THE EFFECTS OF NITROGEN MUSTARD ON PLASMA MEMBRANE FUNCTION

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SUMMARY

The antitumour bifunctional alkylating agent nitrogen mustard (HN2) inhibited the unidirectional influx of the potassium congener, ^{86}rubidium, into murine PC6A plasmacytoma cells and L1210 leukaemia cells. The proliferation of L1210 cells *in-vitro* was characterised and shown to be sensitive to HN2.

^{86}Rubidium influx into cells from rapidly-dividing cultures was more sensitive to inhibition by HN2 than that of cells from stationary cultures. Three components of unidirectional $^{86}Rb^+$ & K^+ influx into proliferating L1210 cells were identified pharmacologically: approximately 40% was sensitive to the Na^+K^+ATPase inhibitor ouabain (10^{-3}M), 40% was sensitive to the 'loop' diuretics bumetanide (10^{-4}M) and furosemide (10^{-3}M) and the remainder was insensitive to both ouabain and furosemide. HN2 (10^{-5}M) selectively inhibited the diuretic-sensitive component, which was entirely dependent upon extracellular Na^+ and Cl^- ions, and was presumed to represent $Na^+K^+Cl^-$cotransport activity. The system did not mediate K^+/K^+ exchange or unidirectional $^{86}Rb^+$ efflux; accordingly, $^{86}Rb^+$ efflux was insensitive to HN2. Inhibition of $^{86}Rb^+$ & K^+ influx by 10^{-5}M HN2 was accompanied by approximately 35% reduction of cell volume under isosmotic conditions; thus intracellular Na^+ and K^+ concentrations remained unchanged.

These effects followed lethal damage to the cells but preceeded actual cell death; other cellular functions were maintained including accumulation of cycloleucine, transmembrane potential, permeability to trypan blue, intracellular pH, total intracellular glutathione and calcium concentrations. No evidence was found that elevated cAMP levels or reduced ATP levels were involved in modulation of $^{86}Rb^+$ & K^+ influx. However, the Na^+- dependent transport of an amino acid was inhibited in a manner which appeared to be independent of $^{86}Rb^+$ & K^+ influx.

An HN2-resistant L1210R cell line was also resistant to furosemide, and lacked a component of $^{86}Rb^+$ & K^+ influx which was sensitive to furosemide (10^{-3}M).

The results strongly suggest that the $Na^+K^+Cl^-$ cotransporter of L1210 cells is a cellular target for HN2. This lesion is discussed with reference to the cytotoxic effects of the agent.

Key words: nitrogen mustard; tumour cell membranes; cell membrane transport; $Na^+K^+Cl^-$ cotransport; cell volume regulation.

To my Father,
with love.

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ABBREVIATIONS AND SYMBOLS

ATP	adenosine 5'triphosphate
∝AIB	∝-aminoisobutyric acid
BA	benzoic acid
cAMP	adenosine 3'5'-cyclic monophosphate
$[Ca^{++}]_i$	intracellular calcium concentration
$[Ca^{++}]_o$	extracellular calcium concentration.
CDDP	cis-diaminodichloroplatinum (II); cisplatin
Ci	curies
cpm	counts per minute (corrected for background radioactivity)
cycloleucine	1-aminocyclopentane-1-carboxylic acid
dbcAMP	N^6, 2'-O-dibutyryladenosine 3'5'-cyclic monophosphate
DIDS	4,4'di-isothiocyanatostilbene-2, 2'-disulphonic acid
DMO	dimethyloxazolidine-2, 4-dione
DMSO	dimethylsulphoxide
DTNB	5,5-dithiobis-2-(2-nitrobenzoic acid)
EBSS	Earles buffered saline solution
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor.
EGTA	ethyleneglycol-bis-(β-aminoethylether) N,N, tetraacetic acid.
FCS	foetal calf serum
g	acceleration due to gravity
GSH	reduced glutathione
GSSG	oxidised glutathione
h	hours
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid

HN2	nitrogen mustard; mustine hydrochloride; mechlorethamine; bis-(2-chloroethyl)methylamine
HN2-1	monofunctional analogue of nitrogen mustard; 2-chloroethyldimethylamine; β-dimethylaminoethylchloride hydrochloride.
HS	horse serum
IC_{50}	concentration of drug which inhibits 50% of cell growth *in vitro*
ip	intraperitoneal
$[K^+]_i$	intracellular potassium concentration
$[K^+]_o$	extracellular potassium concentration
KR	Krebs-Ringer bicarbonate buffer, pH7.4
log	logarithm
min	minute
n	number of experiments
$[Na^+]_i$	intracellular sodium concentration
$[Na^+]_o$	extracellular sodium concentration
NADH	reduced nicotine adenine dinucleotide
NADPH	reduced nicotine adenine dinuceotide phosphate
NEM	N-ethyl maleimide
p	probability
pg	page
PC6A	Adj-PC6A murine plasmacytoma ascites cells
pcmbs	*p*-chloromercuribenzene sulphonate
psi	pounds per square inch
rpm	revolutions per minute
RPMI	RPMI 1640 medium
RVD	regulatory volume decrease
RVI	regulatory volume increase

s	seconds
S.D.	standard deviation
t	time
TDDP	trans-diaminodichloroplatinum (II)
TNB	5-thio2-nitrobenzoate
TMA^+	tetramethylammonium ion
$TPMP^+$	triphenylmethylphosphonium ion
$\bar{X}$	mean of a set of samples

All other abbreviations refer to SI units.

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INTRODUCTION

1.1 THE ANTITUMOUR ACTIVITY OF NITROGEN MUSTARD

1.1.1 The problem of cancer chemotherapy

The requirement for major advances in antitumour chemotherapy is evident from the statistics gathered annually by the Office of Population Statistics*; there are approximately 200,000 new cases of, and approximately 140,000 deaths each year from, malignant disease in England and Wales alone. The metastatic nature of malignant tumours often necessitates a systemic chemotherapy to supplement the local measures of surgery and radiotherapy in order to control the disease.

In the search for suitable agents many thousands of compounds have been synthesized and screened for antitumour activity, but only a very small number of them have been of clinical value, so emphasising the need for a rational, rather than random, approach to their design (Ross, 1974; Heidelberger, 1969). The rational design of truly selective antitumour agents must depend upon the identification of susceptible biochemical targets within tumour cells; further, such targets should represent metabolic abnormalities of the transformed phenotype. The majority of classical anticancer compounds in fact possess non-specific antiproliferative, rather than selective antitumoural, activity, because they are directed towards the inhibition of DNA synthesis; yet, despite extensive study, their pharmacological effects have not been defined at the molecular level (Hickman, 1982; Tritton and Hickman, 1984; Connors, 1980).

Thus it is possible to identify two areas of investigation which are likely to yield improvement in the design of novel chemotherapeutic agents. Firstly, the biochemical differences

*Crown Office
Office of Population Statistics: Mortality statistics in 1984 in England and Wales: (Malignant neoplams: 138,326 deaths):
and Cancer Statistics registrations: Cases of diagnosed cancer in England and Wales in 1982: (New cases registered: 197,125)

between normal cells and their transformed counterparts have been the subject of numerous studies. In particular, the elucidation of the functions of oncogene products clearly will have potential for pharmacological intervention. Secondly, the dissection of the molecular events involved in the cytotoxic mechanism of existing antitumour drugs will aid the identification of the susceptible loci within tumour cells. This thesis documents a study toward this end.

HN2 in physiological buffers is rapidly converted to the aziridinium ion, which readily reacts with any available nucleophilic centre (see 1.1.4.1 below). In biological systems, the target is most likely to be N, O, or S atoms which may be found in numerous cellular components. Thus, whilst the early work on nitrogen mustard and other alkylating agents suggested that DNA was the site of action of these agents (see 1.1.4.2 below), their mechanism of cytotoxicity is likely to be multimodal (Hickman, 1982: Tritton and Hickman, 1984). A number of studies have demonstrated that anticancer drugs have deleterious effects upon the tumour cell membrane (Tritton and Hickman, 1984), a key point of control of cell multiplication. The present study examines the possibility that the cytotoxicity of nitrogen mustard may involve perturbations of tumour cell membrane function.

1.1.2 The history of nitrogen mustard

It is one hundred years since the cytotoxic effects of alkylating agents were first reported (Meyer, 1887; Ehrlich, 1898). The observation that derivatives of mustard gas were particularly toxic toward lymphoid tissue, producing severe leukopenia, led to the clinical trial of nitrogen mustard (HN2) in the treatment of lymphatic leukaemia (Gilman and Philips, 1946), and thus began the chemotherapy of cancer. Clinical trials of alkylating agents in the

treatment of other neoplasms rapidly followed (Spurr et al, 1947; Goodman, et al 1947; Karnofsky et al, 1947), and several thousand derivatives were prepared and tested (Connors, 1980 ; Ross, 1974; Ochoa and Hirschberg, 1967); several such compounds have been employed in the treatment of a range of human cancers (Zubrod, 1974).

However, the cytotoxicity of these agents against a broad spectrum of tumours (Zubrod, 1974; Ross, 1974) is accompanied by a similar activity against a wide range of rapidly-proliferating normal tissue (Ross, 1974; Connors, 1980); the view has emerged that in order to reduce the effects on normal tissue, agents must be sought which are toxic to some neoplasm in a unique manner (Schmidt et al, 1965; Zubrod, 1974; Ross, 1974). In addition, recognition of the carcinogenic properties of the alkylating agents (e.g., Walpole, 1958) has reduced their clinical use in more recent years (Connors, 1980).

1.1.3 The metabolism of nitrogen mustard

The binding of HN2 to tumour cells is dependent on temperature, and HN2 is rapidly accumulated in cells, mostly in a non-diffusible form (Kessel et al, 1969; Wolpert and Ruddon, 1969). Uptake of HN2 into neoplastic cells in vitro is mediated by an active transport mechanism (Goldenberg et al, 1970; 1971; Wolpert and Ruddon, 1969), with the intact plasma membrane providing a barrier to uptake (Yamada et al, 1963). Goldenberg et al (1971) demonstrated, by mutual inhibition experiments, that both HN2 and its hydrolysis product (2-bis(hydroxyethyl)methylamine - see figure 2), which is not an alkylating agent, employ the membrane transport system for choline.

Choline bears some structural homology to these species. The transport of choline into different tissues has been shown to depend on components of diffusion and active transport, the latter being

Na^+-dependent in some cases (Hakala, 1974). There is no competition for transport between HN2 or choline and other structural analogues of HN2 such as chlorambucil, cyclophosphamide or L-phenylalanine mustard, demonstrating chemical specificity for the transport system (Goldenberg et al, 1970). Although all the compounds are substituted bis-(2-chloroethyl)amines, they lack a methyl group on the nitrogen atom.

^{14}C-labelled HN2 has been used to discover the metabolic fate of HN2. Nadkarni et al (1959) showed that after injection of [^{14}C-methyl]-HN2, radioactivity was rapidly cleared from the blood of mice. A fraction of [^{14}C-methyl]-HN2 was oxidised to CO_2 and exhaled (Skipper et al, 1951). The methyl group may be liberated and oxidised to formaldelyde by microsomal enzymes (Trams and Nadkarni, 1956), or detached and incorporated into cellular consitituents (Mandel, 1959). Nitrogen mustard may be demethylated to form nor-nitrogen mustard in the liver (Smith et al, 1958), by a microsomal enzyme (Trams and Nadkarni, 1956). The enzyme-catalysed reaction between nor-nitrogen mustard and CO_2 in the blood may form substituted oxazolidinones, which are not alkylating agents (Williamson et al, 1966). A number of ^{14}C-labelled compounds have been found in urine but have not been identified (Guarino and Litterst, 1974). Metabolism of the β-chloroethyl groups has been suggested since acetaldehyde has been found in the medium after incubation of microsomes with HN2 (Trams and Nadkarni, 1956).

It has been calculated that at tumour-inhibitory doses of HN2, the concentration of HN2 achieved in vivo is approximately $10^{-5}M$ (Johnson and Ruddon, 1967; Rutman et al, 1961).

Resistance to alkylating agents may arise in a number of ways; altered cell permeability, increased cellular concentration of

protective agents, increased capacity for repair of toxic biochemical lesions, or decreased amounts of susceptible target molecules, which may involve large changes in cell metabolism.

Several studies have shown decreased uptake of labelled HN2, and decreased binding of the drug to cellular macromolecules, in resistant mammalian cells (Rutman et al, 1968; Wolpert and Ruddon, 1969; Chun et al, 1969; Goldenberg et al, 1970); in contrast, other workers have found no such differences (Wheeler and Alexander, 1964).

Nitrogen mustard may react with cellular sulphydryl groups (introduction 1.1.1), and sulphydryl-containing compounds have been implicated in protecting susceptible targets from the drug (Brockman, 1974; Connors, 1966). Elevated protein sulphydryl levels have been found in cells resistant to HN2 (Calcutt and Connors, 1963; Ball et al, 1966; Ujhazy and Winkler, 1965; Goldenberg, 1969; Hirono, 1960; 1961). Resistance to an analogue of nitrogen mustard, melphalan (L-phenylalanine mustard), corresponded to increased cellular glutathione content (Suzukake et al, 1982; 1983; Begleiter et al, 1983) and glutathione was involved in dechlorination of the agent in resistant L1210 cells (Suzukake et al, 1983). Resistance to another analogue, chlorambucil, was associated with raised cellular concentrations of the cysteine-rich polypeptides, the metallothioneins (Endresen et al, 1983). However, several studies have revealed that the relationship between increased cellular sulphydryl content and increased resistance to alkylating agents is not universal (Brockman, 1974; Connors, 1966; Reid and Walker, 1966).

Since DNA has been considered to be the major cellular target for the toxicity of nitrogen mustard, many attempts to explain drug resistance have been centred around the ability of cells to repair damage caused by alkylation of DNA (introduction 1.1.4.2). The

repair of such damage has been frequently compared to the repair of damage to DNA following ultraviolet or X-irradiation (Brockman, 1974; Roberts, 1972; Roberts et al, 1971a; 1971b). Alkylated products are excised from DNA (Crathorn and Roberts, 1966; Lawley, 1966), and DNA synthesis occurs to repair the lesion (Roberts et al, 1968a, 1968b; Ayad et al, 1969). However, it is possible that the repair of DNA damage may be influenced by metabolic processes occurring outside the nucleus (Tritton and Hickman, 1984; Van Ankeren and Wheeler, 1985); thus, resistance to alkylating agents may be influenced by changes in one or more of these processes.

1.1.4 The mechanism of action of nitrogen mustard

1.1.1.4 Alkylation

Alkylation may be defined as the covalent attachment of a simple or substituted alkyl group to a second molecule via a nucleophilic substitution reaction. The reaction may be monomolecular (SN1) or bimolecular (SN2). An SN1 mechanism requires the formation of a reactive, positively-charged carbonium ion to attack the nucleophilic centre; this process may occur slowly enough to be rate-limiting for the entire process. The SN2 mechanism involves attack by the intact alkylating agent upon the nucleophilic centre, thus producing an unstable transition state prior to removal of the leaving group, leaving the alkylated product. The transition product breaks down rapidly, so the rate-limiting step for the whole process is the formation of this intermediate.

Nitrogen mustard is thought to rapidly form a cyclic aziridinium ion, via the SN1 mechanism; this species is unstable and highly reactive, and may attack cellular molecules containing nitrogen, oxygen and sulphur (Ross, 1962).

1.1.4.2 Reaction with nucleic acids

The mutagenic properties of alkylating agents led Auerbach and Robson (1946) to propose that DNA was a susceptible target. Subsequently it was shown that the N7 atoms of guanine bases were particularly susceptible to alkylation because of their orientation in the helical DNA structure (Brooks and Lawley, 1961). Then, in 1969, Price and his colleagues showed that alkylating agents with more than one functional group were capable of cross-linking DNA molecules. The crosslinks were formed between adjacent bases on the same (intra-strand linkage) or opposite (inter-strand linkage) chains of the DNA double helix (Price et al, 1969). This work, together with the earlier observation that only bifunctional agents possessed pharmacological activity, indicating that a crosslinking event was an essential step, led to the acceptance of earlier suggestions that a crosslink of DNA was the cellular target of these agents (Alexander and Lett, 1960; Goldacre et al, 1949). In addition, nitrogen mustard was observed to have gross effects on nuclear morphology (Wheeler, 1962). The interaction of the alkylating agents with DNA was also thought to interfere with DNA replication (Connors, 1974).

More recently, monofunctional antitumour agents have been synthesised and their activity questions the hypothesis that a crosslinking of DNA is essential for cytotoxicity. The view has emerged that monofunctional alkylation by the bifunctional agents may explain their mutagenic properties (Connors, 1974).

Alkylation of DNA could conceivably interfere with the interaction of the molecule with essential enzymes and regulatory peptides involved in the synthesis and transcription of DNA, thus disrupting mitosis (Kohn and Ewig, 1979). Alkylating agents have also been shown to produce crosslinkages between DNA and nuclear

proteins (Thomas et al, 1978; Klatt et al, 1969); the latter group may potentially include the same important enzymes. However, these lesions may be unimportant to the cytotoxicity, since cellular systems exist for their repair (Ewig and Kohn, 1977); although, if these systems become saturated by high doses of the drug, it is possible that the lesion may contribute to cytotoxicity.

It has been shown that the majority of the enzymes involved in DNA metabolism are unaffected by pharmacological concentrations of nitrogen or sulphur mustards (Drysdale et al, 1958; Smellie et al, 1955; Papirmeister, 1961; Ruddon and Johnson, 1968), although Tomisek et al (1966) reported inhibition of DNA polymerase and thymidilate kinase, and Needham (1948) reported that some phosphokinases are sensitive to alkylation. Wheeler and Alexander (1964) suggested that alkylating agents interfered with the de novo synthesis of ribonculeotides.

Several studies have shown that nitrogen and sulphur mustards inhibit DNA synthesis at concentrations which leave RNA and protein synthesis unaffected for prolonged periods (Brewer et al, 1961; Levis et al, 1963; Caspersson et al, 1963; Herriot, 1950; Bodenstein and Kondritzer, 1948; Goldthwait, 1952). However, Drysdale et al (1958) found no evidence for a specific effect of either compound on DNA synthesis in murine Ehrlich or MC1M ascites cells, a conclusion in agreement with studies by others (Davidson and Freeman, 1955; LePage and Greenlees, 1955; Heidelberger and Keller, 1955). In cultured L cells, and in the tissues of rabbits treated in vivo, Drysdale et al (1958) found that $^{32}PO_4^{3-}$ incorporation into DNA was depressed by a greater amount than incorporation into RNA, in agreement with Lowrance and Carter (1950). In the cultured cell line, the content of RNA increased at a faster rate than the content of DNA, which

result was in general agreement with the studies mentioned above. Drysdale et al (1958) concluded that there was no evidence that HN2 at any concentration exercises its effects solely on DNA metabolism.

The formation of interstrand crosslinkages in alkylated DNA may prevent the physical separation of the two strands during semiconservative replication (Kohn and Ewig, 1979). The treatment of DNA with alkylating agents in vitro caused changes in the physical properties of DNA, including the sedimentation and diffusion constants, electrophoretic mobility, and susceptibility to thermal denaturation, suggesting that crosslinkages were formed (Wheeler, 1962; Butler and James, 1951; Butler et al, 1951, Conway et al, 1950). More recently, Kohn et al (1966) demonstrated that bacterial DNA acquired an increased resistance to alkaline denaturation after incubation with HN2 at concentrations which would theoretically react each DNA molecule with only a single molecule of HN2. Brewer et al (1961) suggested that crosslinkage of DNA by HN2 may affect an early step of mitosis, such as prevention of condensation and separation of the chromatids.

No such changes in the physical properties of DNA were found after treatment with alkylating agents in vivo (Wheeler and Stephens, 1965; Golder et al, 1964), leading one of the authors to speculate that the lesion was unimportant for cytotoxicity (Wheeler, 1967). Latterly, Kohn et al (1976) demonstrated that alkylating agents produced DNA interstrand cross-links both in vitro and in vivo; the technique of alkaline elution was used, and the earlier failure to detect the lesion in vivo may be due to technique (Ochoa and Hirschberg, 1967).

Kohn and Green (1966) found that transforming activity was retained by bacteria in which the DNA molecule had suffered two

crosslinkages by HN2, suggesting that these were without effect on the biological activity of the molecule. After incubation of DNA *in vitro* for 24h with HN2, 35-45% of the alkylated bases had been deleted, but the inhibition of DNA template activity was unchanged during this period (Ruddon and Johnson, 1968). Similarly, destruction or deletion of bases in irradiated cells was without effect on the priming activity of DNA (Weiss and Wheeler, 1967).

Nitrogen mustard may also induce breaks in one or both strands of the DNA, leading to disorganised replication (Kohn *et al*, 1981). Cellular repair mechanisms exist for the excision of alkylated bases (Ludlam, 1975), but the mechanism by which double-strand breaks may be repaired is uncertain (Kohn *et al*, 1981). Spurgin (1981) suggested that the extension of the period between mitoses, observed by Roberts *et al* (1968), may be explained by a delay caused by repair occurring only at specific points in the cell cycle.

Examination of the effects of nitrogen mustard during the cell cycle allows a number of objections to be raised against the proposal that DNA is the only pharmacologically important target for nitrogen mustard. Brox *et al* (1980) found that cell division was blocked in G_2 by alkylating agents. At low concentrations, the block was temporary and DNA crosslinkages were repaired. If crosslinking prevents DNA synthesis, this result does not explain how the cells achieved the duplication of DNA. Dean and Fox (1984) showed that HN2-treated cells were delayed in S phase, then accumulated in G_2. At higher concentrations of HN2, cells became blocked in G_2. However, despite the delay in S phase, the cells were able to achieve the premitotic complement of DNA, suggesting that DNA damage was repaired; this was supported by observations in other studies, that the longer the S phase delay, the better the survival (Scott and

Zampetti-Bosseler, 1982; Dean and Fox, 1983; Graham and Fox, 1983). However, the duration of S phase delay exceeded the time required for removal of crosslinks, suggesting that other factors were important (Dean and Fox, 1984). Other studies, however, conflicted with this conclusion and have demonstrated that the greater the delay in S phase the more sensitive are the cells (Alabaster and Burnage, 1976; Rosenburg et al, 1976; White and Creighton, 1976; Scott, 1975; Scott and Marshall, 1978; Scott et al, 1974a; 1974b; Scott and Zampetti-Bosseler, 1981; Edgar and Creighton, 1982; Kaiser et al, 1981).

Mauro and Madoc-Jones (1970) found that proliferating mammalian cells were most sensitive to nitrogen mustard during mitosis and at the transition from G_1 to S phase. Inhibition of mitosis has been noted in many other studies (Brewer et al, 1961; Caspersson et al, 1965; Layde and Baserga, 1964; Levis et al, 1963; 1965). Walker and Helleiner (1963) also found the S phase to be the most sensitive. DeCosse and Gelfant (1970) found a primary effect of the agent to occur in G_2 and suggested that a major biological action was independent of DNA replication. Further, Rutman et al (1961) compared the effects of HN2 and chloroquine mustard on Ehrlich ascites cells and found disparity between the extent of alkylation of DNA and the extent of cell damage. A similar conclusion was reached by Clarkson and Mitchell (1981), who found that cells in midG_1 phase were most sensitive to HN2, with resistance increasing as the cells progressed through the cell cycle; however, this observation was not accounted for by different levels of DNA damage or repair at different stages of the cell cycle.

