection of the phosphoinositides.

The failure to find agonist-stimulated inositol phosphate metabolism may be due to either inappropriate choice of agonists for the lymphocytes, transformation-induced alterations in the PtdIns system, too low labelling of the cells, and/or too few cells for adequate detection of the inositol phosphates. Further studies should involve the use of more cells, more agonists and if possible, increased labelling. The finding of differences between the control and bipolar cell lines may also be true for the inositol phosphates if investigated.

How the differences between bipolar and control cell lines account for the nature of the illness and the therapeutic effects

of lithium is not clear until, for example, the extent of isotope equilibration is unambiguously determined. If the difference is in phosphoinositide pool size, it is possible that a smaller signal will be generated for each agonist event, depending on the efficiencies of the enzymes responsible for the resynthesis of the phosphoinositides, and/or that repeated stimulation may lead more quickly to a depletion of phosphoinositide pools in bipolar subjects. The same scenario may result if the differences are due to abnormalities in the synthesis enzymes. If it is the case that the illness is primarily due to a lower capacity for 2nd messenger production, it is difficult to see how the known actions of lithium in inhibiting IP phosphatase would produce a therapeutic effect unless the IP accumulated exerts a feedback inhibition on the enzyme responsible for dephosphorylating IP₃, thus prolonging the IP₃ signal and compensating for the lower signal generation. However this would be eventually expected to result in inositol depletion and a subsequent further decrease in 2nd messenger production.

In contrast to the original suggestion of Berridge et al. (1982) that lithium may cause a depletion of cellular inositol levels and hence the phosphoinositides, preferentially in hyperactive pathways which may be responsible for the illness, levels of PtdIns(4,5)P₂ and presumably IP₃ and DAG have been shown to be maintained even in conditions of inositol depletion in rat parotid acinar cells (Downes and Stone 1986). This is presumably due to some homeostatic mechanism. However if the levels or synthesis of phosphoinositides were also initially decreased as shown in the bipolar cell lines, this together with

lithium may overcome any homeostatic mechanisms and eventually result in a reduced signalling capability. This hypothesis therefore assumes that not only is there a defect in levels or rate of synthesis of phosphoinositides in the illness but that hyperactive pathways exist which are primarily responsible for the illness. Although reduced phosphoinositide levels are present these would not normally contribute to the illness and would be maintained even in the hyperactive pathways, due to a homeostatic mechanism. However in the presence of lithium these would be depleted, unlike the controls where the initial levels are higher, and would account for the therapeutic effect of lithium in correcting the hyperactive pathways.

An extension of this would be to conclude that the decreased phosphoinositide levels or rates of synthesis have occurred as part of a "down-regulation" or "desensitisation" process in response to neuronal hyperactivity. Alternatively down-regulation in response to a primary defect in the activity of neuronal pathways may be present, and an accumulation of IP caused by lithium treatment may compete with IP_3 for the receptor which mediates the Ca^{2+} release. Spat et al. (1986) have demonstrated no effect of 10uM IP on IP_3 binding but it is possible that higher IP concentrations may be produced with chronic lithium treatment and may produce some effect.

The possibility of an involvement of Na^+/K^+ -ATPase in the illness is also not ruled out and may occur secondarily to alterations in the PtdIns system as PtdIns is known to be a specific requirement in the normal activation of the sodium pump (Roelofsen and Van Linde-Sibenius Trip 1981). For example the

sodium pump number and/or activity has been shown to be decreased in inositol-depleted KB cells (Charalampous 1971). This would bring in the possibility that bipolars have an increased pump number or activity and that lithium acts by decreasing the inositol and hence PtdIns levels and corrects the defect. In this study an increased pump number per cell was found in the bipolar cell lines compared with controls but the activity was not ascertained.

In summary therefore, it would appear that there is no difference between bipolar and control subjects regarding inositol uptake. There is however a significantly reduced percentage incorporation of the inositol into the phosphoinositides of the bipolar cell lines, although whether this reflects differences in pool sizes or enzyme activity has yet to be determined. Further studies would involve a determination of isotopic equilibration. Possible hypotheses have been discussed which attempt to integrate the findings of this study with the therapeutic action of lithium. The results need to be validated using age and sex-matched controls and repeated using larger sample sizes, together with family members. Whether there are differences between lithium responders and non-responders would also be of interest. The explanation for the phasic nature of the illness if the PtdIns system is involved in the aetiology of the illness is not apparent.