DNA synthesis has often been examined by measurement of incorporation of radiolabelled precursors. A number of studies have shown that alkylating agents inhibit the incorporation of these

precursors into DNA (Roberts, 1975; Crathorn and Roberts, 1966; Wheeler, 1962; Brewer et al, 1961). However, the use of precursors to measure synthesis of macromolecules may not be reliable (e.g., Kay and Handmaker, 1970). Mitomycin C inhibited protein synthesis before any effect of [^{14}C]-leucine incorporation in chick embryos (McCann et al, 1971). Conversley, chlorambucil inhibited [^{3}H]-thymidine incorporation without an effect on DNA synthesis (Riches and Harrap, 1973). In HeLa cells, chlorambucil apparently inhibited DNA synthesis whilst DNA content continued to increase (Roberts,1975). Dean and Fox (1984) found that [^{3}H]-thymidine incorporation into human lymphoblastoid cells did not reflect the actual rate of DNA synthesis. Grunicke et al (1975) showed that Trenimon (2,3,5-Trisethyleneiminobenzoquinone) reduced [^{3}H]-thymidine incorporation into Ehrlich ascites cells without an effect on DNA synthesis. They further demonstrated that this result was accounted for by an effect of the alkylating agent on the transport of [^{3}H]-thymidine across the cell membrane.

Whilst bifunctional alkylating agents possess widespread antiproliferative activity (introduction 1.1.1), a small degree of selectivity is apparent, and different experimental tumours are sensitive to different alkylating agents (Elson et al, 1958; Elson, 1958; Ross, 1962). Tumours are not necessarily cross-resistant or cross-sensitive to different analogues of alkylating agents (Ross, 1962). It is difficult to explain these observations if a crosslink between adjacent guanine residues is accepted as the primary cytotoxic lesion. DNA would contain many potential sites for the formation of such a cross-linkage. Any bifunctional alkylating agent with suitable stearic arrangement of the active groups would be able to interact with the DNA. However, in vivo, different analogues of

the drug may be transported (see introduction 1.1.3) or metabolised differently. The existence of resistant cell lines may arise as a point mutation; mutations to alter all the potentially sensitive sites within the DNA may have great effects on the phenotype of the cell and may even be lethal.

Examination of cell lines with different sensitivities to cytotoxic agents has often found no difference in DNA (Wheeler and Stephens, 1965; Ball and Roberts, 1970), DNA synthesis, rates of removal of crosslinks, excision repair, or initial or final strand breaks (Hesslewood, 1978; Scott et al, 1974a; 1974b; Fox and Fox, 1973a; 1973b; Yin et al, 1973; Ball and Roberts, 1970). Other studies have revealed differences, but of a magnitude which was inadequate to account for the difference in sensitivities (Dean and Fox, 1984; Fox and Fox, 1973c; Walker and Reid, 1971, Yin et al, 1973; Strandberg et al, 1982; Micetich et al, 1983; Zwelling et al, 1981).

Dean and Fox (1984) have suggested that the different sensitivity of cell lines may be due to different ability to restore DNA metabolism, and not to differences in DNA repair. Erickson et al (1978) suggested that the resistance of a human carcinoma cell line to methylCCNU (1-2chloroethyl-3-(4transmethylcyclohexyl-1-nitrosourea)) in vivo was associated with the reduced formation of crosslinkages in comparison to the sensitive counterpart. Differences in vitro were thought to arise from differences in removal of the crosslinkages. Micetich et al (1983) examined the repair of crosslinkages in sensitive and resistant L1210 cells after treatment with cisplatin. In resistant cells, the period over which monoadducts progressed to form crosslinkages was reduced by a quenching mechanism, allowing the more rapid removal of the

crosslinkages. Such a mechanism did not appear to explain the different sensitivities of two human lymphoblastoid lines to nitrogen mustard; in both cell lines, the formation of crosslinkages reached a maximum within 30 minutes. The authors interpreted this result to suggest that a differentially active quenching mechanism would not have sufficient time to operate (Dean and Fox, 1984).

An attempt has been made to explain the different sensitivities to alkylating agents using the DNA model. Chlorambucil induced phosphorylation of nuclear proteins in sensitive, but not resistant Yoshida cells, which was correlated with the loss of condensed chromatin and with cytotoxicity (Riches and Harrap, 1973; Riches et al, 1977). The loss of condensed chromatin would allow increased drug binding, and the repair of the lesion would be prevented by the interaction between the drug and the chromatin. Thus, resistance to alkylating agents was suggested to arise from the failure of the agent to elevate phosphorylation of nuclear proteins, together with the formation of repairable DNA crosslinkages and the unwinding of condensed chromatin (Wilkinson et al, 1979).

Ord and Danielli conducted experiments on amoebae treated with nitrogen mustard; they transferred treated nuclei to untreated cytoplasm, and vice versa. The nucleus was found to be more sensitive to HN2 than the cytoplasm. However, damage to the cytoplasm was more likely to prevent cell division than nuclear damage; amoebae with treated nuclei, but not treated cytoplasm, were able to divide several times before death. The authors concluded that the cytotoxic effects of HN2 upon nucleus and cytoplasm were independent of each other (Ord, 1956; Ord and Danielli, 1956).

More recently, two cytotoxic drugs have been linked to polymeric beads which cannot enter the cell. The anititumour antibiotic

adriamycin (see Tritton and Yee, 1982) and the alkylating agent chlorambucil (see Grunicke et al, 1979) were both cytotoxic to dividing cells in this immobilised form, suggesting that events at the plasma membrane may mediate cytotoxicity of drugs previously thought to have their effect in the nucleus.

In conclusion, whilst it is certain that bifunctional alkylating agents such as nitrogen mustard may crosslink DNA in vivo and in vitro, there is evidence that this lesion is not unique in mediating their cytotoxicity.

Johnson and Ruddon (1967) demonstrated a loss of coding ability in poly-RNA molecules treated with HN2 in a cell-free protein-synthesising system. Methylation of bases in such molecules has been shown to inhibit coding ability (Ludlum et al, 1965). The reactivity of the bases with alkylating agents is in the sequence:guanine > adenine > cytosine >> thymine or uracil (Lawley, 1957), from which Johnson and Ruddon (1967) predicted that the sensitivity of the poly RNA molecules would be in the sequence: polyA>polyC>>polyU. However, the observed sequence of sensitivity to HN2 was polyC>polyU>polyA, from which they concluded that another site, distinct from the bases in the molecule, was sensitive to alkylation. Methylation of RNA caused esterification of internal phosphate groups, leading to chain breaks (Kriek and Emmelot, 1963), and sulphur mustard reacted with the 5'terminal PO_4^{3-} group of poly-RNA (Abell et al, 1965), so Johnson and Ruddon (1967) suggested that the second site may be PO_4^{3-}groups. Nitrogen mustard was without effect on the binding of the poly RNA or the t-RNA to the ribosome, although base-pairing has been shown to be inhibited by 10^{-3}M HN2 (Ludlum et al, 1964).

Johnson and Ruddon (1967) observed inhibition of poly-RNA coding ability only at 10^{-4}M HN2 or more, which exceeds, by at least ten

times, the therapeutic pharmacological concentration *in vivo*. At 10^{-6}-10^{-4}M, HN2 enhanced protein synthesis in the absence of an effect on the ribosome. It was suggested that this was due to inhibition of ribosomal nucleases and consequent prolongation of the life of RNA molecules.

In a subsequent study the same authors examined the binding of [^{14}C]-HN2 to DNA and its effect upon DNA template activity. After incubation with $5x10^{-7}$M HN2, there was significant inhibition of calf thymus DNA template activity for RNA synthesis, but up to 100 fold more HN2 was required to produce equivalent inhibition of template activity for DNA synthesis (Ruddon and Johnson, 1968), which result concurred with with the finding of Chmielewicz *et al* (1967). Ruddon and Johnson (1968) also found evidence that DNA in its nucleoprotein complex *in vivo* was much less sensitive to the effects of HN2 than the purified DNA *in vitro*; they noted that the cellular DNA may not be the most sensitive target to alkylation, and that other targets may be important in the cytotoxicity of HN2.

A number of workers have studied the effects of nitrogen mustard on nucleic acids and likened them to the effects of X-irradiation (Brewer *et al*, 1961; Brookes, 1964; Alexander and Mikulski, 1961; Levis *et al*, 1963, Ross, 1962). Thus, Sakomoto and Elkind (1969) expected to find that lesions in DNA caused by irradiation would interact with those caused by alkylating agents; instead, the lesions acted independently, suggesting different action at the same site, or action at different sites. The authors also found evidence that a single "hit" was required to kill cells, suggesting that sublethal damage is not involved in the mechanism of action of nitrogen mustard. This conclusion is in direct contrast with the view that alkylating agents have a multimodal mechanism of action (Tritton and

Hickman, 1984); further, it has been shown that repair of lesions in DNA following irradiation may be inhibited by lesions at the plasma membrane (Bertsche, 1984) or changes in other metabolic processes (Van Ankeren and Wheeler, 1985).

1.1.4.3 Reaction with essential enzymes

In 1936 Sir Rudolph Peters proposed that the cytotoxicity of alkylating agents involved the inactivation of enzymes essential to cellular metabolism (Peters, 1936). Studies on the effects of HN2 on enzymes were often conducted *in vitro*, and in many cases it was found that concentrations required to inhibit enzyme activity were disimilar to the concentrations which arose *in vivo* after administration of therapeutic doses (Ross, 1962; Philips, 1950). Although the activity of many enzymes was inhibited by alkylating agents, it was proposed that inhibition arose as an indirect effect of alkylation at other sites (Connors, 1975).

Nitrogen mustard has been shown to depress protein synthesis *in vivo* and *in vitro*, although relatively high concentrations are necessary (Wheeler, 1962). Some effects have been noted on enzymes concerned with metabolism of nucleic acids (see introduction 1.1.4.2). Alkylating agents have also been shown to inhibit the processes of respiration and glycolysis (Ochoa and Hirschberg, 1967), and effects on enzymes in the plasma membrane have been documented (see introduction 1.1.4.6, 1.3; and also Tritton and Hickman, 1984; Baxter *et al*, 1982; Tisdale and Phillips, 1975b; Grunicke *et al*, 1982; 1983; 1985). Nitrogen mustard may also affect the activity of enzymes as a secondary effect of alterations in intracellular mediators (Hickman, 1982; introduction 1.1.4.5) or coenzymes (Connors, 1975; introduction 1.1.4.4).

In conclusion, whilst there is evidence against the reaction of HN2 with enzymes being the single cause of cytotoxicity, the possibilities cannot be discounted that these reactions may contribute to a multmodal mechanism of action, or, that a single, vital enzyme system is especially sensitive (Ross, 1962).

1.1.4.4 Effect of nitrogen mustard on cellular NAD^+ levels

Connors (1975) suggested that the effects of alkylating agents on cellular NAD^+ levels may contribute to their cytotoxicity. Alkylating agents reduced NAD^+ levels in tumour cells (Ochoa and Hirschberg, 1967; Wheeler, 1962; Dold et al, 1962; Liss and Palme, 1964), although results differ in whether this effect occurs at concentrations which leave other processes unaffected (see Liss and Palme, 1964).

Perturbation of NAD^+ levels in cells exposed to alkylating agents may have effects upon enzymes involved in glycolysis and respiration which require NAD^+ as a cofactor (Ochoa and Hirschberg, 1967). It has been suggested that changes in cellular NAD^+ levels may arise through an increase in the activity of DNA repair enzymes following alkylation damage to DNA (Smulson et al, 1977; 1979)

1.1.4.5 Effects of nitrogen mustard on cellular cAMP levels

Cyclic adenosine monophosphate is a major regulator of many cellular activities (Sutherland, 1972). Intracellular accumulation of cAMP, whether exogenously supplied or endogenously generated, inhibits the growth of normal and transformed cells both in vivo and in vitro (Pastan et al, 1975; Cho-Chung, 1979; 1982). CyclicAMP, its derivatives, and agents which raise cAMP have been shown to possess antitumoural activity in vivo, and to induce differentiation of transformed cells in vitro and in vivo (Cho-Chung, 1982). Intracellular cAMP concentrations of tumour cells are frequently

altered in comparison with their normal counterparts, but levels may be higher or lower. It is thought that cAMP in conjunction with cGMP may make an important contribution to cellular transformation, but the precise role has not been elucidated, and conflicting reports exist (Cho-Chung, 1982; Whitfield et al, 1976; Otten et al, 1971).

Although elevation of cAMP levels may signal cessation of cell growth, the relationship does not hold for all cell types. Unrestrained growth is not consistently associated with lower cAMP levels, and there is conflicting evidence on whether cells in stationary cultures have elevated cAMP levels compared to rapidly dividing counterparts (Cho-Chung, 1982).

The action of cAMP in mammalian tissues is mediated by interaction of cAMP with its receptor protein, which is the regulatory subunit associated with cAMP-dependent protein kinase (Kuo and Greengard, 1969). The binding of cAMP to the regulatory unit releases and thus activates the catalytic kinase to phosphorylate specific proteins (Cho-Chung, 1982; Tisdale and Phillips, 1976b). Tisdale and Phillips (1975c) showed that incubation in vitro for 24 hours with 10^{-6}M nitrogen mustard increased by 100% the cAMP levels of rat Walker carcinoma cells. Similar effects were noticed after treatment of Walker cells with cisplatin (cis-diaminodichloroplatinum (II)), merophan (D,L-O-(bis-2-chloroethylamino)phenylalanine) and chlorambucil; the last of these was investigated in detail.

Chlorambucil (p-(bis-2-chloroethylamino)-phenylbutyric acid) inhibited the activity of cyclic-3'5-nucleotide phosphodiesterase (EC. 3.1.4.17) isolated from beef heart (Tisdale, 1974). The enzyme exists in two forms, a membrane-bound, high affinity (low k_m) form, and a cytoplasmic low affinity (high K_m) form (Russell and Pastan, 1973). The two forms were similarly sensitive to alkylation by

iodoacetate, but the membrane-bound low k_m form was more sensitive to inhibition by chlorambucil (Tisdale 1974). Subsequently, it was shown that growth-inhibitory concentrations of chlorambucil rapidly elevated cAMP levels in Walker cells *in vitro* (Tisdale and Phillips 1975b; 1975c), which was similar to the effect of phosphodiesterase inhibitors; these agents have also been shown to inhibit tumour growth (Cho-Chung, 1982; Webb *et al*, 1972; Keller, 1972) and induce differentiation (Prasad and Sheppard, 1972).

Elevation of cellular cAMP levels was due to inhibition of the phosphodiesterase enzyme; the membrane-bound adenylate cyclase was unaffected (Tisdale and Phillips, 1976a). The effects of chlorambucil on protein kinase activity were reproduced by addition of cytotoxic concentrations of dibutyrylcAMP, a lipophilic analogue of cAMP which more readily enters cells (Tisdale and Phillips, 1976b).

The increase in cAMP concentration increased the phosphorylation of specific target molecules, which may include histone and non-histone proteins (Wilkinson *et al*, 1979). Other susceptible targets may include the enzymes involved in DNA synthesis (Tisdale, 1980), although Hickman (1982) pointed out that, whilst this effect may explain the retardation of DNA synthesis after exposure to alkylating agents, such lesions are unlikely to be wholly responsible for cytotoxicity (see also introduction 1.1.4.2). Changes in cellular cAMP concentrations may also affect the activity of transmembrane ion transporter proteins (McManus and Schmidt, 1983; Palfrey and Rao, 1983; Tritton and Hickman, 1984; Moszik, 1969; Palfrey and Greengard, 1981), which may in turn have widespread effects upon cell metabolism (see introduction 1.2).

The proposal that inhibition of cAMP phosphodiesterase was a principle mechanism of chlorambucil cytotoxicity was able to account for the requirement of bifunctionality for antitumour activity, the existence of resistant cells, and the senstitivity of some normal tissues. A monofunctional (N-ethyl) analogue of chlorambucil, which lacks pharmacological activity, was found to be without effect on cAMP levels or either form of phosphodiesterase in Walker cells in vitro, even when cytotoxic concentrations were applied; this result suggested that cell death after incubation with this agent was due to an independent mechanism (Tisdale and Phillips, 1975b).

Rodent tumours exhibiting different sensitivities to chlorambucil were found to possess different activities of the low k_m form of phosphodiesterase, in accordance with their altered sensitivities (Tisdale and Phillips, 1975a). In a resistant Walker cell line, the low k_m form of the phosphodiesterase contributed a lower proportion of total phosphodiesterase activity (Tisdale and Phillips, 1975a). Resistant cell lines displayed cross-resistance to dbcAMP, and reduced activity of the cAMP binding protein (Tisdale and Phillips, 1976c); reductions in cAMP receptor protein or the protein kinase can confer resistance to dbcAMP (Cho-Chung, 1982).

Normal tissues, including bone marrow and gut mucosa, which are susceptible to chlorambucil toxicity, were found to have phosphodiesterase activity where a relatively large proportion of activity was due to the low k_m form of the enzyme (Tisdale and Phillips, 1975c).

There is controversy over which phase of the cell cycle an elevation of cAMP acts upon to inhibit proliferation (Pastan et al, 1975); although, this is somewhat concordant with the conflicting evidence regarding the phase in which alkylating agents may be

considered to act (introduction 1.1.4.2). Changes in cAMP level observed after exposure to chlorambucil may not be sufficient to fully explain the cytotoxic effects of different concentrations of the agent (Hickman, 1982). Finally, Coffino and his coworkers concluded that cAMP levels did not exert absolute control over progression through the cell cycle, since a mutant murine lymphoma cell line, deficient in cAMP-dependent protein kinase, grew normally (Coffino et al, 1975).

1.1.4.6 Effects of nitrogen mustard on the plasma membrane

One of the earliest suggestions regarding the cellular locus of action of alkylating agents was the proposal of Peters (1947) that sulphur mustard acted at the cell membrane to cause cell death by alterations of membrane permeability. Subsequent studies have revealed effects of other alkylating agents upon the physical properties of the cell membrane. Levy (1965) found that the swelling of nucleated chicken erythrocytes, their ghosts, and rabbit lung and bone marrow cells, was inhibited by nitrogen and sulphur mustards at concentrations which were without detectable effect on nucleic acids. The resealed erythrocyte membrane ghosts were more sensitive to the effect of the mustards than the intact erythrocytes, and the cellular protein haemoglobin was found to have a protective effect. Swelling was not inhibited by iodoacetic acid, suggesting that a crosslinking reaction was important. Levy concluded that the mustards react with components of the cell membrane and cytoplasm to alter the elastic properties of cells, and that this action may account for the cytostatic and cytotoxic effects of these agents.

Chlorambucil was shown to react with nucleophilic molecules in the membrane of the human erythrocyte; it was suggested that some component was removed from the membrane, so weakening it and causing cell lysis (Linford et al, 1963).

Human red blood cells are useful in the study of the effects of agents on the plasma membrane since the membrane components are well defined and effects mediated by attack on DNA are eliminated. Wildenauer and his colleagues studied the effect of the alkylating antitumour agent tris(2-chloroethyl)amine on the human erythrocyte membrane. After incubation with this agent ($1\text{-}2\times10^{-3}$M), metabolically-induced shape changes in the erythrocytes were inhibited. The membrane proteins were analysed by polyacrylamide gel electrophoresis, and it was found that the membrane protein spectrin was lost after incubation with the mustard. Evidence was found for the crosslinkage, by the mustard, of spectrin molecules with each other and with other proteins of the membrane or cytoplasm. Quantitative studies on the binding of radiolabelled tris(2-chloroethyl)amine revealed that other membrane proteins were alkylated; these included glycophorin and others (Wildenauer and Weger, 1979; Wildenauer et al, 1980). Spectrin, in conjunction with actin, is an important component of the cytoskeleton (Nicholson, 1976). The cytoskeleton is important in the maintenance of cell shape, the mobility of membrane components and ultimately, in the control of cell proliferation (Folkman and Moscona, 1978; Kulesh and Green, 1986).

Whilst these studies suggested that mustards rigidified the plasma membrane, there is conflicting evidence regarding the effects of these agents on the movement of individual membrane components, i.e., the membrane fluidity. Maintenance of optimum membrane fluidity is crucial to membrane function (Pang et al, 1979); several antitumour agents have been shown to perturb membrane fluidity (Tritton and Hickman, 1984).

Grunicke and his colleagues have conducted a series of experiments to investigate the effects of nitrogen mustard on membrane fluidity (Grunicke et al, 1982; 1985). In the earlier study, the fluorescence polarisation of diphenylhexatriene was monitored. This lipophilic molecule inserts into the lipid bilayer, and is then excited with polarised light. If the membrane environment is fluid the molecule is free to rotate, so the light emitted becomes less polarised. Conversely, if the emitted light is highly polarised, a more rigid membrane environment is indicated. Although they noted some methodological difficulties, Grunicke et al (1982) obtained data which suggested that HN2 caused a decrease in the membrane fluidity of Ehrlich ascites tumour cells, and that this action was related to inhibition of cell growth. No change in fluidity was found in a cell line resistant to HN2.

The interpretation of data from fluorescence polarisation of dephenylhexatriene is controversial (Jahnig, 1979; Bouchy et al, 1981), and in their later study, Grunicke et al (1985) employed two further methods to examine mobility of membrane components. Firstly, Ehrlich ascites cells, which had been incubated with and without 10^{-4}M HN2 for 30 minutes, were labelled with diiodofluorescein-iodoacetamide, which marks integral membrane proteins. The fluorescence polarisation technique was then used to assess the mobility of these proteins (Greinert et al, 1982), which would be expected to respond to changes in membrane fluidity. Again, some methodological difficulty was reported, but it was found that the proteins in HN2-treated cells were consistently more mobile, a result in direct contradiction of the earlier finding.

Grunicke et al (1985) further investigated the lateral mobility of the lipid components of the Ehrlich ascites cell membrane after

incubation with HN2, using a method based upon the quenching reaction between the lipophilic cation cetylpyridinium, and the fluorescence probe, pyrene. Cetylpyridinium moves in the plane of the lipid bilayer, and the speed of this movement depends upon the fluidity of the membrane. After incubation for one hour with 10^{-4}M HN2, there was no difference in the lateral mobility of the probe within Ehrlich cell membrane vesicles. Thus, the effect of HN2 on membrane fluidity is unclear; however, it should be noted that all the methods described above give a mean value for the relative mobility of the membrane components. The possibility cannot be ruled out that membrane components in selected individual domains are crosslinked and immobilised by HN2.

Nitrogen mustard and other alkylating agents have been shown to affect transport functions of tumour cell membranes; these observations form the basis of the work documented in this thesis, and as such are described in detail in introduction 1.3.

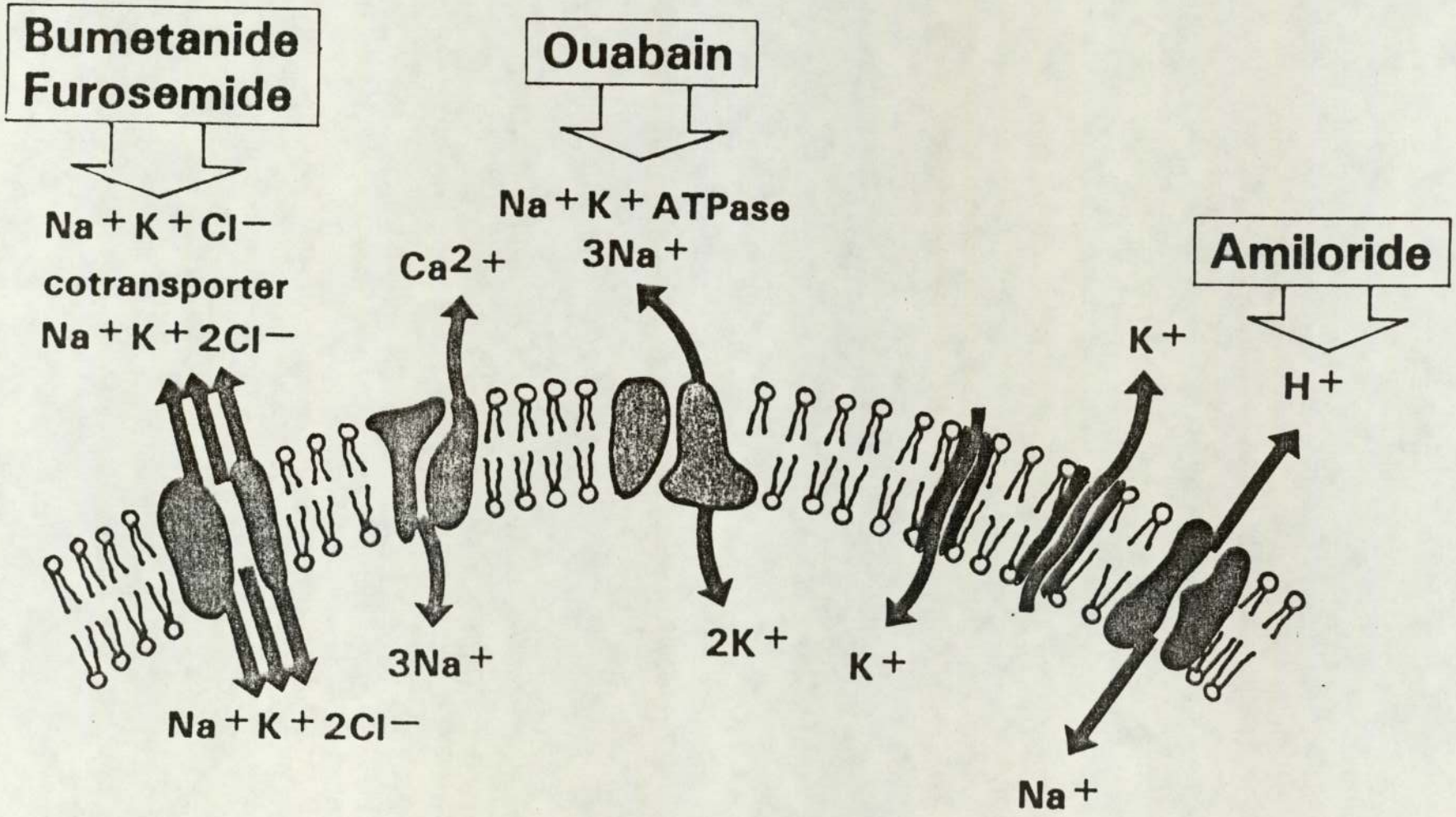
1.2 THE TRANSPORT OF SODIUM AND POTASSIUM ACROSS THE PLASMA MEMBRANE

1.2.1 Specific transport systems

A number of specific mechanisms exist for the transmembrane transport of Na^+ and K^+ ions (see figure 1). Certain of these systems mediate active transport, and this allows the maintenance of a concentration gradient between the cell cytoplasm and the extracellular medium, even though the membrane is permeable to both Na^+ and K^+ and these ions may diffuse across the membrane down their concentration gradients. Intracellular K^+ is maintained at approximately 120-140mM and extracellular K^+ in tissue fluid is at a concentration of approximately 5-10mM; thus, there is passive movement of K^+ out of the cell. Conversely, intracellular Na^+ concentrations are approximately 20mM, and extracellular concentrations are approximately 140mM, so the cell expends energy to maintain the concentration gradient across the membrane against the inward diffusion of Na^+ ions (Moyer et al, 1982).

The maintenance of the concentration gradients of Na^+ and K^+ ions across the plasma membrane requires metabolic energy, and may consume up to one third of the energy generated by the cell (Segel et al, 1978). Active and passive transport of Na^+ and K^+ was demonstrated by Schatzman (1953), who showed that only the active transport was sensitive to ouabain. Subsequently, an enzyme was purified, from plasma membranes, which was found to hydrolyze ATP and to require Na^+ and K^+ for this activity (Skou, 1957), and was sensitive to ouabain (Skou, 1960). The enzyme was subsequently identified as the cellular component responsible for the active transport of Na^+ and K^+ across the plasma membrane (Glynn, 1964) and was thus named Na^+ and K^+ activated adenosine triphosphatase (Na^+K^+ATPase) by Bonting et al (1961).

Figure 1 Transport of ions across the cell membrane



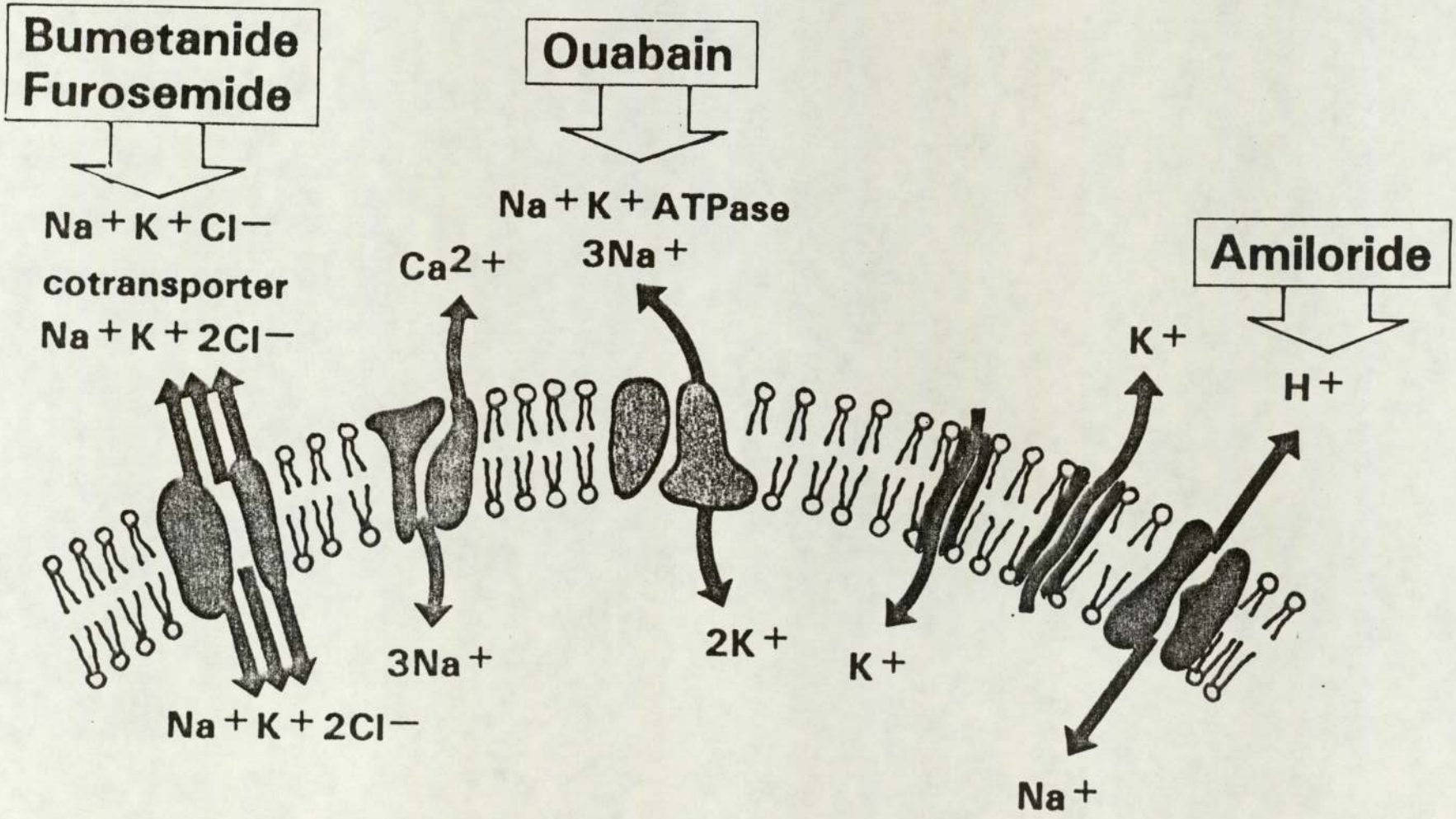
(Drawn by Graham Smith)

Table 1

PHARMACOLOGICAL AGENTS USED TO IDENTIFY THE ACTIVITY OF MEMBRANE SYSTEMS FOR SODIUM AND POTASSIUM TRANSPORT.

SYSTEM	AGENT	REFERENCE
Na^+K^+ATPase	Ouabain	Skou (1960)
$Na^+K^+Cl^-$ cotransporter	'loop' diuretics: furosemide, bumetanide	Palfrey *et al* (1980)
Na^+/H^+ exchanger	amiloride	Cragoe (1979)
Na^+/Ca^{++} exchange	amiloride	Swanson *et al* (1983) Dethmers *et al* (1983)
Ca^{++} stimulated K^+ channel	quinine	Lew and Ferreira (1978)

Figure 1 Transport of ions across the cell membrane



(Drawn by Graham Smith)

The enzyme spans the plasma membrane and its activity may be modulated by its interactions with its lipid environment (Fenster and Copenhaver, 1967). It is a subunit enzyme, composed of two chains: the catalytic α chain, which binds ouabain at the K^+ binding site and is phosphorylated by ATP (Forbush and Hoffman, 1979); and the glycosylated β chain. The enzyme is thought to exist as two dimers (Stein, 1974; Huang and Askari, 1979), although other structures have been suggested (Esmann et al, 1979; Schwartz et al, 1975; Hastings and Reynolds, 1979).

The activity of the enzyme depends on some of its sulphydryl groups (Schoot et al, 1978; 1979), which are involved in the binding of the ATP molecule (Patzelt-Wenczler et al, 1975) and the K^+ phosphatase activity (Schoot et al, 1978). It is therefore inhibited by sulphydryl reagents (Skou, 1963). The two subunits undergo a conformational change relative to each other during the reaction sequence, which is inhibited by crosslinking reagents such as copper ions, copper phenanthroline (Huang and Askari, 1979), or 3,3-dithiobispropionimidate (dePont, 1979), and may render it susceptible to crosslinkage by bifunctional alkylating agents (Sweadner, 1977).

Sensitivity to ouabain is most frequently used to identify the activity of Na^+K^+ATPase (Skou, 1960), although this approach may not be without problems, since ouabain may not be absolutely specific for Na^+K^+ATPase (Hammerstrom and Smith, 1979) and may have a relatively low affinity for rodent ATPase (Detweiler, 1967).

The model for the catalytic sequence of action of the enzyme was proposed by Siegal and Allens (1967). The first step is phosphorylation by ATP which is dependent upon both Na^+ and Mg^{++} ions. The Mg^{++} ions are also essential for the following

conformational change, during which Na^+ is released and so transported across the membrane. The K^+ ion is bound at the next step, the hydrolysis of the phosphate group from the enzyme, which is again followed by a conformational change leading to K^+ transport. The K^+ phosphatase activity is sometimes exploited to measure the activity of the enzyme, by monitoring phosphate release from compounds such as p-nitrophenylphosphate. It is thought that during each catalytic cycle of the enzyme, three Na^+ ions are transported out of the cell and two K^+ ions are transported inwards across the membrane (Glynn, 1964); therefore, both of these ions are transported in the direction opposing that of their passive diffusion across the membrane, and the transmembrane ion gradients are maintained. The imbalance in the ion exchange results in a net loss of ions; thus, the action of the enzyme may potentially regulate the concentration of intracellular ions (introduction 1.2.2) or intracellular water. The imbalance in the net charge of the ions transported, results in a net increase in the negative charge across the cell membrane, thus allowing the action of the enzyme to regulate transmembrane potential (introduction 1.2.2).

The activity of Na^+K^+ATPase cannot alone account for the total carrier-mediated movement of Na^+ and K^+ across the plasma membrane, and it was thought that the Na^+K^+ATPase did not always operate to mediate Na^+/K^+ exchange with the fixed stoichiometry of $3Na^+$: $2K^+$. It was proposed that under certain conditions the enzyme could also mediate net Na^+ efflux, independently of K^+ movement, or self-exchange of both Na^+ and K^+ (Glynn and Karlish, 1975). More recently, a second Na^+ K^+ transport system has been defined by its operational characteristics and by pharmacological distinction from the Na^+K^+ATPase; this system accounts for the bidirectional movement of ions.

The movement of ions due to this second system has been characterised by its sensitivity to the loop diuretics, which are so named because they act in the loop of Henlé in the kidney. They include ethacrynic acid, piretanide, furosemide and bumetanide (Palfrey et al, 1980). Diuretic-sensitive ion transport has been identified in a vast range of human, avian and mammalian cell types (for examples, see Aiton et al, 1981; 1982; 1984; Aiton and Simmons, 1983; Gardner et al, 1975; Geck et al, 1980; McManus et al, 1985; O'Brien and Krzeminski, 1983; Panet and Atlan, 1980; Palfrey et al, 1980; Owen, 1984; Sussman and O'Brien, 1985; Ueberschar and Bakker-Grunwald, 1983; Owen and Prastein, 1985; Kregenow, 1981; Ellory et al 1982; Hoffman et al, 1983; Hoffman, 1982; Saier and Boyden, 1984; McRoberts et al, 1982; Haas et al, 1982; Amsler et al, 1985; Frelin et al, 1986; Paris and Pouyssegur, 1986; Chipperfield, 1986).

The transport of ions via the system appeared to be an active transport process, since the ions were capable of movement against their concentration gradients (Hoffman and Kregenow, 1966). The operation of this system was detected in the presence of ouabain, providing evidence that it did not represent a separate mode of action of the Na^+K^+ATPase (Beauge and Lew, 1977; Schmidt and McManus, 1977). Schmidt and McManus (1977) suggested that the apparent movement of an ion against its concentration gradient was driven by the movement of the co-ion down its concentration gradient. Such a process depends upon the high degree of coupling between the movement of the ions and upon the high degree of specificity for each ion. The direction in which the ions are moved depends upon the ionic concentrations in the cells and in the extracellular medium (Palfrey and Greengard, 1981; Atlan et al, 1984).

In the diuretic-sensitive systems which have been examined to date, $^{86}Rb^+$ completely substitutes for K^+ for transport into the cell. The movement of $^{86}Rb^+$ or K^+ depends upon the presence of extracellular Na^+ ions. Few other positively charged ions can replace Na^+ and allow transport (Aiton et al, 1980; Owen and Prastein, 1985, Atlan et al, 1984; Geck et al, 1980), although there has been one report of partial replacement of Na^+ by choline$^+$ (Amsler et al, 1985), and various degrees of replacement of Na^+ by Li^+ have been reported (Aiton et al, 1980; Gardner et al, 1975; Geck et al, 1980; Owen and Prastein, 1985).

The activity of the transport system is also dependent upon extracellular Cl^- ions (Aiton et al, 1980; Aiton and Simmons, 1983; Amsler et al, 1985; Schmidt and McManus, 1977; Owen and Prastein 1985), although there is evidence that Cl^- may be replaced, at least in part, by Br^- (Geck et al, 1980; Gardner et al, 1975; Palfrey and Greengard, 1981; Schmidt and McManus, 1977).

Geck et al (1980) examined the diuretic-sensitive net movement of Na^+, K^+, and Cl^- ions into Na^+-loaded, K^+-depleted Ehlrich ascites cells in the presence of ouabain. They calculated that the transport system mediated influx of all three ions into the cell in the ratio of $1Na^+ : 1K^+ : 2Cl^-$. The activity of the system was therefore not electrogenic. The net movement of the ions was driven by the sum of the individual electrochemical gradients across the cell membrane. The chloride ions were transported into the cell against the electrical potential of the membrane, which suggested that, either an active transport system was operating, or, that the electrochemical gradients were sufficient to drive the process. Geck et al (1980) measured the total flow of ions at a range of different ionic concentrations, and calculated that even in the absence of a driving

force supplied by the gradients of the ions, there would still be substantial influx of ions. This calculation suggested that the transporter was directly coupled to a metabolic reaction to generate energy, and was supported by the observation that cotransport was reduced during metabolic inhibition, although this in itself was not conclusive proof of a direct effect on cotransport. Further, stimulation of cotransport was without effect on the rate of ATP turnover, and metabolism of ATP did not appear to be affected by inhibition of cotransport by furosemide. However, when the operation of the cotransporter is examined under different internal and external ion concentrations, the stoichiometric ratio of the transported ions may vary (Schmidt and McManus, 1977; Kregenow, 1977; Dunham et al, 1980; Wiley and Cooper, 1974; Atlan et al, 1984; Chipperfield, 1986). In addition, whilst the ions may be moved in either direction across the membrane, all the ions are not necessarily moved in the same direction under any given conditions (Schmidt and McManus, 1977; Jayme et al, 1981; 1984; Atlan et al, 1984). Diuretic-sensitive self-exchange of Na^+ and K^+ has also been detected (Dunn 1973; Lubowitz and Whittam, 1978; Schmidt and McManus, 1977; Duhm and Gobel, 1984).

Wiater and Dunham (1983) identified a Cl^--dependent K^+ influx in human and avian erythrocytes, which was sensitive to furosemide but independent of Na^+. It was suggested that the cells possessed two distinct furosemide-sensitive transport systems, for $Na^+K^+Cl^-$ and K^+Cl^-.

The direction of net diuretic-sensitive ion fluxes across the plasma membrane varies not only with the intra- and extra-cellular ion concentrations, but also according to the individual type of cell. Systems have been described which mediate no net flux (Tupper,

1975; Bakker-Grunwald, 1978; Garay, 1982; Dunn, 1970; Lubowitz and Whittam, 1969), or net outward flux (Panet and Atlan, 1980; Atlan et al, 1984) under physiological conditions.

The membrane proteins responsible for cotransport of $Na^+K^+Cl^-$ have not been isolated and identified, and the precise mechanism by which means ions are transported through the membrane is not known. Binding of the diuretics to the cotransporter has been used in attempts to identify the molecule(s) responsible (Forbush and Palfrey, 1983; Jorgensen et al, 1985). Jorgensen et al (1985) found that a protein of 34000 Dalton molecular weight was bound by bumetanide, and that this protein was associated with components of the cytoskeleton. Similarly, little is known about the mechanism by which the loop diuretics inhibit the cotransport system, although studies of binding of these agents to dog kidney membranes suggest that they associate reversibly with one of the anion sites, thus forming a complex between the transporter, the diuretic, $1Na^+$. $1K^+$ and $1Cl^-$, which is unable to transport ions (Forbush and Palfrey, 1983). Bumetanide has a greater affinity for the transporter than does furosemide (Palfrey et al, 1980); furosemide, but not bumetanide, has been observed to inhibit anion self-exchange (Palfrey et al, 1980; Brazy et al, 1976), phosphate transport (Walter, 1981), glucose transport (Jung and Mookerjee, 1976) and ATPases (Ludens et al, 1980). Wiater and Dunham (1983) suggested that there may be differences in the nature of the binding of furosemide and bumetanide to the contransporter of avian and human erythrocytes; bumetanide binding required Na^+, K^+ and Cl^- ions, but furosemide binding occurred in the absence of Na^+ and Cl^-, although it may not be fully effective in inhibiting transport under such conditions.

A protein of 36000 molecular weight which is phosphorylated

during transformation of cells with avian sarcoma viruses (Kobayashi et al, 1981; Kobayashi and Kaji, 1980; Erikson and Erikson, 1980) has also been shown to be associated with cytoskeletal components of the plasma membrane (Radke et al, 1983; Amini and Kaji, 1983). Whilst the similar molecular weights do not necessarily imply that the protein is related to the protein binding to the diuretics, it would be of interest to compare the binding patterns of labelled bumetanide and the antibody to the phosphorylated p36 protein (Radke et al, 1983).

The role of specific transport systems in the maintenance of Na^+ and K^+ homeostasis has been questioned (Ling, 1965; 1966; 1978; Negendank and Shaller, 1979a; 1979b). If the concentration gradients of Na^+ and K^+ across the cell membrane are maintained by the action of transport systems, the intracellular Na^+ and K^+ ions must be in a free state. However, it has been argued that Na^+ and K^+ ions may associate with anionic sites on cell macromolecules, and that the electron density on the site determines whether it has a greater affinity for Na^+ or K^+. The binding of K^+ is favoured over the binding of Na^+, so up to 99% of cellular K^+, but only 50% of cellular Na^+, may be in a bound state. Thus K^+ is preferably concentrated in cells (Ling 1966).

A second mechanism has been proposed to account for the exclusion of Na^+ from cells. Negendank and Shaller (1979a) proposed that a large proportion of the cell water was in a structured state capable of excluding ions. Ling (1966, 1978) has suggested that this is the major mechanism by which intracellular Na^+ is maintained at lower concentrations than in the extracellular environment.

The eukaryotic cell membrane contains a Na^+/H^+ exchange mechanism capable of catalysing inward or outward movement of Na^+

ions without affecting the membrane potential. The concomitant opposing movement of H^+ ions has a major role in the regulation of cellular pH (Roos and Boron, 1981; Boron, 1983; Moolenaar et al, 1984; L'Allemain et al, 1984b). It is sensitive to amiloride (3,5,diamino-6-chloro-N-(diaminomethylene)pyrazine-carboxamide; Cragoe, 1979; Vigne et al, 1982; 1983; Frelin et al, 1983), which competes for the site of binding of Na^+ ions; therefore inhibition occurs only at millimolar concentrations of amiloride. Although it is used to define the activity of the Na^+/H^+ system, amiloride is relatively nonspecific, and has been shown to interfere with activity of Na^+K^+ATPase (Soltoff and Mandel, 1983) and Na^+/Ca^{++} exchange (Smith et al, 1982).

The mechanism for Na^+/Ca^{++} exchange was suggested from observations that the extrusion of Ca^{++} from cardiac muscle and neuronal tissue was dependent upon extracellular Na^+ (Blaustein and Nelson, 1982). It has since been found in many types of cell, with the notable exception of the human erythrocyte (Schatzmann and Vincenzi, 1969), and may have a major role in maintaining the Ca^{++} concentration gradient across the cell membrane. The movement of Ca^{++} out of the cell against its concentration gradient is driven by the movement of Na^+ into the cell down its concentration gradient. Na^+ ions are then extruded by the Na^+K^+ATPase. Under certain conditions, the system may reverse, so mediating Na^+ efflux and Ca^{++} influx. Blaustein and Nelson (1982) reviewed evidence regarding the stoichiometry of Na^+/Ca^{++} exchange and concluded that in both 'forward' and 'reverse' modes of action, the stoichiometry of exchange was likely to be $3Na^+ : 1Ca^{++}$. Therefore, the process is electrogenic and may regulate, or be regulated by, the cell membrane potential.