CHAPTER 8. CONCLUDING REMARKS

From the general review of the symptoms, classification, occurrence and possible aetiologies of bipolar manic-depressive psychosis presented in Chapter 1, it is clear that the illness is complex and the underlying pathology poorly understood. Although the weight of evidence strongly implicates a genetic aetiology, the mode of inheritance is far from clear. This is also true for the corresponding biological defect(s) which is(are) responsible for the resulting manifestation of the illness.

The main areas in which the defect has been postulated to occur are in neurotransmission, the endocrine system, and in cation transport. Naylor has proposed that manic-depressive patients possess a genetic defect in the sodium pump (Na^+/K^+ -ATPase), which results in an increased susceptibility to an endogenous "regulating factor". This factor has been tentatively identified as vanadium, following the finding of increased plasma/serum vanadium levels in affected individuals, and the demonstration of the potent inhibition of isolated Na^+/K^+ -ATPase enzyme preparations by vanadate (V^{5+}). This thesis has addressed this possibility by firstly determining the effects of vanadate on cation transport in whole cells, using the well-characterised HeLa cell line. Following this, cation transport in virally-transformed lymphoblastoid cell lines from 13 bipolar manic-depressive patients and 13 control subjects has been examined, under normal conditions and after treatment for 24 hours with lithium, ouabain or vanadate. In addition, the possibility that the defect may lie in the phosphatidylinositol system which is involved in 2nd messenger production, has also

been investigated. The findings of the various parts of this study and their interpretation in conjunction with each other, are summarised below.

In HeLa cells, vanadate only produced significant alterations in the K^+ influx pathways at extracellular levels of vanadate where toxic effects were also apparent. The lack of effect of vanadate at concentrations less than $10^{-5}M$ was most likely the result of the intracellular reduction of vanadate to the less biologically active, vanadyl (V^{4+}), as shown using ESR. It is also likely that the changes in cation flux, which were generalised and not specific to any one transport pathway, occurred secondarily to the toxicity. Whether the toxicity is due to vanadyl, either directly or indirectly, or is due to, for example, a depletion of GSH as a result of the intracellular reduction, is not known. No change in energy charge was seen with vanadate treatment although ATP levels were significantly decreased by 40% at $10^{-4}M$ vanadate. This change also seemed to occur as a consequence of the toxicity. From these results, it would appear that vanadate, at the concentrations normally present in the blood, would have no effect on cation transport in whole cells, due to its reduction to vanadyl. At increased levels, toxicity prevails. This would therefore indicate that if vanadate is responsible for the perturbations in cation transport described in manic-depressive psychosis, affected individuals must have some defect which makes them more susceptible to vanadate. Possible examples of such a defect could be a decreased reducing ability or an increased

susceptibility of the sodium pump to the inhibitory actions of either vanadate or vanadyl.

With the lymphoblastoid cell lines, the only difference seen between the bipolar and control cell lines regarding cation transport was in the number of sodium pumps per cell. The mean value for the bipolar group of cell lines is approximately 30% greater than that for the control group. The ouabain-sensitive K^+ influx was similar in both groups although there was considerable variation between cell lines with respect to flux values. Treatment with lithium ($10^{-3}M$) or vanadate ($10^{-6}M$) produced either no change or inconsistent actions on sodium pump site number or cation flux. Ouabain ($10^{-8}M$) produced a decrease in sodium pump numbers in all the cell lines. The decrease was to 54.3% of basal values in the control cell lines, and to 59.5% in the bipolar group, a non-significant difference. Only half of the cell lines in both groups showed a concomitant decrease in the ouabain-sensitive flux per cell.

The conclusion to be drawn from these results would seem to be that there is no defect in the sodium pumps of manic-depressive patients which would render them more susceptible to the actions of vanadate, even when present at levels much greater than those normally present in plasma. In addition treatment with ouabain has also revealed no differences in response, and lithium treatment at therapeutic levels did not alter the sodium pump activity in either group of cells. It is possible that the kinetics of activation of the sodium pump may be different between the two groups but this was impossible to determine without measurement of intracellular ions. Another possibility

is that a longer time period for treatment is needed before a defect becomes apparent. For example there may be differences in the up-regulation of sodium pumps in response to chronic, partial inhibition. It is unlikely that the basal difference in pump number between the two groups is responsible for the illness since the cation fluxes were similar. These differences may be important if occurring in conjunction with some other defect, as yet not elucidated.

When the phosphatidylinositol system was examined, the uptake of inositol appeared to be similar in both groups, with the exception of one control cell line (Tony). However the percentage incorporation into the phosphoinositide pools was significantly lower in bipolar individuals than in controls. Whether this reflects differences in pool size or differences in enzyme activity can not be defined without further studies to determine the degree of isotopic equilibration between the various inositol-containing pools. The possible implications of these findings have been discussed in depth in the preceding chapter.