Calcium ions may also regulate K^+ movements across the cell membrane. A Ca^{++}-sensitive channel for K^+ efflux was first demonstrated by Gardos (1958). The channel may be electrogenic (Hickman et al, 1984) and is important in K^+ homeostasis and regulation of cell volume in a number of cell types (Hoffmann, 1985a; 1985b; Grinstein et al, 1984). Activation of the channel is inhibited by cetiedil (Berkowitz and Orringer, 1982), oligomycin (Blum and Hoffman, 1971; Riordan and Passow, 1971), Ba^{++} ions (Lambert et al, 1984) and quinine (Lew and Ferreira, 1978). Agents which inhibit calmodulin may also affect the activity of the channel (Plishker et al, 1980; Grinstein et al, 1982c; Hoffmann et al, 1980; 1984; Lackington and Orrego, 1981) but the role of calmodulin is unclear (Lew et al, 1982; Lassen et al, 1980).

Quinine is most frequently used to define K^+ movements due to the Ca^{++}-stimulated channel (Lew and Ferreria, 1978). It is thought to displace K^+ from a binding site essential for maintaining the channel in an 'open' state (Reichstein and Rothstein, 1981).

In conclusion, from the list of systems for Na^+ and K^+ transport, although by no means exhaustive and complete, it is evident that mammalian cells possess a number of mechanisms which may interact with each other to maintain Na^+ and K^+ homeostasis.

1.2.2 The role of sodium and potassium ions in cell replication

The role of changes in ion fluxes across the plasma membrane in the initiation and control of cell replication has been investigated in detail (Kaplan, 1979; Leffert, 1980). The mitogenic transformation of lymphocytes has been extensively used as a model system (Hume and Weidemann, 1980), and changes in cation movement have been implicated in the mitogenic process and the maintenance of the transformed state (Quastel and Kaplan 1968; Kaplan 1979).

Reduction in intracellular K^+ was shown to inhibit cell proliferation (Lubin, 1967; Lamb and McCall, 1972; Ledbetter and Lubin, 1977; Shank and Smith, 1976; McDonald et al, 1972, Quissel and Suttie 1973), and attention was focussed on the role of K^+ ions and the activity of K^+ transport systems in mitogenesis. Ouabain, the inhibitor of Na^+K^+ATPase (introduction 1.2.1) was shown to block the transformation of lymphocytes (Quastel and Kaplan, 1968) and fibroblasts (Rozengurt and Heppel, 1975). However, the activity of the Na^+K^+ATPase was not increased in all transformed cells, and both increases and decreases in the activity have been associated with transformed cells (Elligsen et al, 1974; Banerjee and Bosmann, 1976; Kasarov and Friedman, 1974; Kimelburg and Mayhew, 1975; Spaggiare et al, 1976).

Ledbetter and Lubin (1977) suggested that K^+ ions regulated cell metabolism by regulating the rate of protein synthesis, which, in turn, would affect metabolism of DNA and RNA. It was known that K^+ ions are required for protein synthesis, since the ribosomal subunit structure is stabilised by K^+. Removal of K^+ was shown to inhibit the translation of mRNA in vitro (Naslund and Hutton, 1971).

Ouabain was shown to reduce cellular K^+ content and the rate of proliferation (Mayhew and Levinson, 1968; Adam et al, 1979; Cuff and Lichtman, 1975a; 1975b; 1975c; Quastel and Kaplan, 1970). The effect of ouabain was not immediate (Quastel and Kaplan, 1970; Cuff and Lichtman, 1975c), with the inhibition of DNA and RNA synthesis lagging behind inhibition of protein synthesis, judged by incorporation of radiolabelled precursors (Quastel and Kaplan 1970). Cuff and Lichtman (1975c) found that, whilst all parts of the cell cycle were sensitive to ouabain, greatest sensitivity was found in S phase, which appeared to have the greatest dependence upon

correct ion homeostasis. Adam et al (1979) showed protein synthesis to be inhibited by reduction in K^+ content before RNA synthesis was affected.

The effects of K^+ ions on protein synthesis may not be due to changes in cellular K^+ content. Herzberg et al (1974) found evidence that the inhibition of protein synthesis by K^+ ionophores was mediated by their effects on the plasma membrane. Subsequently, ouabain-resistant K^+ transport was shown to be sensitive to K^+ ionophores. It was suggested that this inhibition interfered with the cellular ATP/ADP ratio which, in turn, inhibited protein synthesis (Panet and Atlan, 1979). However, Andersson (1979) pointed out that, whilst changes in cellular K^+ content might regulate protein synthesis, the cessation of DNA synthesis was unlikely to be due wholly to changes in cellular K^+ concentrations, since DNA synthesis was still maintained at low K^+ concentrations.

Sodium ions may be a major regulatory factor in cell replication (Leffert, 1980). Cone (1974) investigated Na^+ transport in relation to growth of cells in vitro. He noted that actively dividing cells, in the exponential phase of the culture, had a relatively low trans-membrane electrical potential, which increased as the culture reached stationary phase. He suggested that control of cell division might be exerted via changes in the activity of Na^+ transport systems in the cell membrane. In the dividing cells, the membrane was more permeable to Na^+, or, the systems to extrude Na^+ were less active. Thus, the membrane potential was relatively low, and intracellular Na^+ content was high, which allowed synthesis of messenger RNA involved in the process of cell division. The converse was true for quiescent cells (Cone and Tongier, 1973).

Cone's theory was supported by a number of observations; high concentrations of Na^+ stimulated RNA synthesis. Cone (1971) predicted that tumour cells and rapidly-dividing normal cells would have higher intracellular Na^+ concentrations; this was confirmed by Cameron et al (1980), Smith et al, (1981), and Zs-Nagy et al (1981). However, a more recent comparative study failed to find consistent changes in Na^+ and K^+ concentrations to be associated with malignant transformation (Moyer et al, 1982). Cone and Cone (1978) showed that ouabain activated DNA synthesis and nuclear division. This observation was consistent with Cone's theory (Cone, 1974) and the proposal that Na^+ was a mitogen (Stillwell et al, 1973), since ouabain would be expected to elevate intracellular Na^+ concentrations. Indeed, elevated intracellular Na^+ concentrations have been associated with stimulation of cell division (Rozengurt and Mendoza, 1980; Koch and Leffert, 1979). However, in most other cases, ouabain has been reported to block mitogenesis (Lamb and McCall, 1972; Cuff and Lichtman, 1975a).

There is also contradictory evidence regarding the proposal of Cone (1974) that membrane potential was involved in the regulation of cell division (Boonstra, 1982; Kiefer et al, 1980; Deutsch and Price, 1982; Mikkelson and Koch, 1981). Changes in transmembrane potential of synchronised cells as they progressed through the cell cycle were not those predicted from Cone's suggestions (Starnbrook et al, 1975). Similarly, the value of the membrane potential of tumour cells, whilst it was more dependent on Na^+ ions, was not consistently different from that of normal cells in the manner predicted by Cone (Shen et al, 1978). A direct causal role of membrane potential in control of replication is considered unlikely (Deutsch and Price, 1982).

Table 2

MEMBRANE SYSTEMS FOR THE TRANSPORT OF AMINO ACIDS

System					
A	**ASC**	**GLY**	**β**	**N**	**L**
Substrate Transported:					
most, especially Alanine Glycine Proline AIB	Alanine Serine Cystine	Glycine Sarcosine	β-alanine Taurine	Asparagine Glutamine Histidine	most, especially Leucine Isoleucine Valine Phenylalanine
Specific substrate:					
MethylAIB	Cystine Threonine	Glycine	Taurine	Glutamine	BCH[1]
Na^+ dependent:					
yes	yes	yes	yes	yes	no
Stereospecifity:					
moderate	high	-	?	high	moderate
Effect of low pH:					
inhibition	variable	none	?	inhibition	stimulation
Regulation by amino acid limitation:					
yes	no	no	no	yes	yes
Trans effects:					
inhibited	stimulated	?	?	?	stimulated
Regulation by hormones;					
yes	no	no	no	no	no
Cell distribution:					
most; not erythyrocytes reticulocytes	ubiquitous	rat hepatocyte + hepatoma pigeon erythrocyte rabbit retuculocyte	rat Ehrlich ascites cells	rat hepatocyte	ubiquitous hepatocyte

[1]BCH is 2-aminobicyclo-(2,2,1)-heptane-2-2-carboxylic acid

Taken from Shotwell et al (1983)

Sodium ions have an important role in the transport of nutrients across the plasma membrane, since the cell exploits the concentration gradient of Na^+ across the plasma membrane to provide the energy to accummulate nutrients. A number of membrane systems for the transport of neutral amino acids have been identified in mammalian cells, and many of these are directly dependent on the cotransport of extracellular Na^+ down its concentration gradient (Table 2). Thus, the transport of many neutral amino acids depends upon the maintenance of the Na^+ concentration gradient by the action of Na^+K^+ATPase (Schultz and Curran, 1970). It was suggested by Shank and Smith (1976) that cellular amino acid transport is finely regulated at critical levels, so that the transport might represent a point of control of cell metabolism. However, there is evidence against this proposal. The amino acid content of quiescent cells is often elevated compared to that of their dividing counterparts, as a consequence of reduced protein synthesis in quiescent cells (Oxender et al, 1977). It is therefore unlikely that fine control of metabolism and replication is exerted by limitations of supply of amino acids (Oxender et al, 1977; Pardee et al, 1978).

Further evidence that Na^+ ions may have a role in the control of replication comes from the observations of Kroeger (1963), who found that Na^+ can alter the selective transcription of genes. An elevation in the intracellular Na^+ content of mature, differentiated cells was shown to be associated with an increase in the transcription of genes whose products were more common in their immature progenitor cells.

The combined content of Na^+ and K^+, or the ratio of Na^+ and K^+ ions, within the cell may also be the determining factor in the effect of Na^+ and K^+ on cell metabolism (Kimelburg and Mayhew, 1975;

Fossel and Solomon, 1979). Variation in Na^+ and K^+ concentrations were found to induce preferential translation of certain mRNA polymers in a cell-free system (Carrasco and Smith, 1976). The removal of Na^+ and K^+ from certain anionic groups on proteins may represent a point of control during cell division, and may be particularly involved in the change in DNA structure prior to replication (Kellermayer and Hazelwood, 1979), Changes in intracellular Na^+ and K^+ concentrations have been associated with protein turnover (Shinohara and Piatigorsky, 1977), although it is uncertain whether a direct relationship exists (Piatigorsky et al, 1978).

Moscatelli et al (1979) induced drastic changes in intracellular Na^+ and K^+ concentrations of chick embryo fibroblasts, by variation of extracellular ion concentrations, yet they observed no change in the rate of incorporation of radiolabelled precursors into DNA. In conclusion they stated that there was evidence against a direct casual relationship between intracellular ion concentrations and DNA synthesis in the control of cell proliferation.

It was proposed that upon treatment of lymphocytes with phytohaemagglutinin (PHA), the mitogenic transformation depends upon an increase in the Na^+K^+ATPase activity, which causes the increase in cellular K^+ concentrations responsible for stimulation of DNA synthesis (Kaplan et al, 1976); however, Kaplan (1979) subsequently showed that there was a decrease in cellular K^+ content upon PHA treatment, and Segel et al (1978) demonstrated that K^+/K^+ exchange was increased, and K^+ content was unchanged (Segel et al, 1976a). Thus an increase in K^+ influx was matched by an increase in K^+ efflux (Segel et al, 1976b; Kaplan, 1979; Iverson, 1976). These observations forced reconsideration of the role of Na^+K^+ATPase in

mitogenesis. It was suggested that the process required an increase in Na^+K^+ATPase activity in order to allow an increase in amino acid transport for protein synthesis (Kaplan, 1979). Further to the suggestion that cell division required reduced concentrations of cAMP (introduction 1.1.4.5) it was proposed that the increased Na^+K^+ATPase activity was required to deplete the ATP supply for cAMP synthesis (Lelievre et al, 1977).

Changes in intracellular K^+ concentration may arise through changes in the transport of water as well as through changes in K^+ transport (introduction 1.2.3). Holien et al (1979) found no change in the K^+ content of lymphocytes upon stimulation with PHA. However, there was an immediate decrease in the volume of lymphocytes, with a concomitant transient increase in K^+ concentration. It was suggested that the regulation of cell volume may be involved in the control of proliferation.

The $Na^+K^+Cl^-$ cotransporter is thought to make a major contribution to the regulation of cell volume in a number of cell types (MacKnight and Leaf, 1985; Geck and Pfeiffer, 1985). The identification of K^+ transport due to this system by use of the loop diuretics has allowed more precise characterisation of the systems responsible for K^+ fluxes, and the effect of mitogens on $Na^+K^+Cl^-$ activity has been the subject of recent study (O'Brien and Krzeminski, 1983; Paris and Pouyssegur, 1986; Owen, 1985; Sussman et al, 1985).

The phorbol ester phorbol-12-myristate-13-acetate (PMA;TPA) stimulates Na^+K^+ATPase activity in quiescent fibroblast cells leading to an increased $^{86}Rb^+$ influx (Moroney et al, 1978; Dicker and Rozengurt, 1981). In contrast O'Brien and Krzeminski (1983) found a rapid, but not immediate, inhibition of activity of the $Na^+K^+Cl^-$

cotransporter in actively-dividing cells. Both the unidirectional influx and efflux of K^+ were inhibited, and in contrast to earlier reports the intracellular K^+ content was increased. There was no effect upon Na^+K^+ATPase activity, also in contrast to earlier reports (Dicker and Rozengurt, 1981; Moroney et al, 1978). Owen (1984) reported that TPA stimulated $Na^+K^+Cl^-$ cotransport. However, in a subsequent study it was noted that the results were not reproducible with different lots of TPA and evidence was presented that $Na^+K^+Cl^-$ cotransport was inhibited by TPA (Owen 1985), in agreement with the results of O'Brien and Krzeminski (1983). TPA was found to exert 40% maximal inhibition of $Na^+K^+Cl^-$ cotransport activity in vascular smooth muscle cells, but it was capable of 100% inhibition of the stimulation of $Na^+K^+Cl^-$ cotransport activity observed after treatment with epidermal growth factors (Owen, 1985).

TPA was also shown to inhibit $Na^+K^+Cl^-$ cotransport in Balb/C 3T3 cells by Sussman and her coworkers (Sussman and O'Brien, 1985; Sussman et al, 1985). Intracellular K^+ concentrations were increased and the enzyme ornithine decarboxylase was induced (Sussman et al, 1985; O'Brien et al, 1979), in common with the effects of TPA in other cells (Wu and Byus, 1981; Yuspa et al, 1976; Kensler et al, 1978; O'Brien et al, 1975). In a mutant cell line deficient in $Na^+K^+Cl^-$ cotransport (Sussman and O'Brien, 1985) TPA failed to alter K^+ fluxes or induce the enzyme ornithine decarboxylase (Sussman et al, 1985). The authors concluded that the ionic signal required to induce ornithine decarboxylase was not generated in the mutant defective in $Na^+K^+Cl^-$ cotransport.

Phorbol esters bind to specific receptors in the cell membrane (Dicker and Rozengurt, 1981; Rossof et al, 1984), which may be associated with the phospholipid- and calcium-dependent protein

kinase C (Kikkawa et al, 1983; Nishizuka, 1984; Castanga et al, 1982) with the phorbol ester acting as a substitute for endogenous diacylglycerol (Nishizuka, 1984; Castagna et al, 1982). The substrates for the protein kinase C activity are not all known, but may include the receptor for epidermal growth factor (EGF; Moon et al, 1984; Davies and Czeck, 1984; Iwashita and Fox, 1984; Cochect et al, 1984; also Whiteley et al, 1984). TPA was shown to inhibit EGF-stimulated processes of Na^+ influx, $Na^+K^+Cl^-$ cotransport and DNA synthesis (Owen, 1985). It was suggested that TPA activated protein kinase C and the subsequent phosphorylation of the EGF receptor might alter the affinity for EGF, and thus alter EGF-stimulated processes (Owen, 1985).

The binding of EGF to its receptor and the binding of TPA to its receptor have effects on the same cellular processes. EGF induces mitogenesis (Taylor et al, 1972) and changes $Na^+K^+Cl^-$ cotransport activity (Owen, 1984; Owen and Prastein, 1985; Paris and Pouyssegur, 1986) and Na^+/H^+ exchange activity (Owen and Villereal, 1983a; 1983b; 1983c; Burns and Rozengurt, 1984; Rothenberg et al, 1983; Rozengurt, 1981) leading to stimulation of DNA synthesis (Rozengurt, 1981; Burns and Rozengurt, 1981). TPA stimulates Na^+ influx and DNA synthesis (Sussman et al, 1985, Cuatrecasas, 1984; Moolenaar et al, 1984; Rossoff et al, 1984; Dicker and Rozengurt, 1981; Besterman and Cuatrecasas, 1984; Swann and Whitaker, 1985). TPA can also affect movement of Na^+ through epithelial Na^+ channels (Civan et al, 1985). However, the action of TPA and growth factors upon the transport systems may be very different. Thus, whilst TPA and EGF both stimulated Na^+/H^+ exchange in vascular smooth muscle cells, their effects were not additive, and TPA inhibited the EGF-stimulated Na^+ flux: similarly, both agents stimulated DNA synthesis, but TPA inhibited EGF-stimulated DNA synthesis (Owen 1985).

There is evidence that EGF and other mitogens, including serum growth factors, stimulate $Na^+K^+Cl^-$ cotransport activity (Amsler et al, 1985; Smith and Rozengurt, 1978; Rozengurt and Heppel, 1975; Panet et al, 1982; 1983; Paris and Pouyssegur, 1986; Owen, 1985; Owen and Prastein 1985) but serum alone failed to stimulate $Na^+K^+Cl^-$ cotransport in cultured human fibroblasts (Owen and Prastein, 1985). TPA was thought to inhibit Na^+ K^+Cl^- cotransport (see above); however, besides the discredited report of Owen (1984), there is a recent report of stimulation of cotransport activity in response to TPA. Paris and Pouyssegur (1986) reported that EGF, TPA and the mitogenic hormone thrombin, were all found to stimulate $Na^+K^+Cl^-$ cotransport activity in a Chinese hamster fibroblast line, which was a mutant lacking Na^+/H^+ antiport activity. The authors proposed that these results were suggestive of an involvement of phosphorylation reactions in the stimulation of $Na^+K^+Cl^-$ cotransport (Paris and Pouyssegur, 1986), on the basis of the following evidence: Firstly, TPA activates protein kinase C (see above); secondly, EGF binds to a receptor which has a tyrosine kinase activity (Carpenter, 1984); thirdly, thrombin also stimulates phosphatidylinositol turnover (Paris and Pouyssegur, 1986), which can activate phosphorylation reactions (Berridge, 1984), although the effect of thrombin on $Na^+K^+Cl^-$ cotransport may not be wholly dependent upon this pathway (Paris and Pouyssegur 1986).

Serum was shown to stimulate the influx of $^{86}Rb^+$ into 3T3 cells without changing the affinity for the ion (Panet et al, 1982), and Paris and Pouyssegur (1986) found no change in the affinity for extracellular Na^+ and K^+ upon stimulation of $Na^+K^+Cl^-$ contransport with thrombin; however, they speculated that there may be a change in the affinity for intracellular ions, similar to that detected in the

Na^+/H^+ antiport after mitogenic stimulation (Paris and Pouyssegur, 1984). This suggestion is supported by the observations of Paris and Pouyssegur (1986) that the rapid effect on cotransport activity suggested a direct effect of the mitogen on the cotransporter. The effects did not appear to be mediated by changes in intracellular cAMP concentration, which may regulate $Na^+K^+Cl^-$ cotransport activity in certain cell types (introduction 1.2.1). The activation of $Na^+K^+Cl^-$ cotransport was not due to activation of a Na^+/H^+ antiporter in the deficient mutant cell line. Modification of the mechanism of action of $Na^+K^+Cl^-$ cotransporter in response to mitogens was suggested from the results of Panet (1985). It was shown that serum growth factors caused the $Na^+K^+Cl^-$ cotransporter to mediate net K^+ influx instead of K^+/K^+ exchange in 3T3 cells.

Paris and Pouyssegur (1986) found evidence that EGF and TPA stimulated cotransport activity by different mechanisms. EGF, in contrast to TPA, does not stimulate phosphatidylinositol turnover, and thus does not activate protein kinase C (see also Vara and Rozengurt, 1985). The effects of EGF and TPA on the stimulation of $Na^+K^+Cl^-$ cotransport were found to be additive.

Preincubation with TPA was found to inhibit EGF- and thrombin-stimulated $Na^+K^+Cl^-$ cotransport, and it was suggested that this effect was consistent with negative feedback inhibition by activated protein kinase C (Paris and Pouyssegur, 1986). The authors also concluded, in agreement with Amsler et al (1985), that $Na^+K^+Cl^-$ cotransport was unlikely to be a major control point for the ionic events of mitogenesis, since complete blockage of the transporter by bumetanide failed to completely inhibit DNA synthesis associated with mitogenic stimulation.

Changes in intracellular pH, pH_i, have also been associated with mitogenesis (Moolenaar et al, 1984; Paris and Pouyssegur, 1986; Pouyssegur et al, 1985; L'Allemain et al, 1984b). It has been proposed that small changes in pH_i may cause changes in the activity of enzymes at key points in the control of cell replication. Mitogens which stimulate Na^+/H^+ exchange (see above) lead to alkalinisation of the intracellular pH, which is essential for progression of mitogenesis (Moolenaar et al, 1984; Pouyssegur et al, 1985). In the mutant hamster fibroblast line deficient in Na^+/H^+ antiport, cellular alkalinisation did not occur in response to thrombin (Paris and Pouyssegur, 1986; L'Allemain et al, 1985). However, acidification of the intracellular pH was found to inhibit $Na^+K^+Cl^-$ cotransport activity in the presence and absence of thrombin, providing evidence that the $Na^+K^+Cl^-$ contransport system is sensitive to changes in cellular pH.

In summary, whilst it is clear that the movement of ions across the plasma membrane may direct the events of cell division, the precise biochemical reactions at which control may be exerted have yet to be fully elucidated, and different events may be involved in the response to different stimuli in different cell types.

1.3 WORK PROMPTING THE PRESENT STUDY

Ever since the earliest investigations of the pharmacological activity of the alkylating agents, effects on the plasma membrane have been recorded (Peters, 1947; introduction 1.1.4.6). The present study was undertaken to investigate in more detail the effects of nitrogen mustard on plasma membrane function, with particular reference to ion transport. The regulation of ion transport is important for correct cellular metabolism and replication (introduction 1.2.2) and as such may represent a potential target for the chemotherapy of cancer (Tritton and Hickman, 1984). A number of biochemical differences exist between the surfaces of neoplastic cells and their normal counterparts which may be exploited by immunological methods to direct anticancer drugs and thus improve their selectivity (Baldwin and Byers, 1982).

Grunicke et al showed that administration of 10^{-6} moles per kilogram body weight of Trenimon (2,3,4,tris(ethyleneimino)-benzoquinone) to tumour-bearing mice interfered with transport functions of the plasma membrane of Ehrlich ascites tumour cells (Grunicke et al, 1979; Ihlenfeldt et al, 1981). Four hours after administration of Trenimon, the initial rates of transport of 3-O-methyl-D-glucose, cycloleucine, and aminoisobutyric acid into tumour cells were inhibited by approximately 80%, 70% and 50% respectively. Transport of thymidine was similarly inhibited, but DNA synthesis continued (Grunicke et al, 1979). The transport of 3-O-methyl-d-glucose was carrier mediated and was still maximally inhibited 12 hours after a single injection of Trenimon. The transport of the two amino acid analogues was inhibited by a similar extent, even though they were selected to represent two different amino acid transport systems. Aminoisobutyric acid is taken up

largely by the Na^+-dependent 'A' system (Oxender and Christensen, 1963; Van den Berg and Betel, 1973; introduction 1.2.1). The effects were not due to inhibition of protein synthesis. Ihlenfeldt *et al* (1981) concluded that these results indicated that Trenimon was inhibiting Na^+-dependent transport processes, possibly *via* an interaction with the Na^+K^+ATPase enzyme.

The activity of Na^+K^+ATPase was examined by measurement of the uptake of ^{86}rubidium ions. ^{86}Rubidium is widely used as a congener for potassium, since it has a longer half life and is thus easier to handle than the radioisotopes of potassium (Lowe and Burch 1953; Banerjee and Bosman, 1976; Beauge and Ortiz, 1970; Banerjee *et al*, 1977; Geny *et al*, 1979; Brown and Lamb, 1978). Trenimon was found to inhibit the influx of $^{86}Rb^+$ into Ehrlich ascites cells within 30 minutes of administration of 10^{-6} mole kg^{-1} to tumour-bearing mice. Further, $^{86}Rb^+$ uptake was found to be wholly sensitive to $5x10^{-4}$M ouabain, the cardiac glycoside inhibitor of Na^+K^+ATPase. The transport of aminoisobutyric acid was also inhibited by ouabain; however, although Ihlenfeldt *et al* recognised that cycloleucine may be transported at least in part by a Na^+-dependent system, the susceptibility to ouabain was not investigated. At a higher concentration of Trenimon (10^{-4}M), which maximally inhibited the Rb^+ transport system *in vivo*, there was inhibition of transport within 30 seconds of addition of this agent to Ehrlich ascites cells *in vitro*; it was suggested that this result implied that the effects upon $^{86}Rb^+$ transport were due to a direct and immediate interaction with Na^+K^+ATPase. The authors proposed that inhibition of transport of glucose, amino acids or thymidine (Grunicke *et al*, 1975) was unlikely to limit cell growth, and that inhibition of Na^+K^+ATPase was involved in the cytotoxic mechanism of Trenimon (Ihlenfeldt *et al*, 1981).

Subsequently, the same group of workers demonstrated an effect of nitrogen mustard on $^{86}Rb^{+}$ transport in Ehrlich cells (Grunicke et al, 1982; 1983).

Nitrogen mustard was injected i.p. into tumour bearing mice. The animals were sacrificed four hours later and the cells were harvested. The influx of $^{86}Rb^{+}$ into the Ehrich ascites cells was presumed to represent the activity of $Na^{+}K^{+}$ATPase, since it was earlier shown to be completely inhibited by ouabain (Ihlenfeldt et al, 1981). All concentrations of HN2 which were able to inhibit further proliferation of the tumour cells were shown to depress $^{86}Rb^{+}$ transport. Similar effects were noted for Trenimon, confirming earlier observations (Grunicke et al, 1979; Ihlenfeldt et al, 1981) and for phosphamide mustard (N,N-bis(2chloroethyl)diamidophosphoric acid) which does not penetrate the membrane of Ehrlich ascites cells (Lenssen and Hohorst, 1979). Although the $^{86}Rb^{+}$ influx was never fully inhibited, the authors suggested that the results provided good evidence that alkylation of the tumour membrane, and the interference with $Na^{+}K^{+}$ATPase activity, may be important in the mechanism of action of certain alkylating agents (Grunicke et al, 1982; 1983). The same study showed that chlorambucil was without effect on $^{86}Rb^{+}$ transport, although it was shown in a preliminary study that this agent was cytotoxic to cells when it was linked to large polymer molecules (Grunicke et al, 1979). Thus, the role, if any, of inhibition of ion transport in the mechanism of action of HN2 does not apply to all alkylating agents.

Baxter et al (1982) and Spurgin (1981) found similar effects of HN2 on the transport of $^{86}Rb^{+}$ into PC6A murine plasmacytoma ascites cells. PC6A cells were incubated in vitro with 10^{-5}M HN2, which concentration produced greater than 99% cell kill in an in vitro -

in vivo bioassay. Briefly, $2x10^7$ PC6A cells per ml, were incubated in tissue culture medium RPMI 1640 with various concentrations of HN2. Animals were injected i.p. with 10^7 cells, and upon sacrifice seven days later the cells were harvested from the peritoneum and counted. The approximate number of cells killed was calculated by comparison with the number of cells harvested from animals which received cells which had been incubated in the absence of drug (Gescher *et al*, 1981).

After incubation for 4 hours with 10^{-5}M HN2, $^{86}Rb^+$ transport into PC6A cells was inhibited by 45%. No immediate effect of 10^{-5}M HN2 on $^{86}Rb^+$ transport was detected, although higher concentrations of HN2 (10^{-3} and 10^{-2}M) immediately depressed $^{86}Rb^+$ uptake (Baxter *et al*, 1982). In the same series of experiments, preincubation for 4 hours with 10^{-5}M HN2 was shown to selectively inhibit Na^+-dependent transport of amino acids: transport of aminoisobutyric acid was inhibited by 19%, whereas transport of cycloleucine was inhibited by only 5%.

A monofunctional analogue of HN2 ((2chloroethyl)dimethylamine) which was without effect on the growth of the PC6A ascites tumour, was also without effect on $^{86}Rb^+$ transport; thus a crosslinkage appeared to be important for the inhibition. The requirement of bifunctionality for antitumour activity was satisfied, suggesting that inhibition of $^{86}Rb^+$ uptake was important for the cytotoxicity of nitrogen mustard.

Incubation of PC6A cells with 10^{-5}M HN2 was calculated to represent a slight overestimate of the likely concentration of drug following administration of a therapeutic dose *in vitro*. A dose of $0.8mgkg^{-1}$ HN2, representing a concentration of $4x10^{-6}$M if equal distribution was assumed, caused 90% inhibition of PC6A tumour growth (Baxter *et al*, 1982).

Baxter et al (1982) proposed that the locus for the inhibition of $^{86}Rb^{+}$ transport into PC6A cells by HN2 was the $Na^{+}K^{+}$ATPase enzyme, which was known to be susceptible to crosslinkage and alkylation (Skou, 1963; Sweadner, 1977). An investigation was made into the effect of HN2 on the activity of $Na^{+}K^{+}$ATPase in vitro. In a crude PC6A cell membrane preparation, where mitochondrial Mg^{++}ATPases were selectively inhibited by 40nM dicyclohexlycarbodiimide (Beechey et al, 1967), the total phosphatase activity was 91±7% sensitive to 10^{-3}M ouabain. After incubation at 37°for 30 minutes with 10^{-3}M HN2, ouabain-sensitive phosphatase activity was inhibited by approximately 44%, but ouabain-insensitive phosphatase activity was reduced by only 9%. However, it was stressed that there was a large variation in the rate of enzyme activity in different membrane preparations, and it was suggested that further purification of $Na^{+}K^{+}$ATPase was required to allow further study.

The work of Spurgin (1981) and Baxter et al (1982) therefore gave rise to a number of observations regarding the cytotoxic mechanism of nitrogen mustard which were the basis of further investigation.

A number of problems were associated with the use of the PC6A tumour as the system in which to study the effect of HN2 on $Na^{+}K^{+}$ATPase. The cell line is passaged in mice and has only a limited survival time in vitro (Spurgin, 1981), which in turn has obvious limitations on prolonged experimentation. However, it also prevents experimentation with cells in defined phases of the cell cycle, except after lengthy separation procedures. Such experiments are of interest; any biochemical effect of HN2 must explain the increased susceptibility of dividing cells in comparison with their quiescent counterparts (introduction 1.1.1) if it is to be considered

relevant to the cytotoxic action. Error may arise in the *in vitro-in vivo* bioassay technique (Gescher *et al*, 1981), because it is carried out by killing mice and determining cell death only at one single time point; this also follows shortly after the exposure of cells to the drug *in vitro*, also for a limited, single period. Therefore the assay does not distinguish between the reduction in cell numbers due to the death of cells and the reduction due to delayed growth (Spurgin, 1981). The colony forming assay for cells plated in soft agar (Tew and Wang, 1982) allows this distinction, but is prevented by poor survival of PC6A cells *in vitro*.

In view of these problems, the L1210 murine lymphoma was chosen as the system in which to investigate further the effects of HN2 on membrane function. The cell line may be passaged as an ascitic tumour in mice, but is readily maintained *in vitro* in suspension culture, thus allowing direct comparison between effects observed *in vitro* and *in vivo*. The L1210 tumour *in vivo* is used more frequently than the PC6A tumour to screen for activity in potential antitumour compounds, and it is susceptible to alkylating agents. Maintenance of the cell line *in vitro* allows accurate definition of the rate of cell proliferation, and the identification of rapidly dividing and stationary cell populations. Experiments are not limited in time by short survival *in vitro* or death of a host animal.

The aims of the present study are summarised as follows:-

(a) to confirm the observations of Baxter *et al* (1982) on the effects of HN2 on ^{86}rubidium transport in PC6A cells.

(b) to characterise the proliferation of L1210 cells *in vitro* and the sensitivity of proliferation to inhibition by HN2;

(c) to investigate the effects of HN2 on $^{86}Rb^{+}$ transport into L1210 cells; to identify the cellular target of HN2 which gives rise to the observed effects; to investigate what consequences the inhibition of $^{86}Rb^{+}$ transport may have for cell metabolism and replication, and to investigate its role, if any, in the mechanism of action of nitrogen mustard;

(d) to investigate the relationship between the effects of HN2 on $^{86}Rb^{+}$ transport and the pharmacological activity of HN2; to account for the susceptibility of rapidly dividing cells, the existence of resistant cells, and the requirement of bifunctionality for antitumour activity.

Section 2

MATERIALS

2.1 Equipment

The Heraeus Christ Labofuge 6000 centrifuge was purchased via LKB Ltd, Croydon, Surrey, U.K. The Beckman Microfuge B microcentrifuge and the Beckman LS230 liquid scintillation counter were purchased from Beckman-RIIC Ltd, High Wycombe, Bucks, U.K. The MSE Hi-Spin 21 and the Pegasus AP ultracentrifuges were purchased from MSE Scientific, Crowley, Sussex, U.K. The Packard Tri-Carb 2660 liquid scintillation counter and glass scintillation vials with caps were purchased from Packard Instruments Ltd, Caversham, Berks., U.K. The ICN Tracerlab Gamma Set 500 gamma detector was purchased from Tracerlab Services Ltd., Twickenham, Middlesex, U.K. Gamma detector sample tubes (LP3) and tops (SP3) were purchased from Luckham Ltd., London, U.K. Polypropylene Microfuge tubes (0.4ml and 1.5ml) were purchased from Alpha Laboratories, Eastleigh, Hants., U.K.

2.2 Tissue culture equipment

All sterile procedures were carried out in an Envair Class II Microbiological safety cabinet, with unidirectional laminar downflow (Envair Ltd., Rossendale, Lancs., U.K.). Cell cultures were incubated in a Flow Laboratories CO_2 incubator (Flow Laboratories, Irvine, Scotland, U.K). Cell suspensions were routinely centrifuged in the Heraeus Christ Labofuge 6000 centrifuge (see above, 2.1) unless otherwise stated. Sterile disposable tissue culture plastics - 30ml universal containers, 7ml bijou bottles, petri dishes, 50ml and 300ml culture flasks, and pipettes - were purchased from A/C Nunc, Kamstrup, Denmark (via Gibco, Paisley, Scotland) and Sterilin Ltd, Teddington, Middlesex, U.K. (via Baird and Tatlock Ltd, Chadwell, Essex, U.K.) The Coulter Counter and Channelyzer, and Isoton were purchased from Coulter Electronics Ltd., Luton., Beds, U.K.)

2.3 Cell Lines

L1210 murine lymphoid leukaemia cells (Law et al, 1949) derived from ascitic fluid in DBA/2 mice, (Hutchinson et al, 1966) were purchased from Flow Laboratories, Irvine, Scotland, U.K. L1210R murine leukaemia cells made resistant to nitrogen mustard were the kind gift of Dr. Neil Gibson, National Cancer Institute, Bethesda, Maryland, U.S.A.

L1210 murine leukaemia cells nominally sensitive to cisplatin were the kind gift of Prof. K. Harrap, Institute of Cancer Research, Sutton, Surrey, U.K. K562 human erythroleukaemia cells (Lozzio and Lozzio, 1975) were obtained from Dr. Michael Tisdale, CRC Experimental Chemotherapy Research Group, Aston University.

The L1210 murine leukaemia in vivo cell line, and the Adj-PC6A murine plasmacytoma (Potter and Roberston, 1960) were cell lines routinely passaged as ascites in the peritoneal cavity of mice, in the Animal Unit, Department of Pharmaceutical Sciences, Aston University.

2.4 Buffers and Tissue Culture Media

RPMI 1640 medium containing 25mM HEPES, with L-glutamine, Fischers medium (with L-glutamine, without antibiotics), horse serum (mycoplasma screened), and foetal calf serum (virus and mycoplasma screened) were purchased from Gibco, Paisley, Scotland, U.K. Noble agar was purchased from Difco Laboratories Ltd., East Molesley, Surrey, U.K. Phosphate buffered saline, pH7.3, was made from Oxoid Buffer Tablets (Dulbecco A), purchased from Oxoid Ltd., Basingstoke, Hants, U.K. Krebs-Ringer bicarbonate buffer pH7.4 (KR), consisted of 118mM NaCl, 5mM KCl, 25mM $NaHCO_3$, 1.18mM $MgSO_4 7H_2O$, 1.17mM KH_2PO_4, 5.5mM glucose, except where modified as stated in the text. Phenol red was added to $10mgl^{-1}$ to serve as pH indicator. The buffer was

stored at 4° and incubated at the required temperature for 40 minutes before use and with continuous gassing, with 5% CO_2 in air. For the last 20 minutes 1.27mM $CaCl_2$ was added from a standard 1M solution (BDH see below). Modified (Parker, 1961) Earles Buffered Saline Solution (EBSS; Earle et al, 1943) consisted of 5.40mM KCl, 117.24mM NaCl, 26.19mM $NaHCO_3$, 1.08mM NaH_2PO_4, 0.81mM $MgSO_47H_2O$ and 5.56mM glucose. Phenol red was added ($10mgl^{-1}$) to serve as pH indicator. The buffer was stored at 4° and incubated before use, as for Krebs-Ringer buffer (above). $CaCl_2$ was added to a final concentration of 1.81mM.

2.5 Chemicals, Biochemicals and Pharmacological Agents

All chemicals were of analytical grade and purchased from BDH., Poole, Dorset, (U.K.) or Fisher Chemical Co, (U.S.A.), except where listed below. The following chemicals were purchased from Sigma Chemical Co, Poole, Dorset, U.K.: β-dimethylaminoethylchloride hydrochloride (HN2-1); ouabain octahydrate; furosemide (4-chloro-N-furfuryl-5-sulphamoyl-anthranilic acid); calcium ionophore A23187, free acid; monensin, sodium salt; DIDS (4,4 Diisothiocyanatostilbene 2,2-disulphonic acid), disodium salt; dbcAMP (N^6, 2'-O-dibutyryladenosine 3'5' cyclic monophosphate), sodium salt; amiloride hydrochloride; ATP (adenosine 5' triphosphate), disodium salt; reduced and oxidised glutathione; NADPH, tetrasodium salt; cycloleucine (1-aminocyclopentane carboxylic acid), α-aminoisobutyric acid; L-methionine; L-arginine; DTNB (5,5-dithiobis-(2-nitrobenzoic acid); firefly luciferase-luciferin reagent; glutathione reductase, Type III from Bakers Yeast (122 Units mg^{-1}); trypan blue; pararosanilin; methyl green; lanthanum chloride; choline chloride; polyethylene glycol 6000; dextran T500; α-naphthyl acetate; EGTA (ethylene glycol-bis-(β-amino ethylether) N, N'tetra acetic acid),

free acid; HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), sodium salt.

Rubidium chloride, choline bromide, quinine hydrochloride, and gluconic acid, sodium salt were purchased from Sigma Chemical Co., St Louis, Missouri, USA. Hexylene glycol was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Triphenylmethylphosphonium bromide was purchased from K & K laboratories (via Kodak, Liverpool, U.K.). Nitrogen mustard was the generous gift of Dr. V. Holland, Boots plc, Nottingham, U.K. Bumetanide (3-butylamino-4-phenoxy-5-sulphamoyl benzoic acid) was the generous gift of Ms. H. Court, Leo Laboratories Ltd., Aylesbury, Bucks., U.K.) Cis-diaminodichloroplatinum and trans-diaminodichloroplatinum were the generous gifts of the Johnson-Matthey Research Centre, Reading, UK. Chlorambucil was the generous gift of The Wellcome Foundation Ltd., Beckenham, Kent, UK.

2.6 Radiochemicals and Scintillation Fluid.

The following radiolabelled compounds were purchased from Amersham plc, Amersham, Bucks., U.K. : ^{86}rubidium chloride (1mCiml^{-1}, 280gml^{-1}); [^{14}C]-α-aminoisobutyric acid (50μCiml^{-1}, 58mCimmol) [^{14}C]-cycloleucine (1-aminocyclo-pentane-[1-^{14}C]-carboxylic acid;50μCiml^{-1}, 59mCimmol^{-1}); ^{22}sodium chloride (200μCiml^{-1}, 0.84 gml^{-1}); inulin-[^{14}C]-carboxylic acid (5mCimmol^{-1}); [^{3}H]-H_2O (5mCiml^{-1}); ^{45}calcium chloride (31mCimg^{-1}Ca^{++}, 1mCiml^{-1}); sodium ^{36}chloride (3.7mCig^{-1} Cl^{-}); sodium [^{51}Cr]-chromate (1mCiml^{-1}, 400mCimg^{-1}Cr); [^{3}H]-methionine (L-[methyl-^{3}H]-methionine; 83μCimmol^{-1}, 1Ciml^{-1}). [^{14}C]-DMO (5,5,-[2-^{14}C]-dimethyloxazolidine-2,4,-dione) (50mCimmol^{-1}), [7-^{14}C]-benzoic acid (19.3mCimmol^{-1}) and [^{3}H]-TPMP^{+} (41.5 Cimmol^{-1}) were purchased from New England Nuclear, Southampton, U.K. ^{42}Potassium chloride (2.67mCiml^{-1}, 373 mM)

and ^{86}rubidium chloride (3.42mCimg^{-1}, 35.281mCiml^{-1}) used in the U.S.A were purchased from New England Nuclear, Boston, Massacheussetts, U.S.A.

Fisofluor 'mpc' scintillation fluid was purchased from Fisons plc, Loughborough, Leics., U.K.

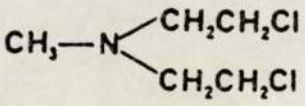
2.7 Gases and Liquid gases

Carbon dioxide, 5% carbon dioxide in air, and liquid nitrogen were purchased from British Oxygen plc, Wolverhampton, U.K.

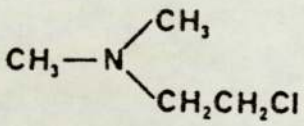
Figure 2

Structures of compounds referred to in this report

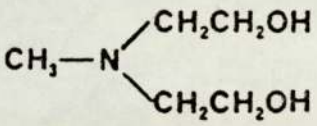
nitrogen mustard



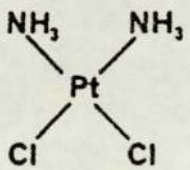
2-chloroethyldimethylamine



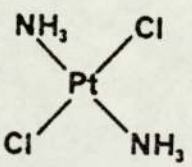
hydrolysed nitrogen mustard



cis-diaminodichloroplatinum (II)



trans-diaminodichloroplatinum (II)



chlorambucil

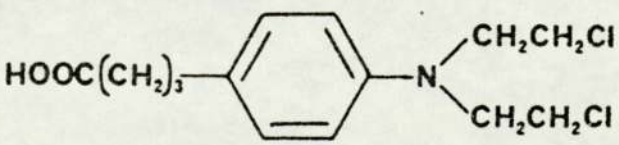
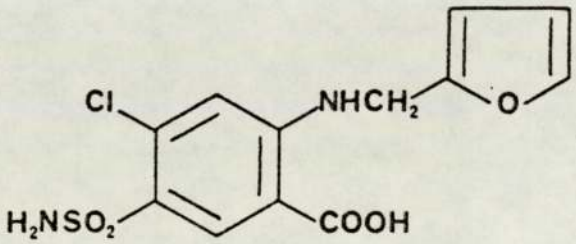
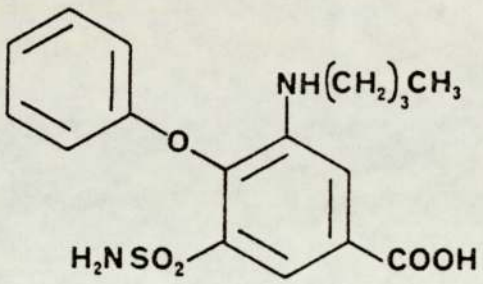


Figure 2 (continued)

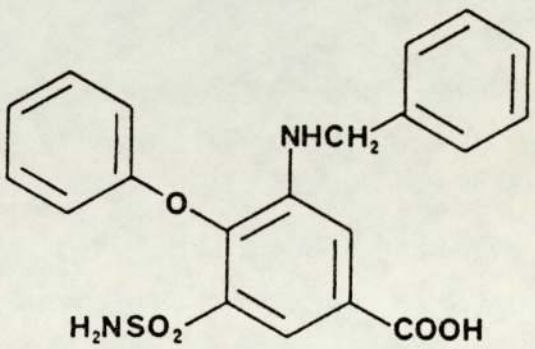
furosemide



bumetanide



benzmetanide



amiloride

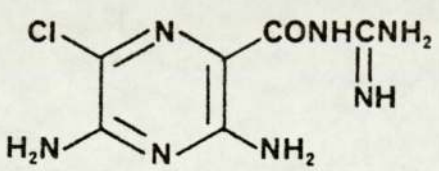
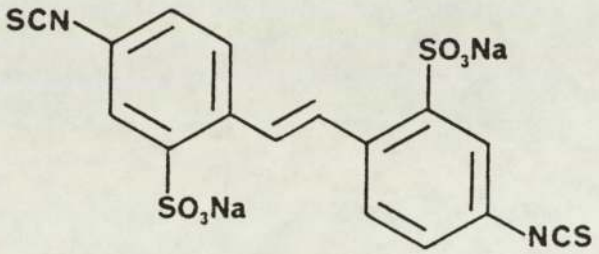
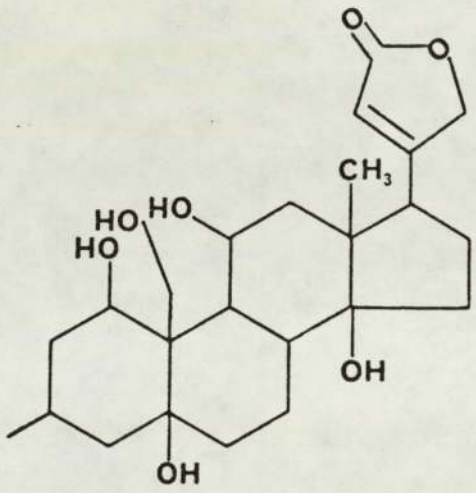


Figure 2 (continued)

DIDS



ouabain



Drawn by Roger Griffin.