The finding for one of the control cell lines (Tony) of a much greater inositol uptake, together with a proportionately much smaller incorporation of inositol into the phosphoinositides, is interesting. The reasons for this difference are not clear but may have possible implications in another field as the individual from whom this cell line was established was a myeloma patient. This warrants further investigation if other such cell lines can be obtained.

The use of lymphoblastoid cell lines to study genetically-determined illnesses has been discussed previously. Potentially, they are also of use in the study of manic-depressive psychosis and have revealed differences between the control and affected subjects. However, the possibility that other differences exist in vivo but have been masked as a result of the lymphocyte transformation process, can not be ruled out. In addition, the results have to be interpreted with caution in view of the lack of age and sex-matched controls. Such cell lines have, however, enabled the investigation of various cation transport parameters and the phosphatidylinositol system, in a model-system free from any effects of medication. The differences revealed between the control and bipolar cell lines warrant further investigation, to include in particular, the use of cell lines from unaffected and affected relatives. This could help to determine the predictive value and possible role of these differences in the aetiology of bipolar manic-depressive psychosis.

APPENDICES

APPENDIX 1

Calculation of intracellular ion contents of HeLa cells (see Chapter 3.II.)

Normally the flame photometer was set to a 50% of scale reading with a standard containing 100 μM Na^+ and K^+ . If the sample reading was A,

$$\text{Ion concentration} = \frac{(\text{vol. water added} + \text{pl. water}) \times 2(\text{A} - \text{blank})}{\text{pl. water} \times 1000}$$

where: ion concentration is in mmol/lcw

vol.water added = 5000 μl

pl. water = the mean plate water for that treatment condition (μl)

A = the sample reading in units

blank = the mean reading from the blank plates

2 = the conversion factor from units on the scale to $\mu\text{mol/l}$ (100 μM standard was set to 50% of scale)

1000 = the factor needed to convert $\mu\text{mol/l}$ to mmol/l

A further dilution factor could be introduced into the calculation if the sample needed diluting before being measured. The calculation was repeated for each ion and each plate in turn, using the appropriate plate water in each case as ascertained from the plates run in parallel.

APPENDIX 2

Calculation of ouabain binding values of HeLa cells (see Chapter 3.II.)

$$\text{Binding} = \frac{A \times \text{ratio} \times \text{Avogadro's no.} \times [\text{ouabain}] \times \text{std. vol.}}{\text{cell no.} \times B}$$

where: binding is in molecules/cell.

A = counts per minute (cpm) per plate sample minus the mean plate blank (cpm)

$$\text{ratio} = \frac{\text{total soln. volume on plate}}{\text{volume taken for radioactive counting}} = 3$$

Avogadro's no. = 6.02×10^{23} (molecules/mole)

[ouabain] = 2.12×10^{-7} M (200 nM is cold ouabain and 12 nM is ^3H -ouabain)

std. vol. = the volume of standard taken for radioactive counting (litres) i.e. 10^{-4}

cell no. = the number of cells per plate

B = mean std. counts (cpm) minus the mean machine blank (cpm)

This was carried out for each of the plates in turn using the appropriate standard counts depending upon whether incubated in 15K or K-free Krebs, and the appropriate cell numbers. Within each treatment group, the mean of the 15K (non-specific) binding results was subtracted from each of the correspondingly treated K-free plates yielding the specific ouabain binding figures which were then meaned within each treatment group.

APPENDIX 3

Calculation of the K^+ influx in HeLa cells (see Chapter 3.II.)

$$K^+ \text{ influx} = \frac{A \times \text{std. vol.} \times \text{ratio} \times [K^+]_0}{\text{flux time} \times B \times \text{plate cell water}}$$

where: influx is in mmol K^+ /lcw/min. (lcw is litres of cell water)

A = cpm per plate sample minus the mean plate blank (cpm)

std. vol. = the volume of standard taken for counting (litres) i.e. 10^{-4}

ratio = $\frac{\text{total soln. volume on plate}}{\text{volume taken for radioactive counting}} = 3$

$[K^+]_0$ = potassium concentration of the Krebs or NO_3^- -
Krebs incubation medium i.e. 5.7mM, checked
by flame photometry. The contribution from the
 ^{86}Rb was negligible.

Flux time = 4.5 (minutes)

B = mean std counts (cpm) minus the mean machine blank (cpm)

plate cell water = the total volume of the cell water for the plate in question, in litres.