Section 3

METHODS

3.1 MAINTENANCE OF CELL LINES

3.1.1 MAINTENANCE AND HARVEST OF CELL LINES *in vivo*

3.1.1.1 Passage and harvest of Adj/PC6A murine plasmacytoma cells in Balb/C mice

The Adj/PC6A murine plasmacytoma was passaged in female 20g Balb/C mice by weekly intraperitoneal injection of $5x10^5$ ascites cells. After 10 passages, a new line was established from cells which were stored in liquid nitrogen (methods 3.1.3). Seven days after tumour transplantation, the animals were killed by cervical dislocation. The skin of the abdomen was opened and 1ml of 0.9% (w/v) NaCl solution was injected into the peritoneum. Tumour cells were collected in a sterile 1ml syringe and placed in 10ml 0.9% NaCl solution on ice. The abdomen was opened and examined for solid tumours. The cell suspension was centrifuged at 350g (1500rpm) for 5 minutes. Erythrocytes were removed by repeated, alternate washes in 0.9% NaCl and Boyle's blood cell lysis medium (Boyle, 1968) consisting of 0.016M Tris-HCl, 0.0142M NH_4Cl, pH7.2, at 37^o. The cells were resuspended in 10ml 0.9% NaCl and the cell number was estimated (methods 3.1.6). The cells were finally washed and resuspended to $5x10^6$ cells per ml in RPMI.

3.1.1.2 Passage and harvest of L1210 murine leukaemia cells in DBA/2 mice

The L1210 murine leukaemia was passaged in female 20g DBA/2 mice by weekly intraperitoneal injection of $2x10^5$ ascites cells. After every 10th passage a new line was established from cells frozen in liquid nitrogen (methods 3.1.3). Four days after tumour transplantation the animals were injected with nitrogen mustard or 0.9% NaCl solution. Three hours later the animals were killed by cervical dislocation and the tumour cells were removed from the peritoneum and washed in the manner described in methods 3.1.1.1.

3.1.2 MAINTENANCE OF CELL LINES *in vitro*

3.1.2.1 Culture of K562 human erythroleukaemia cells

K562 Human erythroleukaemia cells were subcultured every three days to an initial cell density of $5x10^4$ per ml in 10% foetal calf serum in RPMI 1640 medium at 37^o in 50 or 300 ml culture flasks (Nunc) gassed with 10% CO_2 in air. New cultures were established from frozen stocks every 3-4 months (methods 3.1.4).

3.1.2.2 Culture of L1210 murine leukaemia cells

L1210 murine leukaemia cells were obtained as a suspension culture derived from ascites cells in male DBA/2 mice, and incubated at 37^o until a density of 10^6 cells per ml was attained. Cells were subcultured 2-3 times weekly in 10% horse serum in RPMI 1640 medium and incubated at 37^o in 50 or 300ml culture flasks (Nunc) gassed with 10% CO_2 in air. New cultures were established from frozen stocks every 3-4 months (methods 3.1.4).

3.1.3 Culture of L1210R murine leukaemia cells resistant to nitrogen mustard

L1210R murine leukaemia cells resistant to nitrogen mustard were maintained in the same manner as L1210 cells (methods 3.1.2.2).

3.1.3 STORAGE OF CELLS IN LIQUID NITROGEN

Cultured cells which were in the exponential phase of growth (results 4.1.2) or freshly-isolated passage cells (methods 3.1.1) were centrifuged at 350g (1500rpm) for 5 minutes. The cells were washed once in RPMI then resuspended in 90% of the volume of RPMI required to suspend cells to $2x10^6$ per ml, and cooled on ice for 10 minutes. DMSO was added to 10% final volume and 1ml aliquots of the suspension were dispensed into sterile plastic vials (Nunc). The vials were cooled overnight in a Union Carbide BF-5 Biological Freezer (via Jencons (Scientific), Leighton Buzzard, Beds., U.K.) at

a rate of 1^{o} per minute and afterwards stored under liquid nitrogen until required.

3.1.4 ESTABLISHMENT OF NEW CULTURES FROM FROZEN CELLS

A vial containing $2x10^{6}$ frozen cells (methods 3.1.3) was warmed to room temperature and the contents pipetted into a culture flask (Nunc) which contained 20ml of 20% foetal calf serum in RPMI. The final cell number was 10^{5} per ml and the final DMSO concentration was 0.5%. The cells were incubated for 3-4 days until the culture reached stationary phase (results 4.1.2). The cells were then subcultured daily for 5 days to a density of $2x10^{5}$ cells per ml in normal growth medium and thereafter handled as described in section 4.1.2. After each new culture was established, the mean generation time was determined (methods 3.1.5).

3.1.5 ESTIMATION OF MEAN GENERATION TIME OF CULTURES

A range of suspensions of 10^{4} - $5x10^{5}$ cells per ml in normal growth medium was prepared. Aliquots (2ml) were dispensed into Multiwell dishes (Nunc) and incubated at 37^{o} with full humidity in an atmosphere of 5% CO_2 in air. At each time point cells in duplicate wells were mixed by rapid pipetting 20 times in a Pasteur pipette. 1ml samples were removed and the cell number was determined (methods 3.1.6). An estimate was obtained of the time taken for the population of cells to double in number.

3.1.6 ESTIMATION OF CELL NUMBER

A 0.2-1.0ml sample of the cell suspension was diluted to 20ml with Isoton and the cell number was determined in the Z_{BI} Coulter Counter attached to a Coulter Channelyzer (Coulter Electronics Ltd; Luton, Beds., U.K.) with the appropriate settings as detailed below. The count was corrected for coincidence from the chart supplied with the instrument.

3.1.6.1 Coulter Counter settings for PC6A cells

Amplification	4
Threshold : Lower	10
Upper	>110
Aperture Current	0.5

3.1.6.2 Coulter Counter settings for L1210 *in vivo*, L1210 *in vitro* and L1210R *in vitro* cell lines.

Amplification	4
Threshold: Lower	10
Upper	80
Aperture current	0.5

3.1.6.3 Coulter Counter settings for K562 cells

Amplification	1
Threshold: Lower	18
Upper	>110
Aperture current	0.066

3.1.7 ATTEMPTED FORMATION OF L1210 MURINE LEUKAEMIA CELLS RESISTANT TO NITROGEN MUSTARD FROM THE *in vitro* L1210 CELL LINE

3.1.7.1 Method 1

L1210 murine leukaemia cells which were in the late exponential phase of growth in culture (results 4.1.2) were subcultured to an initial density of $5x10^4$ cells per ml in 10% HS in RPMI, to which was added 10^{-8}M nitrogen mustard. The cells were incubated at 37^o until a growth plateau was obtained (results 4.1.2). The subculture was repeated until the mean generation time of the culture was identical to that of untreated cells (methods 3.1.5). A portion of cells was stored in liquid nitrogen (methods 3.1.3) The cells were subcultured in a two-fold concentration of nitrogen mustard. The process was repeated until a limit of resistance was reached at a concentration

of nitrogen mustard which totally prevented cell growth. A culture was established from the frozen cells from the previous culture.

3.1.7.2 Method 2

L1210 murine leukaemia cells which were in the late exponential phase of growth in culture (results 4.1.2) were incubated for 1 hour at 37^{o} in RPMI medium containing 10^{-4}M nitrogen mustard under an atmosphere of 5% CO_2 in air. The cells were centrifuged at 350g (1500rpm) for 5 minutes, washed once and resuspended to 10^7 - 10^8 cells per ml in 10% HS in RPMI. The medium was changed daily and the population viability, based on the exclusion of trypan blue (methods 3.3.1) was monitored. The initial cell number per ml was decreased twofold with each successive subculture until a culture was established at an initial density 3-5x10^4 cells per ml. This culture was allowed to reach late exponential phase of growth (results 4.1.2) and the process repeated twice. The effect of nitrogen mustard on the growth of the culture was estimated (methods 3.3.3).

3.1.8 ATTEMPTED CULTURE OF L1210C MURINE LEUKAEMIA CELLS WITH INCREASED SENSITIVITY TO CISPLATIN

L1210C murine leukaemia cells nominally sensitive to cisplatin (materials 2.3) were subcultured daily to an initial density of 2x10^5 cells per ml in 10% horse serum in Fischers medium and the mean generation time of the culture (methods 3.1.5) was estimated. The effect of cisplatin on the growth of the cells during 72h in culture was measured (methods 3.3.3).

3.2 PREPARATION OF DRUG SOLUTIONS

3.2.1 PREPARATION OF SOLUTIONS OF ANTITUMOUR DRUGS

3.2.1.1 Preparation of solutions of nitrogen mustard and its monofunctional analogue

Nitrogen mustard (HN2) and a monofunctional analogue (HN2-1; 2-chloroethyldimethylamine) were dissolved in ice-cold 0.9% NaCl at 500 times the required final concentration in cell suspensions. The appropriate volume of drug solution was added to the cell suspension within 20 seconds.

3.2.1.2 Preparation of solutions of chlorambucil

Chlorambucil was dissolved in DMSO at 500 times the required final concentration in cell suspensions. The appropriate volume of drug solution was added immediately to the cell suspension.

3.2.1.3 Preparation of solutions of *cis*- and *trans*-diaminodichloroplatinum(II)

Cis- and *trans*-diaminodichloroplatinum(II) were dissolved in DMSO then diluted to 1% DMSO by addition of ice-cold 0.9% NaCl solution to 500 times the required final concentration in cell suspensions. The solutions were prepared freshly or 24 hours in advance (results 4.19) and stored at room temperature. The appropriate volume of drug solution was added to the cell suspension.

3.2.1.4 Preparation of solutions of aquated derivatives of *cis*- and *trans*-diaminodichloroplatinum(II)

Mono-aquated and di-aquated derivatives of *cis*- and *trans*-diaminodichloroplatinum(II) were prepared by the method of Scanlon *et al* (1983). A 1ml portion of a 0.1M solution of CDDP or TDDP which had been dissolved in 5% DMSO in distilled water was then mixed with either 9ml distilled water to create mono-aquated derivatives or 9ml 0.22M $AgNO_3$ to create di-aquated derivatives. The solutions were

adjusted to pH4 with concentrated nitric acid and allowed to stand at room temperature for 48 hours. Solutions were spun at 3000g (4500rpm) for 10 minutes in a Heraeus Christ Labofuge 6000 centrifuge. The appropriate volume of the supernatent fluid was added to cell suspensions to give the required final concentration.

3.2.2 PREPARATION OF OTHER SOLUTIONS FOR ADDITION TO CELL SUSPENSIONS

3.2.2.1 Preparation of solutions of ouabain, bumetanide and furosemide

Ouabain, furosemide and bumetanide were all dissolved in DMSO to 200 times the required final concentration in cell suspensions. An equal volume of cold 0.9% NaCl solution was added and the appropriate volume added immediately to cell suspensions.

3.2.2.2 Preparation of solutions of dibutyryladenosine cyclic monophosphate

Dibutyryladenosine cyclic-3,5-monophosphate was dissolved in ice-cold 0.9% NaCl solution to 100 times the required final concentration in cell suspensions. The appropriate volume was added immediately to cell suspensions.

3.2.2.3 Preparation of solutions of sodium ionophore monensin

Monensin was dissolved in absolute ethanol to 200 times the required final concentration in cell suspensions. An equal volume of ice-cold 0.9% NaCl solution was added and the appropriate volume was added immediately to cell suspensions.

3.2.2.4 Preparation of solutions of calcium ionophore A23187

Calcium ionophore A23187 was dissolved in DMSO to 1000 times the required final concentration in cell suspensions. Nine volumes of ice-cold 0.9% NaCl were added and the appropriate volume was added immediately to cell suspensions.

3.2.2.5 Preparation of solutions of quinine

Quinine was dissolved in absolute ethanol to 500 times the required final concentration in cell suspensions. The appropriate volume was added to cell suspensions.

3.2.2.6 Preparation of solutions of amiloride

Amiloride was dissolved in DMSO to 200 times the required final concentration in cell suspensions. An equal volume of 0.9% NaCl was added and the appropriate volume added immediately to cell suspensions.

3.2.2.7 Preparation of solutions of diisothiocyanatostilbene disulphonic acid (DIDS)

DIDS was dissolved in DMSO to 200 times the required final concentration in cell suspensions. An equal volume of 0.9% NaCl was added and the appropriate volume added immediately to cell suspensions.

3.3 ESTIMATION OF CYTOTOXICITY OF ANTITUMOUR DRUGS TO TUMOUR CELLS in vitro

3.3.1 PERMEABILITY OF CELLS TO TRYPAN BLUE

Samples (200µl) of cell suspensions were mixed with 50ul filtered 0.5% trypan blue in 0.9% NaCl solution. After one minute the suspension was introduced through a pasteur pipette into a haemocytometer, and examined using an Olympus (Tokyo) CK Microscope. The number of cells which excluded trypan blue was counted, and expressed as a percentage of the total cell number.

3.3.2 FLOW CYTOFLUORIMETRY OF L1210 CELLS

L1210 murine leukaemia cells were incubated with and without 10^{-5}M HN2 in RPMI for 3 hours at 37^{o} under an atmosphere of 5% CO_2 in air. The cells were prepared for flow cytofluorimetry by use of a method similar to that of Gray and Coffino (1979). L1210 cells were

centrifuged at 350g (1500rpm) for 5 minutes, washed once in 10ml 0.9% NaCl solution and resuspended to 10^6 cells per ml in ice-cold 70% ethanol in water. Aliquots of 1ml were stored in the dark at 4^o. Each aliquot was centrifuged (11600g) for 1 minute in the Beckman Microfuge B and the cells resuspended in $1mgml^{-1}$ of ribonuclease A in phosphate buffer pH7.0, consisting of 1.689g Na_2HPO_4 and 0.7429g $Na_2HPO_4.12H_2O$ made up to 100ml with distilled water. After incubation for 30 minutes at 37^o the nuclei were resuspended in 1ml of $50\mu gml^{-1}$ propidium iodide solution in 0.1% sodium citrate and 0.1% Triton X100 for 30 minutes. The nuclei were immediately transferred to sample tubes (Falcon 2054, via Becton Dickinson, Cowley, Oxon, U.K.) and placed in the Becton Dickinson FACS 440 fluorescence activated cell sorter with Becton Dickinson consort 40 computer and Tektronix 4612 printer. Forward scatter was recorded to give a profile of cell size and red fluorescence (excitation 540nm and emission 625nm) was recorded to give a profile of DNA content.

3.3.3 MEASUREMENT OF INHIBITION OF GROWTH OF CULTURED CELLS

Cultured cells were harvested by centrifugation at 350g (1500rpm) for 5 minutes and resuspended in sterile incubation medium (for details see results). The cells were incubated with and without drug at 37^o under an atmosphere of 5% CO_2 in air for the appropriate time. Alternatively, for continuous exposure to the drug during growth, drug was added to growth medium. The cells were washed twice and resusupended in the appropriate growth medium (methods 3.1.2) to an initial cell number per ml which achieved a stationary phase of growth after 72 hours in control cultures (for details see results). Duplicate 2ml aliquots were dispensed into Multiwell dishes and incubated for 72 hours in a incubator at 37^o with full humidity in an atmosphere of 5% CO_2 in air. The cells in each well

were mixed by rapid pipetting 20 times in a pasteur pipette. Samples of 1ml were removed from each well and cell number was determined (methods 3.1.6) The increase in cell number per ml in drug-treated cells was expressed as a percentage of the increase in cell number per ml of untreated cells and the percentage inhibition of cell growth caused by each concentration of drug was thus calculated.

3.3.4 MEASUREMENT OF COLONY FORMING UNITS OF L1210 AND L1210R CELLS

3.3.4.1 Method 1

The procedure described below (Dr. Neil Gibson, personal communication) is based on the method of Chu and Fischer (1968).

Noble agar was washed with 0.1M EDTA at pH7.4 daily for four days with continuous stirring, followed by daily rinsing with distilled water for four days. The agar was stirred while drying at 37^{o} and stored in an airtight bottle until use. Washed Noble agar (67mg) was dissolved in 5ml distilled water and autoclaved at 120^{o}, 15psi for 15 minutes. A 45ml portion of 20% HS in RPMI was warmed to 48^{o} and 5ml mixed with the autoclaved agar. The mixture was returned to the bulk of the medium and mixed well. Portions of 3ml were dispensed into sterile plastic tubes (Falcon 2058, via Becton Dickinson, Cowley, Oxon, U.K.) which were kept at 37^{o} until the addition of cell suspension. L1210 cells which had been incubated with and without drug were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended in 20% HS in RPMI, such that 1ml suspension contained the required number of cells per cloning tube. The cells were added to the agar and the tubes were inverted to mix. The final agar concentration was 0.1%. The tubes were placed upright in a rack in iced water for 15 minutes to set, then allowed to stand for 45 minutes at room temperature. The air space

above the agar in each tube was gassed with 5% CO_2 in air and the top sealed tightly. The tubes were incubated in a padded rack at 37° for 10-14 days. The number of colonies in each tube was counted. The "plating efficiency" was calculated as the number of colonies formed as a percentage of the number of cells present initially. The "surviving fraction" was calculated as the number of colonies formed from drug-treated cells as a fraction of the number of colonies formed from untreated cells.

3.3.4.2 Method 2

The procedure described below (Dr. J. A. Hickman, personal communication) is a modification of the method of Chu and Fischer (1968).

Noble agar (0.11g) was placed in a 100ml sterile glass flat-bottomed bottle (GIBCO) and 5.2ml sterile distilled water was carefully added. The agar was autoclaved at 120°, 15psi for 15 minutes with slow exhaust, then stored at 44° in a water bath. A 50ml portion of 20% HS in RPMI was warmed to 37°. After incubation with and without drug, L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended in 20% HS in RPMI such that 2ml suspension contained the required number of cells per cloning tube. Three tubes were set up for each condition tested. The agar solution was cooled to 37° and mixed with the prepared medium. The pH of the mixture was adjusted to 6.5 (estimated with pH paper) with 0.1M HCl, to compensate for the alkaline pH drift during cloning. A 3ml portion of agar mixture was placed in each cloning tube (Falcon 2058, *via* Becton Dickinson, Cowley, Oxon, U.K.), 2ml cell suspension was added and the tubes were inverted and rotated gently to mix with minimum formation of air bubbles. The final agar concentration was 0.12%. The tubes were placed upright in iced water

for 2 minutes, then allowed to stand at room temperature for 15 minutes. The air space above the agar was gassed with 5% CO_2 in air and the tubes were sealed tightly. The tubes were incubated in a padded rack at 37° for 15 days and the number of colonies in each tube was recorded. Plating efficiency and surviving fractions were calculated as described in section 3.3.4.1.

3.3.4.3 Method 3

The procedure described below is identical to a method for the assay of colony forming units of Walker cells (Dr. J. M. Walling, personal communication) and is a modification of the method described by Tew and Wang (1982).

A 5% solution of Noble agar in distilled water in a McCartney bottle was autoclaved for 20 minutes at 120°, 15psi with slow exhaust and stored until required, when it was placed in a boiling water bath to melt. One volume of molten agar was mixed with 9 volumes of 15% FCS in RPMI at 44°. Portions (19.6ml) of this medium were dispensed into sterile plastic 30ml universal tubes (Sterilin) which were stored at 44° until use. After incubation with and without drug for the required time, L1210 or L1210R cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended in 15% FCS in RPMI such that 1ml suspension contained 10 times the required number of cells per cloning dish. Cell suspension (0.4ml) was mixed with each tube of agar mixture by gentle inversion and rotation to minimise formation of air bubbles. The final agar concentration was 0.49%. Triplicate 5ml portions were dispensed into bacterial grade 4.5cm triple-vented petri dishes (Sterilin), distributed evenly and allowed to set at room temperature for 5 minutes. The dishes were placed in sterile cake boxes with loose-fitting lids, and incubated in an incubator at 37° with full humidity in an atmosphere of 5% CO_2

in air. Dishes were examined under an Olympus (Tokyo) CK microscope with x40 magnification and colonies of 50 cells or greater were counted. Plating efficiency and surviving fractions were calculated as described in section 3.3.4.1 above.

3.4 CONFIRMATORY STUDIES : THE EFFECT OF NITROGEN MUSTARD ON PLASMA MEMBRANE FUNCTIONS OF PC6A MURINE PLASMACYTOMA CELLS

3.4.1 MEASUREMENT OF ^{86}RUBIDIUM TRANSPORT INTO PC6A MURINE PLASMACYTOMA CELLS

The method used for the measurement of $^{86}Rb^{+}$ influx into PC6A cells was as described previously (Baxter et al, 1982): PC6A cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^{6}$ cells per ml in RPMI. The cells were incubated for 30 minutes under an atmosphere of 5% CO_2 in air at 37^{o} in a water bath shaking at 50 oscillations per minute. Incubation was continued with or without nitrogen mustard for the required time (for details see results 4.1.1). ^{86}Rubidium ($5\mu Ci ml^{-1}$) was added to begin the measurement of influx. Triplicate 200µl aliquots were carefully layered over oil barriers in 400ul microfuge tubes and spun at 11600g in the Beckman Microfuge B for 30 seconds. The microfuge tubes contained 100ul of a mixture of 10 parts Dow-Corning 550 silicon oil (Hopkin and Williams, Romford, Essex, U.K.) with 3 parts pure corn oil (Mazola), supported by 50ul 90% formic acid and were previously spun in the Microfuge for 30 seconds to ensure separation of the two phases. The tubes were frozen in liquid nitrogen and cut with wire cutters at the boundary between the acid and oil phases. Spurgin (1981) showed that the cells were contained in the acid layer, leaving unabsorbed radiolabel in the aqueous supernatant. The two fragments were placed in separate plastic sample tubes (Luckham LP3), thawed, mixed and counted in the ICN Tracerlab Gamma Set 500 gamma

detector, set with mimimum threshold and maximum window, gain 32. The "fractional uptake" of $^{86}Rb^{+}$ was determined as the number of counts in the formic acid, which contained cell fragments, as a fraction of the total counts in acid and supernatant layers.

3.4.2 MEASUREMENT OF AMINO ACID TRANSPORT INTO PC6A MURINE PLASMACYTOMA CELLS

3.4.2.1 Measurement of [^{14}C]-α-aminoisobutyric acid transport into PC6A murine plasmacytoma cells

The method used for measurement of [^{14}C]-α-AIB influx into PC6A cells was as described previously (Baxter et al, 1982): PC6A cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in RPMI. The cells were incubated for 30 minutes under an atmosphere of 5% CO_2 in air at 37^o, in a waterbath shaking at 50 oscillations per minute. Incubation was continued with or without 10^{-5}M nitrogen mustard for 4 hours. The measurement of influx was begun by the addition of 1μCiml^{-1} [^{14}C]-α-AIB. At each time point, triplicate 200μl samples were layered over oil barriers in 400ul microfuge tubes (methods 3.4.1) and spun at 11600g in the Beckman Microfuge B for 30 seconds. The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in separate glass scintillation vials containing 10ml scintillation fluid, thawed, mixed, and counted in the Packard Tri-Carb 2660 liquid scintillation counter. The "fractional uptake" of [^{14}C]-α-AIB was determined as the number of counts in the acid layer containing the cells, as a fraction of the total counts in acid and supernatant layers.

3.4.2.2 Measurement of [^{14}C]-cycloleucine transport into PC6A murine plasmacytoma cells

The method used for measurement of [^{14}C]-cycloleucine influx into PC6A cells was as described previously (Baxter et al 1982): PC6A cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in RPMI. The cells were incubated for 30 minutes under an atmosphere of 5% CO_2 in air, at 37^o in a water bath shaking at 50 oscillations per minute. Incubation continued with or without 10^{-5}M nitrogen mustard for 4 hours. The measurement of influx was begun by the addition of 1μCiml^{-1} [^{14}C]-cycloleucine. At each time point, triplicate 200μl samples were layered over oil barriers in microfuge tubes (methods 3.4.1) and spun at 11600g in the Beckman Microfuge B for 30 seconds. The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in separate glass scintillation vials containing 10ml scintillation fluid, thawed, mixed, and counted in the Packard Tri-Carb 2660 liquid scintillation counter. The "fractional uptake" of [^{14}C]-cycloleucine was determined as the number of counts in the acid layer, containing the cells, as a fraction of the total counts in acid and supernatant layers.

3.5 MEASUREMENT OF ^{86}RUBIDIUM TRANSPORT ACROSS THE PLASMA MEMBRANE OF L1210 MURINE LEUKAEMIA CELLS

3.5.1 MEASUREMENT OF ^{86}RUBIDIUM INFLUX INTO L1210 MURINE LEUKAEMIA CELLS

3.5.1.1 Measurement of the effect of drugs on ^{86}rubidium influx

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in RPMI, Krebs-Ringer buffer, or a modified Krebs-Ringer buffer (for details, see results). The cells were incubated for 30 minutes under an

atmosphere of 5% CO_2 in air at 37° in a water bath shaking at 40 oscillations per minute. Incubation was continued with and without drug for the required period. The measurement of influx was begun by the addition of 5μCiml^{-1} $^{86}Rb^+$. At each time point triplicate 200μl aliquots were layered over oil barriers in microfuge tubes (methods 3.4.1) and spun at 11600g in the Beckman Microfuge B for 30 seconds. The microfuge tubes were frozen in liquid nitrogen, cut at the acid:oil boundary and the two fragments placed in separate plastic tubes (Luckham LP3). "Fractional uptake" of $^{86}Rb^+$ was determined as described in section 3.4.1.

3.5.1.2 Measurement of ^{51}chromium distribution in cell fractions obtained by centrifugation in the Microfuge

Cultured L1210 cells were centrifuged at 350g (1500 rpm) for 5 minutes, washed twice and resuspended to 5x10^6 cells per ml in RPMI with 2μCiml^{-1} Na-[^{51}Cr]-chromate. The cells were incubated for 2 hours under an atmosphere of 5% CO_2 in air at 37° in a water bath shaking at 40 oscillations per minute. The cells were washed ten times with 20ml RPMI and finally resuspended to 5x10^6 cells per ml in RPMI. Incubation was continued with or without drug for the required time. Triplicate 200μl samples of each incubate were layered over oil barriers in microfuge tubes and centrifuged at 11600g (methods 3.3.1). The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary, and the two fragments were placed in separate plastic tubes (Luckham LP3). ^{51}Chromium was detected in the ICN Tracerlab Gamma Set 500 gamma detector with standard ^{51}Cr window. Further 1ml portions of drug-treated $^{51}Cr^{3+}$-labelled cells were placed in 1.5ml microfuge tubes and centrifuged at 11600g for 30 seconds. The cells were washed 5 times with RPMI. Triplicate 200ul samples were layered over oil barriers in microfuge tubes (methods

3.4.1) and spun in the microfuge for 30 seconds. The tubes were frozen and cut, and the $^{51}Cr^{3+}$ content of cells and supernatent fractions were determined as before.

3.5.1.3 Measurement of [^{14}C]-inulin carboxylic acid distribution in cell fractions obtained by centrifugation in the Microfuge

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in RPMI. The cells were incubated for the required time with and without drug under an atmosphere of 5% CO_2 in air at 37^o in a water bath shaking at 40 oscillations per minute. For the last 15 minutes, the incubation was continued in the presence of 1μCiml^{-1} [^{14}C]-inulin carboxylic acid. Triplicate 200μl portions were then layered on to oil barriers in microfuge tubes and centrifuged at 11600g for 30 seconds (methods 3.4.1) The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in separate glass scintillation vials containing 10ml scintillation fluid, thawed, mixed, and counted in the Packard Tri-Carb 2660 liquid scintillation counter.

3.5.1.4 Measurement of ^{42}potassium and ^{86}rubidium influx

L1210 cells which were in the exponential phase of growth in culture (results 4.1.2) were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in a K^+-free Krebs-Ringer buffer, pH7.4, consisting of 123mM NaCl, 25mM $NaHCO_3$, 1.17mM NaH_2PO_4, 1.18mM $MgSO_4$, 5.5mM glucose, 1.27mM $CaCl_2$, at 37^o. At zero time ^{86}RbCl & RbCl or ^{42}KCl & KCl was added to a final concentration of 0.3mM and 2μCiml^{-1}. Influx was allowed to proceed at 37^o under an atmosphere of 5% CO_2 in air for 15 minutes. Triplicate 200μl portions were layered over oil barriers in microfuge tubes and centrifuged for 30s at 11600g (methods 3.4.1). The tubes

were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in separate plastic tubes (Luckham, LP3) thawed, mixed, and counted in a Packard Auto-gamma counter (Packard Instrument Co., U.S.A). The "fractional uptakes" of $^{86}Rb^+$ & Rb^+ and $^{42}K^+$ & K^+ were determined as described in section 3.4.1.

3.5.1.5 Measurement of ^{86}rubidium transport into L1210 cells incubated in the presence of inhibitors of potassium transport

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in RPMI, Krebs-Ringer buffer or a modified Krebs-Ringer buffer (for details see results). The cells were incubated with or without drug for the required time, under an atmosphere of 5% CO_2 in air, at 37°. Cells were incubated for 5 minutes with one of the following:- 1mM ouabain; 0.1mM bumetanide; 1mM furosemide; or 1mM furosemide plus 1mM ouabain. Drugs were added from freshly-made stock solutions (methods 3.2.2). The final concentration of DMSO was 0.5% (v/v); this was added to control incubations. $^{86}Rb^+$ ($5\mu Ci ml^{-1}$) was added and influx allowed to proceed for 15 minutes. Triplicate 200µl samples were layered over oil barriers in microfuge tubes and the tubes were centrifuged at 11600g for 30 seconds (methods 3.4.1). The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in separate plastic tubes (Luckham, LP3) thawed, mixed and counted in the ICN Tracerlab Gamma Set 500 gamma detector (methods 3.4.1).

3.5.1.6 Measurement of ^{86}rubidium transport into serum-deprived L1210 cells

L1210 cells were harvested under sterile conditions from stationary cultures (results 4.1.2) by centrifugation at 350g (1500rpm) for 5 minutes, washed four times and resuspended to $2x10^6$

cells per ml in serum-free RPMI. The cells were incubated for 18h at 37° in an atmosphere of 5% CO_2 in air, then washed twice and resusupended to $5x10^6$ cells per ml in RPMI. The permeability of the cells to trypan blue (methods 3.3.1) was monitored throughout the procedure. The cells were incubated with or without 10% horse serum for the required time. Inhibitors of potassium transport (methods 3.5.1.5) were added 5 minutes prior to the initiation of measurement of influx, by the addition of $5\mu Ciml^{-1}$ $^{86}Rb^+$ (methods 3.4.1). The initial rate of $^{86}Rb^+$ & K^+ transport was calculated from the $^{86}Rb^+$ uptake after 5 minutes (results 4.2.1) over which period the rate of uptake was constant.

3.5.1.7 Measurement of bumetanide-sensitive ^{86}rubidium transport into L1210 cells after replacement of external sodium by lithium, choline or tetramethylammonium

L1210 cells, which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed once and resuspended to $5x10^6$ cells per ml in a modified Krebs-Ringer buffer pH7.4, which consisted of 123mM NaCl, 25mM $NaHCO_3$, 1.18mM $MgSO_4$, 1.17mM KH_2PO_4, 5.5mM glucose, 1.27mM $CaCl_2$ with 1mM ouabain. A series of buffers with progressive replacement of NaCl by LiCl, choline chloride, or TMACl were warmed to 37° under an atmosphere of 5% CO_2 in air. Portions of cells (1ml) were centrifuged at 11600g in the Beckman Microfuge B for 1 minute, then washed twice in the appropriate buffers. The cells were resuspended in 1ml of buffer which contained $5\mu Ciml^{-1}$ $^{86}Rb^+$, with or without 0.1mM bumetanide. Influx was allowed to proceed at 37° for 15 minutes. Triplicate 200ul portions were layered over oil barriers in microfuge tubes and the tubes spun in the microfuge for 30 seconds (methods 3.4.1). The tubes were frozen, cut, thawed and counted as described in section

3.4.1. Bumetanide-sensitive $^{86}Rb^{+}$ influx was calculated for each concentration of Na^{+}.

3.5.1.8 Measurement of bumetanide-sensitive ^{86}rubidium transport into L1210 cells after replacement of external chloride by bromide, nitrate or gluconate

L1210 cells, which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed once and resuspended to $5x10^{6}$ cells per ml in a modified Krebs-Ringer buffer, pH7.4, which consisted of 123mM NaCl, 25mM $NaHCO_3$, 1.18mM $MgSO_4$, 1.17mM KH_2PO_4, 5.5mM glucose, 1.27mM $CaCl_2$ with 1mM ouabain. A series of buffers, with progressive replacement of NaCl by NaBr, $NaNO_3$ or sodium gluconate, were warmed to 37° under an atmosphere of 5% CO_2 in air. Portions of cells (1ml) were centrifuged at 11600g in the microfuge for 1 minute, then washed twice in the appropriate buffer. Cells were resuspended in 1ml buffer containing $5\mu Ci ml^{-1}$ $^{86}Rb^{+}$, with or without 0.1mM bumetanide. Influx was allowed to proceed at 37° for 15 minutes. Triplicate 200µl portions were layered over oil barriers in microfuge tubes and spun in the microfuge for 30 seconds (methods 3.4.1). The tubes were frozen, cut, thawed, and counted as described in section 3.4.1. Bumetanide-sensitive $^{86}Rb^{+}$ influx was calculated for each concentration of chloride.

3.5.1.9 Measurement of sodium-dependent transport of ^{86}rubidium into drug-treated L1210 cells

L1210 cells which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended at $5x10^{6}$ cells per ml in Krebs-Ringer buffer, pH7.4. The cells were incubated with and without drug for the required time under an atmosphere of 5% CO_2 in air, at 37°, in a

water bath shaking at 40 oscillations per minute. The cells were washed twice and resuspended in either Krebs-Ringer buffer, pH7.4, or a Na^+-free Krebs-Ringer buffer, pH7.4, which consisted of 118mM choline chloride, 5mM KCl, 25mM $KHCO_3$, 1.18mM $MgSO_4$, 1.17mM KH_2PO_4, 5.5mM glucose, 1.27mM $CaCl_2$. Both buffers contained 1mM ouabain. The measurement of influx was begun by the addition of 5μCiml^{-1} $^{86}Rb^+$ and incubation was continued at 37^o under 5% CO_2 in air. At each time point triplicate 200μl samples were layered over oil barriers in microfuge tubes. The tubes were centrifuged for 30 seconds in the microfuge (methods 3.4.1). The tubes were frozen, cut, thawed, and counted and "fractional uptake" of $^{86}Rb^+$ was determined as described in section 3.4.1.

3.5.1.10 Measurement of chloride-dependent transport of ^{86}rubidium into drug-treated L1210 cells.

L1210 cells, which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended to 5x10^6 cells per ml in Krebs-Ringer buffer, pH7.4. The cells were incubated with or without drug under an atmosphere of 5% CO_2 in air for the required time at 37^o in a water bath shaking at 40 oscillations per minute. The cells were washed twice and resuspended in Krebs-Ringer buffer or a Cl^--depleted Krebs-Ringer buffer, pH7.4 which consisted of 118mM $NaNO_3$, 5mM KNO_3, 25mM $NaHCO_3$, 1.18mM $MgSO_4$, 1.17mM KH_2PO_4, 5.5mM glucose and 1.27mM $CaCl_2$. Both buffers contained 1mM ouabain. The measurement of influx was begun by the addition of 5μCiml^{-1} $^{86}Rb^+$ and incubation continued at 37^o, under an atmosphere of 5% CO_2 in air. At each time point, triplicate 200μl samples were layered over oil barriers in microfuge tubes and the tubes were centrfuged in the Microfuge at 11600g for 30 seconds (methods 3.4.1) The tubes were frozen, cut, thawed and counted and "fractional uptake" of $^{86}Rb^+$ was determined.

3.5.1.11 Measurement of ^{86}rubidium transport into L1210 cells with replacement of external bicarbonate by nitrate.

L1210 cells, which were in the exponential phase of growth in culture, were centrifuged at 350g (1500rpm) for 5 minutes. The cells were washed twice and resuspended to $5x10^6$ cells per ml in Krebs-Ringer buffer, pH7.4, and incubated with or without drug, for the required time, under an atmosphere of 5% CO_2 in air, at 37°, in a water bath shaking at 40 oscillations per minute. The cells were washed twice in Krebs-Ringer buffer or HCO_3^--free buffer, pH7.4, which consisted of 118mM NaCl, 5mM KCl, 25mM $NaNO_3$, 1.18mM $MgSO_4$, 1.17mM KH_2PO_4, 5.5mM glucose, 1.27mM $CaCl_2$. Both buffers contained 1mM ouabain. The measurement of influx was begun by the addition of $5\mu Ciml^{-1}$ $^{86}Rb^+$ and incubation continued at 37°, under an atmosphere of 5% CO_2 in air for incubation in Krebs-Ringer, or under air for incubation in HCO_3^--free buffer. At each time point, triplicate 200µl samples were layered over oil barriers in microfuge tubes and the tubes were centrifuged at 11600g for 30 seconds (methods 3.4.1). The tubes were frozen, cut, thawed and counted and "fractional uptake" of $^{86}Rb^+$ determined as described in section 3.4.1.

3.5.2 MEASUREMENTS OF ^{86}RUBIDIUM EFFLUX FROM L1210 MURINE LEUKAEMIA CELLS

3.5.2.1 Measurement of ^{86}rubidium efflux from L1210 cells incubated in RPMI or Krebs-Ringer buffer

L1210 cells, which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, then washed twice and resusupended at $5x10^6$ cells per ml in RPMI. The cells were incubated for 2 hours with $5\mu Ciml^{-1}$ $^{86}Rb^+$ under an atmosphere of 5% CO_2 in air, at 37^o, in a water bath shaking at 40 oscillations per minute. Incubation was continued with or without drugs for the required time. The cells were washed twice, rapidly, in a Krebs-ringer buffer pH7.4, which contained 123mM KCl, 25mM $KHCO_3$, 1.17mM KH_2PO_4, 1.18mM $MgSO_4$, 5.5mM glucose, 1.27mM $CaCl_2$. To begin efflux the cells were resuspended in the RPMI Krebs-Ringer buffer or a modified Krebs-Ringer buffer (for details, see results). At each time point, triplicate 200µl aliquots were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds (methods 3.4.1) The tubes were frozen, cut, and counted in the ICN Tracerlab Gamma Set 500 gamma counter (section 3.4.1). The fraction of $^{86}Rb^+$ effluxed from the cells was calculated.

3.5.2.2 Measurement of calcium-stimulated ^{86}rubidium efflux

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in RPMI. The cells were incubated for 2 hours with and without $5\mu Ciml^{-1}$ $^{86}Rb^+$ under an atmosphere of 5% CO_2 in air, at 37^o, in a water bath shaking at 40 oscillations per minute. A 1ml portion of cell suspension, with or without $^{86}Rb^+$, was centrifuged at 11600g in the Beckman Microfuge B for 30 seconds. The cells were washed twice rapidly in the efflux buffer, pH7.4, which consisted of 140mMNaCl,

5mM KCl, 5mM glucose, 25mM HEPES, 1mM ouabain. To begin the efflux of $^{86}Rb^{+}$ the cells were resuspended in the efflux buffer containing the additions listed below:-

Incubation	Addition
1	None
2	0.1mM EGTA
3	0.1mM bumetanide
4	0.1mM $MgSO_4$
5	0.3mM quinine
6	10uM A23187 + 0.1mM EGTA
7	10uM A23187 + 0.1mM $CaCl_2$
8	10uM A23187 + 1mM $CaCl_2$
9	10uM A23187 + 1mM $CaCl_2$ + 0.1mM bumetanide
10	10uM A23187 + 1mM $CaCl_2$ + 0.3mM quinine

Cells which had been incubated in the absence of $^{86}Rb^{+}$, but which were treated as above, were incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O to measure cell volume (methods 3.11), after efflux at 37^{o} for 10 minutes. At the same time, cells loaded with $^{86}Rb^{+}$ were centrifuged for 15 seconds at 11600g and 0.5ml of the supernatant was rapidly removed from each tube and counted in a Packard Auto-gamma counter. (Packard Instrument Co, U.S.A.). The remaining contents of incubation 1 (see above) were also counted, so that the initial amount of $^{86}Rb^{+}$ in the cells was calculable. The fraction of $^{86}Rb^{+}$ effluxed from the cells in 10 minutes was determined under each condition as detailed above.

3.6 MEASUREMENT OF SODIUM TRANSPORT INTO L1210 MURINE LEUKAEMIA CELLS

3.6.1. METHOD 1: CENTRIFUGATION THROUGH AN OIL BARRIER

L1210 cells which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended to $5x10^6$ cells per ml in the appropriate incubation buffer (for details see results). Incubation was continued with or without drug, for the required time, at 37^o in a water bath shaking at 40 oscillations per minute. The cells were washed and resuspended in RPMI, Krebs-Ringer buffer or modified Krebs-Ringer buffer (for details, see results) containing $1\mu Ciml^{-1}$ $^{22}Na^+$. At each time point, triplicate 200µl samples were layered over oil barriers in microfuge tubes which were centrifuged at 11600g (methods 3.4.1) The tubes were frozen in liquid nitrogen, and cut at the acid:oil boundary, and the two fragments placed in separate plastic tubes (Luckham, LP3). The tubes were counted in the ICN Tracerlab Gamma Set 500 gammma detector set with minimum threshold and maximum window, gain 32. The "fractional uptake" of $^{22}Na^+$ was determined as the number of counts in the acid layer, which contained the cells as a fraction of the total counts in acid and supernatent layers.

3.6.2 METHOD 2: ABSORPTION FILTRATION

L1210 cells which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended to $5x10^6$ cells per ml in RPMI. The cells were incubated with or without drug, for the required period, under an atmosphere of 5% CO_2 in air at 37^o, in a water bath shaking at 40 oscillations per minute. The measurement of influx was begun by the addition of $1\mu Ciml^{-1}$ $^{22}Na^+$. At each time point, duplicate 200µl

samples were filtered through 0.45μM pore filters (Millipore HAWP, Millipore SA, Molsheim, France) which had been presoaked in RPMI. The filters were rinsed 3 times with 5ml ice-cold RPMI then placed into glass scintillation vials, containing 10ml scintillation fluid, Radioactivity was determined in the Beckman LS230 scintillation counter set with minimum threshold and maximum window.

3.7 MEASUREMENT OF CHLORIDE TRANSPORT INTO L1210 MURINE LEUKAEMIA CELLS

L1210 cells, which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended to $5x10^6$ cells per ml in RPMI. The cells were incubated with and without drug for the required period under an atmosphere of 5% CO_2 in air at 37^o in a waterbath shaking at 40 oscillations per minute. Influx measurement was begun by the addition of $2\mu Ciml^{-1}$ $^{36}Cl^-$. At each time point, triplicate 200μl samples were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds (methods 3.4.1). The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in separate glass scintillation vials containing 10ml scintillation fluid. The contents were mixed and radioactivity was determined in the Beckman LS230 scintillation counter set with minimum threshold and maximum window.

3.8 ESTIMATION OF SODIUM AND POTASSIUM CONTENTS OF L1210 MURINE LEUKAEMIA CELLS

3.8.1 ESTIMATION OF L1210 CELL POTASSIUM CONTENT BY EQUILIBRIUM DISTRIBUTION OF ^{86}RUBIDIUM

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended to $5x10^6$ cells per ml in RPMI, Krebs-Ringer buffer, or modified Krebs-Ringer buffer (for details see

results). The cells were incubated with or without 5μCiml^{-1} $^{86}Rb^{+}$ under an atmosphere of 5% CO_2 in air for 2 hours at 37^{o} in a water bath shaking at 40 oscillations per minute. The incubation was continued with or without drug for the required time. At each time point, cells which were incubated the absence of $^{86}Rb^{+}$ were incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O for determination of cell volume (methods 3.11). Triplicate 200μl samples of cells incubated with $^{86}Rb^{+}$ were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds (methods 3.4.1). The tubes were frozen, cut, thawed and counted in the ICN Tracerlab Gamma Set 500 gamma detector (section 3.4.1). The counts in cell fragments were corrected for extracellular $^{86}Rb^{+}$, and intracellular concentration of K^{+} was determined.

3.8.2 ESTIMATION OF L1210 CELL SODIUM CONTENT BY EQUILIBRIUM DISTRIBUTION OF ^{22}SODIUM

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended to 5x10^{6} cells per ml in RPMI. The cells were incubated with or without 1μCiml^{-1} $^{22}Na^{+}$ for 30 minutes under an atmosphere of 5% CO_2 in air at 37^{o} in a water bath shaking at 40 oscillations per minute. Incubation was continued with or without drugs for the required time. At each time point, cells which were incubated in the absence of $^{22}Na^{+}$ were incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O for determination of cell volume (methods 3.11). An aliquot of cells incubated in the presence of $^{22}Na^{+}$ was spun in the Beckman Microfuge B (11600g) for 30 seconds and the supernatent liquid was removed with a pasteur pipette and 200μl reserved to be counted. The cells were rapidly washed twice, and resuspended in 1ml ice-cold 150mM choline chloride. Triplicate 200μl samples were rapidly layered over oil barriers in microfuge

tubes which were centrifuged for 30 seconds at 11600g, frozen in liquid nitrogen and cut at the acid:oil boundary (methods 3.4.1). The acid layer was collected in a plastic tube (Luckham, LP3) and counted in the ICN Tracerlab Gamma Set 500 Gamma detector (methods 3.4.1).