This was repeated for each plate in turn using the appropriate standards. The above influx could be converted to nmol K^+ /10⁶ cells/min by multiplying by 0.8 (converts per litre of cell water to per litre of cells assuming that 80% of the cell volume is water), multiplying by the mean cell volume (u^3) and multiplying by 10^{-3} . This latter factor is a composite comprised of a factor of 10^{-15} to convert from per litre to per u^3 , a factor of 10^6 to convert mmoles to nmoles, and a factor of 10^6 to convert from per cell to per 10^6 cells.

Results from triplicate plates were then meaned and by the appropriate subtraction, the magnitudes of the different flux pathways were seen.

APPENDIX 4

Calculation of ouabain binding values of lymphocyte cell lines

(see Chapter 6.II.)

$$\text{Binding} = \frac{A \times \text{ratio} \times \text{Avogadro's no.} \times [\text{ouabain}] \times \text{std. vol.}}{\text{cell no.} \times B}$$

where: binding is in molecules/cell

A = counts per minute (cpm) per 400 ul cell sample minus the mean cell blank

$$\text{ratio} = \frac{\text{total soln. vol in the tube}}{\text{volume taken for radioactive counting}} = 1.875$$

Avogadro's no. = 6.02×10^{23} (molecules/mole)

[ouabain] = 2.25×10^{-7} M (200 nM is cold ouabain and 25 nM is ^3H -ouabain)

std. vol. = the volume of the 1 in 10 dilution of 10x standard taken for radioactive counting (litres) i.e. 10^{-4}

cell no. = the number of cells per tube

B = mean std. counts (cpm) minus the mean machine blank (cpm)

This was carried out for each of the tubes in turn using the appropriate standard counts depending upon whether incubated in 15K or K-free Krebs, and the appropriate cell numbers. Within each treatment group, the mean of the 15K (non-specific) binding results was subtracted from each of the correspondingly treated K-free plates yielding the specific ouabain binding figures which were then meaned within each treatment group.

APPENDIX 5

Calculation of the K^+ influx of lymphocyte cell lines (see Chapter 6.II.)

$$K^+ \text{ influx} = \frac{A \times \text{std. vol.} \times \text{ratio} \times [K^+]_0}{\text{flux time} \times B \times \text{tube cell water}}$$

where: influx is in mmol K^+ /lcw/min. (lcw is litres of cell water)

A = cpm per 100 ul cell sample minus the mean cell blank for that condition

std. vol. = the volume of the 1 in 10 dilution of 10x standard taken for radioactive counting (litres) i.e. 10^{-4}

$$\text{ratio} = \frac{\text{total soln. volume in the tube}}{\text{volume taken for radioactive counting}} = 2$$

$[K^+]_0$ = potassium concentration of the Krebs or NO_3^- - Krebs incubation medium i.e. 5.7mM, checked by flame photometry. The contribution from the ^{86}Rb was negligible.

Flux time = 12 (minutes)

B = mean std. counts (cpm) minus the mean machine blank (cpm)

tube cell water = the total volume of the cell water for the tube in question (litres)

This was repeated for each tube in turn using the appropriate standards and blanks. The above influx could be converted to nmol K^+ /10⁶ cells/min by multiplying by 0.8 (converts per litre of cell water to per litre of cells assuming that 80% of the cell volume is water), multiplying by the mean cell volume (u^3) and multiplying by 10^{-3} . This latter factor is a composite comprised of a factor of 10^{-15} to convert from per litre to per u^3 , a factor of 10^6 to convert mmoles to nmoles, and a factor of 10^6 to convert from per cell to per 10^6 cells.

Results from triplicate tubes were then meaned and by the appropriate subtractions, the magnitudes of the different flux pathways were ascertained.

APPENDIX 6

Calculation of the total inositol uptake in 6 hours of lymphoblastoid cell lines from bipolar manic-depressive patients and control subjects

$$\text{Uptake} = \frac{A \times 10 \times [\text{inositol}] \times \text{std. vol.} \times 10^6}{\text{Cell water} \times B}$$

where: Uptake is in umoles/lcw/6 hours

A = cpm per 100 ul microfuge sample minus the mean blank

B = standard counts minus the mean machine blank

10 = conversion factor to correct the sample counts to per ml of suspension

[inositol] = 10^{-6} (M)

std. vol. = 10^{-4} (litres)

10^6 = conversion factor to convert moles to umoles

Cell water = the cell water (litres) per ml of cell suspension

The above procedure was carried out for each sample in turn using the appropriate values for cell water depending on the sample. The results could be converted to pmoles/ 10^6 cells/6 hours by multiplying by the cell water (litres), dividing by the cell number per ml of suspension, multiplying by 10^6 to convert umoles to pmoles, and multiplying by 10^6 to convert to per 10^6 cells.

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