3.8.3 ESTIMATION OF L1210 CELL SODIUM AND POTASSIUM CONTENTS BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended at $5x10^6$ cells per ml in RPMI. The cells were incubated with or without drug for the required time, under an atmosphere of 5% CO_2 in air, at 37^o in a water bath shaking at 40 oscillations per minute. At each time point, a 1ml portion of cells was incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O for estimation of cell volume (methods 3.11). A second 1ml portion of cells was centrifuged at 11600g in the Beckman Microfuge B and the supernatant removed with a pasteur pipette. The cells were washed twice rapidly and resuspended in 1ml 150mM choline chloride prewarmed to 37^o. Triplicate 200ul samples were quickly layered over oil barriers in 400ul microfuge tubes. The microfuge tubes contained 150µl 12% perchloric acid supporting 50µl of a mixture of 10 parts Dow-Corning 550 silicon oil (Hopkin & Williams, Romford, Essex, U.K.) and 3 parts pure corn oil (Mazola) and were spun previously in the microfuge for 30 seconds to ensure separation of the two phases. This method was similar to that which was described by Gargus and Slayman (1980) and Finkelstein and Adelberg (1977). The microfuge tubes were spun for 30 seconds in the microfuge, then frozen in liquid nitrogen and cut with wire cutters at the 100µl mark on the tube. The tips were collected in plastic tubes (Luckham LP3) and allowed to thaw. The contents of each tube were mixed for 30

seconds, then 50µl lysate was made up to 3ml with 0.1% lanthanum chloride solution in 7ml plastic bijou bottles (Sterilin). Sodium and potassium contents were determined at 589.5nm and 766.5nm, respectively, using a Perkin-Elmer 560 atomic absorption spectrophotometer (Perkin-Elmer, Beaconsfield, Bucks., U.K.) with an air:acetylene flame, and attached to a Perkin-Elmer printer. The machine was calibrated with standards of 0.1 and 0.5ppm ($\mu g ml^{-1}$) Na^+ and 0.5 and 2.0ppm K^+, made from NaCl and KCl in 0.1% $LaCl_3$ in distilled deionised water. The mean of three readings was calculated for each sample, and the cellular concentration of Na^+ and K^+ determined.

3.9 ESTIMATION OF TOTAL CALCIUM CONTENT OF L1210 MURINE LEUKAEMIA CELLS BY EQUILIBRIUM DISTRIBUTION OF ^{45}CALCIUM

L1210 cells which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended at $5x10^6$ cells per ml in RPMI. The cells were incubated with or without $2\mu Ci ml^{-1}$ $^{45}Ca^{++}$ for 5 hours under an atmosphere of 5% CO_2 in air at 37° in a water bath shaking at 40 oscillations per minute. Nitrogen mustard was present for the last 3 hours. At each time point, cells which were incubated in the absence of $^{45}Ca^{++}$ were incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O for determination of cell volume (methods 3.11). An aliquot of cells which were incubated with $^{45}Ca^{++}$ were centrifuged at 11600g for 30 seconds in the Beckman Microfuge B. The supernatent liquid was removed with a pasteur pipette and 200ul reserved to be counted. The cells were washed 6 times and resuspended in 1ml ice-cold Ca^{++}-free Krebs-Ringer buffer pH7.4, which contained 118mM NaCl, 5mM KCl, 25mM $NaHCO_3$, 1.18mM $MgSO_4 7H_2O$, 1.17mM KH_2PO_4, 5.5mM glucose and 2mM EGTA. Triplicate 200µl samples were layered over oil

barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds and frozen in liquid nitrogen (methods 3.4.1). The tubes were cut at the acid:oil boundary and the acid fragments placed in glass scintillation vials containing 10ml scintillation fluid. The vials were counted in the Packard Tri-Carb 2660 liquid scintillation counter and the cellular Ca^{++} content calculated (results 4.9.2).

3.10 CYTOCHEMICAL STAINING OF L1210 MURINE LEUKAEMIA CELLS FOR NON-SPECIFIC ESTERASES

L1210 cells which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resusupended at $2x10^7$ cells per ml in 0.9% NaCl. The cytochemical staining method was described by Yan et al (1971): approximately 10^6 L1210 cells were smeared on to a glass microscope slide and the smears were allowed to dry, then fixed for 30 seconds. The fixative was composed of 20mg NaH_2PO_4, 100mg KH_2PO_4 in 40ml distilled water mixed with 45ml acetone and 25ml formalin. The smears were washed in distilled water and dried for 10-30 minutes, then stained for 1-2 hours at 37^o. The stain consisted of 8.9ml phosphate buffered saline, pH7.3 (materials, 2.4); 0.6ml hexazotised pararosanilin and 10mg α-naphthyl acetate dissolved in 0.5ml ethylene glycol monomethylether. Hexazotisation was achieved by mixing and filtering 1 minute before use a filtered solution of 1g pararosanilin in 25ml 2M HCl with an equal volume of 4% $NaNO_2$ with the pH adjusted to 5.8-6.5 with 1M NaOH. The smears were washed in distilled water then counterstained with 1% methyl green for 30 seconds. After a final wash with distilled water the cells were examined using an Olympus (Tokyo) CK Microscope.

3.11 ESTIMATION OF L1210 MURINE LEUKAEMIA CELL VOLUME

Cultured L1210 cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended at $5x10^6$ cells per ml in the RPMI, Krebs-Ringer buffer or a modified Krebs-Ringer buffer (for details see results). The cells were incubated at 37^o with or without drug for the required time. Portions of cells (1ml) were incubated for 10 minutes at 37^o with $1\mu Ciml^{-1}$ [^{14}C]-inulin carboxylic acid and $1\mu Ciml^{-1}$ [^{3}H]-H_2O, added from a stock solution of $50\mu Ciml^{-1}$ [^{14}C]-inulin carboxylic acid and $50\mu Ciml^{-1}$ [^{3}H]-H_2O, in distilled water. At the required times, triplicate 200µl samples were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds (methods 3.4.1). The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in glass scintillation vials containing 10ml scintillation fluid. ^{14}C and ^{3}H were detected in a dual isotope program in the Packard Tri-Carb 2660 liquid scintillation counter. Total water and extracellular water were calculated and an estimate of cell volume obtained by subtraction.

3.12 ESTIMATION OF L1210 MURINE LEUKAEMIA CELL MEMBRANE POTENTIAL BY EQUILIBRIUM DISTRIBUTION OF [^{3}H]-TRIPHENYLMETHYLPHOSPHONIUM ION.

The method used for determination of membrane potential was as described elsewhere (Chahwala and Hickman, 1985): L1210 cells which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended at $5x10^6$ cells per ml in Krebs-Ringer (KR) buffer, pH7.4. The cells were incubated with or without drug for the required time at 37^o in a water bath shaking at 40 oscillations per minute under an atmosphere of 5% CO_2 in air. The cells from each incubation were divided into 4 portions; 2 portions were resuspended in KR and 2 portions were

resuspended in a high - K^+ KR, pH7.4, which contained 123mM KCl, 1.17mM KH_2PO_4, 25mM $KHCO_3$, 1.18mM $MgSO_4$, 5.5mM glucose, 1.27mM $CaCl_2$. One portion of cells in each buffer was incubated for 1 hour at 37° with 2μCiml^{-1} 2μM [^{3}H]-triphenylmethylphosphonium bromide. Triplicate 200μl samples were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds (methods 3.4.1). The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The fragments were placed in glass scintillation fluid and counted in the Packard Tri-Carb 2660 liquid scintillation counter. A second portion of cells in each buffer was incubated for 1 hour with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O present for the final 10 minutes, for determination of cell volume (methods 3.11). The concentration of $TPMP^+$ inside the cells was calculated and an estimate of the cell membrane potential (E_m) obtained by use of the Nernst equation (Lichtshtein et al, 1979):-

$$E_m = -2.3\frac{RT}{zF} \log \frac{[TPMP^+]^{\text{corrected in}}}{[TPMP^+]^{\text{out}}}$$

where

$$2.3\frac{RT}{zF} = 61 \text{ mV at } 37^o$$

and

$$[TPMP^+]^{\text{corrected in}} = [TPMP^+]^{\text{low}}{}_{in}K^+ - [TPMP^+]^{\text{high}}{}_{in}K^+$$

3.13 ESTIMATION OF INTRACELLUAR pH OF L1210 MURINE LEUKAEMIA CELLS

3.13.1 ESTIMATION OF INTRACELLULAR pH BY EQUILIBRIUM DISTRIBUTION OF [^{14}C]-DIMETHYLOXAZOLIDINE-2,4,-DIONE

L1210 cells, which were in the exponential phase of growth in culture, were centrifuged at 1500rpm (350g) for 5 minutes, washed twice and resuspended to 5x10^6 cells per ml in a bicarbonate-free

medium consisting of 130mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM glucose, 20mM HEPES, adjusted to pH7.4 with concentrated HCl (L'Allemain et al, 1984b). The cells were incubated with or without drug for the required time at 37° in a water bath shaking at 40 oscillations per minute. Fifteen minutes before each time point, a 1ml portion of cells was incubated with 1μCiml^{-1} [^{14}C]-DMO (Roos and Boron, 1981; Waddell and Butler, 1959). A second 1ml portion of cells was incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O for determination of cell volume (methods 3.11). At the various time points, triplicate 200μl samples of cells incubated with [^{14}C]-DMO were layered over oil barriers in microfuge tubes which were centrifuged at 11600g and frozen in liquid nitrogen (methods 3.4.1). The tubes were cut at the acid:oil boundary and the two fragments placed in glass scintillation vials containing 10ml scintillation fluid, and counted in the Packard Tri-Carb 2660 liquid scintillation counter. An estimate of intracellular pH (pH_i) was obtained from the following equation (Roos and Boron, 1981; Jacobs, 1940):-

$$pH_i = pk + \log \left[\frac{[DMO]_{in}^{corrected}}{[DMO]^{out}} (10^{\,pH - pk_{+1}}) - 1 \right]$$

where

$pk = pk_a$ DMO = 6.1 at 37°

pH_0 = external pH

$[DMO]_{in}^{corrected}$ = cpm per μl cell water, corrected for extracellular space

$[DMO]^{out}$ = cpm per μl external medium

3.13.2 ESTIMATION OF INTRACELLULAR pH BY EQUILIBRIUM DISTRIBUTION OF [^{14}C]-BENZOIC ACID.

L1210 cells, which were in the exponential phase of growth in culture, were centrifuged at 350g (1500rpm) for 5 minutes, then washed twice and resuspended at $5x10^6$ cells per ml in bicarbonate-free saline (methods 3.13.1). The cells were incubated with or without drug for the required time at 37^o in a water bath shaking at 40 oscillations per minute. Fifteen minutes before each time point, a 1ml portion of cells was incubated with 1μCiml^{-1} [^{14}C]-benzoic acid (L'Allemain et al, 1984b). A second 1ml portion of cells was incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O for determination of cell volume (methods 3.11). At various times, triplicate 200μl portions of cells, which had been incubated with [^{14}C]-BA, were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds and frozen in liquid nitrogen, then cut at the acid:oil boundary (methods 3.4.1). The two fragments were placed in glass scintillation vials containing 10ml scintillation fluid, and counted in the Packard Tri-Carb 2660 liquid scintillation counter. An estimate of the intracellular pH (pH_i) was obtained from the following equation (L'Allemain et al, 1984b):-

$$pH_i = pH_0 + \log\left[\frac{[BA]^{\text{corrected in}}}{[BA]^{out}}\right]$$

where

pH_0 = external pH

$[BA]^{\text{corrected in}}$ = cpm per μl cell water, corrected for extracellular space

$[BA]^{out}$ = cpm per μl external medium

3.14 ESTIMATION OF INTRACELLULAR ATP CONCENTRATION OF L1210 MURINE LEUKAEMIA CELLS

L1210 cells which were in the exponential phase of growth in culture were centrifuged for 5 minutes at 350g (1500rpm), washed twice and resuspended at $5x10^6$ cells per ml in either RPMI or Krebs-Ringer buffer. The cells were incubated with and without drugs for the required time under an atmosphere of 5% CO_2 in air at 37^o in a water bath shaking at 40 oscillations per minute. At each time point a 1ml portion of cells was incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O, for determination of cell volume (methods 3.11). A 2ml portion of cells was washed twice and resuspended in 1ml ice-cold 0.9% NaCl, and centrifuged at 11600g for 1 minute in the Beckman Microfuge B. The supernatant was removed with a pasteur pipette. The method used for ATP extraction was similar to that described by Cole et al (1967). The cells were resuspended in 0.5 ml ice cold 12% perchloric acid and kept on ice for 15 minutes. The extract was neutralised with 0.5ml 1M KOH, mixed, and kept on ice for 5 minutes. The precipitate was removed by centrifugation at 11600g for 2 minutes. The supernatent liquid was stored at -20^o until analysis. A 50µl sample was made up to 5ml with 45mM glycyl-glycine buffer, pH7.4, and 10µl of this was mixed with 980µl ice-cold buffer and 10µl luciferase-luciferin reagent (20mgml^{-1} in sterile distilled water). After 15 seconds the luminescence was measured against a blank of 10µl luciferase-luciferin reagent in 990µl buffer in an LKB-Wallac 1250-001 Luminometer (LKB, Croydon, Surrey, U.K.) attached to a LKB-Bromma 2210-032 potentiometric chart recorder. ATP content was read from a plot of the relationship between luminescence and ATP content of standard solutions (0-0.8µgml^{-1}) in buffer.

3.15 ESTIMATION OF TOTAL GLUTATHIONE CONCENTRATION OF L1210 MURINE LEUKAEMIA CELLS

L1210 cells, which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended at $5x10^6$ cells per ml in either RPMI or Krebs-Ringer buffer. The cells were incubated with and without nitrogen mustard for the required time under an atmosphere of 5% CO_2 in air at 37° in a water bath shaking at 40 oscillations per minute. At each time point a 1ml portion of cells was incubated with [^{14}C]-inulin carboxylic acid and [^{3}H]-H_2O for determination of cell volume (methods 3.11). A 2 ml portion of cell suspension was washed three times with 1ml ice-cold buffer, pH7.5, which contained 125mM Na_2HPO_4, 6.3mM EDTA, by centrifugation in the Beckman Microfuge B (11600g) for 30 seconds. Total glutathione (GSH and GSSG) was extracted and assayed by a method similar to those described by Griffith (1980) and Akerboom and Sies (1981). L1210 cells (10^7) were resuspended in 1ml 10% metaphosphoric acid and kept on ice for 1 minute. The suspension was centrifuged at 3000g (4500rpm) for 5 minutes, and the supernatant was removed with a pasteur pipette and stored on ice. 300µl of the acid supernatent liquid was neutralised by addition of 120µl hot 50% Na_3PO_4 solution. 140µl of the mixture was placed in a small volume quartz cuvette with 700µl 0.3mM NADPH in buffer, 100µl 6.0mM DTNB in buffer and 10µl glutathione reductase (53 units ml^{-1} in buffer). The change in absorbance was read at 405nm at 30° against a blank of 700µl NADPH, 100µl metaphosphoric acid, 40µl Na_3PO_4, 100µl DTNB and 10µl glutathione reductase in the Beckman DU7 spectrophotometer (Beckman-RIIC Ltd., High Wycombe, Bucks., UK). A standard curve of change in absorbance against glutathione content of solutions of GSSG in buffer (0-50µM) was

constructed. Glutathione content of the samples was computed from the standard curve by the spectrophotometer.

3.16 MEASUREMENT OF AMINO ACID TRANSPORT INTO L1210 MURINE LEUKAEMIA CELLS

3.16.1 MEASUREMENT OF [^{14}C]-α-AMINOISOBUTYRIC ACID TRANSPORT INTO L1210 CELLS

Cultured L1210 murine leukaemia cells were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended at $5x10^6$ cells per ml in Krebs-Ringer bicarbonate buffer, pH7.4. The cells were incubated with or without drug for the required time under an atmosphere of 5% CO_2 in air at 37° in a waterbath shaking at 40 oscillations per minute. The measurement of influx was begun by the addition of $0.1mgml^{-1}$ cycloleucine and $1\mu Ciml^{-1}$ $0.5mgml^{-1}$ [^{14}C]-α-AIB. At appropriate time points, triplicate 200µl samples were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds, frozen in liquid nitrogen and cut at the acid:oil boundary (methods 3.4.1). The two fragments were placed in glass scintillation vials containing 10ml scintillation fluid, and counted in the Packard Tri-Carb 2660 liquid scintillation counter. "Fractional uptake" of [^{14}C]-α-AIB was determined as the number of counts in the acid layer, containing the cells, as a fraction of the total counts in acid and supernatant layers.

3.16.2 MEASUREMENT OF [^{14}C]-CYCLOLEUCINE TRANSPORT INTO L1210 CELLS

L1210 murine leukaemia cells, which were in the exponential phase of growth in culture, were centrifuged at 350g (1500rpm) for 5 minutes, washed twice and resuspended at $5x10^6$ cells per ml in Krebs-Ringer bicarbonate buffer, pH7.4. The cells were incubated with and without drug for the required time under an atmosphere of 5% CO_2 in air in a waterbath shaking at 40 oscillations per minute. The

measurement of influx was begun by the addition of 0.3 $mgml^{-1}$ AIB and 1μCiml^{-1} 0.5$mgml^{-1}$ [^{14}C]-cycloleucine. At appropriate time points, triplicate 200μl samples were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 30 seconds (methods 3.4.1). The tubes were frozen in liquid nitrogen, cut and the radioactivity determined as described in section 3.16.1 above. The "fractional uptake" of [^{14}C]-cycloleucine was determined as the number of counts in the cell fragment as a fraction of the total counts in cell and supernatant fragments.

3.16.3 MEASUREMENT OF [^{3}H]-METHIONINE TRANSPORT INTO L1210 MURINE LEUKAEMIA CELLS

L1210 cells which were in the exponential phase of growth in culture were centrifuged at 350g (1500rpm) for 5 minutes, washed twice, and resuspended at $5x10^{6}$ cells per ml in Krebs-Ringer bicarbonate buffer, pH7.4. The cells were incubated with or without drug for the required time under an atmosphere of 5% CO_2 in air at 37^{o} in a waterbath shaking at 40 oscillations per minute. The cells were resuspended in various buffers for measurement of [^{3}H]-methionine influx (for details, see results). The measurement of influx was begun by the addition of 0.1$mgml^{-1}$cycloleucine and 1μCiml^{-1} [^{3}H]-methionine. At each time point triplicate 200μl samples were layered over oil barriers in microfuge tubes which were centrifuged at 11600g for 15 seconds (see methods 3.4.1). The tubes were frozen in liquid nitrogen and cut at the acid:oil boundary. The two fragments were placed in scintillation vials containing 10ml scintillation fluid and radioactivity counted in the Packard Tri-Carb 2660 liquid scintillation counter. The "fractional uptake" of [^{3}H]-methionine was determined as the number of counts in the acid layer, which contained the cells, as a fraction of the total counts in acid and supernatant layers.

3.17 ISOLATION OF L1210 MURINE LEUKAEMIA CELL MEMBRANES

3.17.1 METHOD 1

L1210 murine leukaemia cells, which were in the exponential phase of growth in culture, were centrifuged at 350g (1500rpm) for 5 minutes. The method for isolation of L1210 cell membranes was described by Hourani et al (1973) after Brunette and Till (1971) and was based on a two-phase polymer system. The two phases were prepared by mixing 103g 30% polyethylene- glycol 6000, 200g 20% dextran T500, 333ml 0.2M sodium phosphate, pH7.2, 172ml distilled water, and 7.2ml 0.5M $MgCl_2$. The mixture was shaken and allowed to separate in a separating funnel at 4^o. The upper (dextran-saturated PEG) and lower (PEG-saturated dextran) phases were collected and stored at 4^o. $5x10^8$ L1210 cells were washed three times in ice-cold 0.9%NaCl solution and resuspended in 10 volumes of a hypotonic lysis solution which consisted of 2mM $NaHCO_3$, 0.2mM $CaCl_2$, 5mM $MgCl_2$, pH6.8. The suspension was homogenised by 50 strokes of a Dounce homogeniser. The suspension was centrifuged at 10000g (11000rpm) for 15 minutes in the MSE Hi-spin 21 ultracentrifuge, and the pellet resuspended in 50ml upper phase by vigorous agitation for 10 minutes. 50ml lower phase was added and the mixture was shaken for a further 10 minutes. Membraneous material was removed from the interface with a pasteur pipette. The two phases were combined and the process repeated twice. The membraneous material was suspended in 5 volumes of 0.05M Tris-HCl buffer, pH7.2, and centrifuged at 48000g (24000rpm) in a Pegasus AP Ultracentrifuge for 15 minutes. The pellet was washed twice and finally resuspended in Tris-HCl buffer, pH7.2.

3.17.2 METHOD 2

L1210 murine leukaemia cells, which were in the exponential phase of growth in culture, were centrifuged at 350g (1500rpm) for 5 minutes. The method used for isolation of L1210 cell membranes was similar to that described by Tsai et al (1975) with modifications by Yang et al (1979). $5x10^8$ cells were washed in 0.9% NaCl solution then washed and resuspended in 10ml ice-cold homogenising medium for homogenisation which consisted of 10mM sodium phosphate, pH7.0, 0.5M hexyleneglycol, 1mM $CaCl_2$ and 1mM $MgCl_2$. The suspension was rapidly frozen in liquid nitrogen, thawed in a water bath at 37^o then subjected to 50 passes of a hand-held Dounce homogeniser. The process was repeated 5 times. The homogenate was spun at 1300g (2600rpm) for 1 minute in a Heraeus Christ Labofuge 6000 centrifuge. The supernatent liquid was decanted, stored on ice and the pellet washed twice in four volumes of homogenising medium. The supernatants were pooled, then made up to 10% sucrose by the addition of an appropriate volume of 60% sucrose and layered on to a discontinuous sucrose gradient of 20ml 30% sucrose overlayed with 5ml 10% sucrose in a 50ml polycarbonate centrifuge tube. The tube was spun at 23000g (17000rpm) for 30 minutes in an MSE Hi-Spin 21 Ultracentrifuge. The material at the interface was collected with a pasteur pipette and resuspended in 5ml 10% sucrose and applied to a second gradient. The material at the interface was resuspended in 10ml 0.9% NaCl and spun at 33000g (20000rpm) for 30 minutes. The pellet was washed in 10ml hypotonic buffer which consisted of 25mM sucrose in 10mM Tris-HCl pH7.4, and finally resuspended in 1ml 150mM sucrose in 50mM potassium phosphate buffer, pH7.4. The pellet was stored at -70^o. Protein content was estimated by the method of Lowry et al (1951).

3.18 STATISTICS

The statistical methods used for the processing of results are summarized below. The mean ($\bar{x}$) and standard deviation from the mean (S.D.) of each set of results was calculated by use of a CASIO FX-702P calculator from the following equations:

$$\bar{x} = \frac{\Sigma x}{n}$$

and

$$S.D. = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

where

$\bar{x}$ = the mean of a number of samples

Σx = the sum of all the samples

Σx^2 = the sum of squares of all samples

S.D. = standard deviation from the mean

n = the number of samples

For the comparison of one set of data with another, the students "t" test was applied (Swinscow, 1978). The factor "t" was calculated by use of a North Star Computer from the following equation.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\frac{(SD_1)^2}{n_1} + \frac{(SD_2)^2}{n_2}}$$

where

$\bar{x}_1$ = the mean of the first set of samples

$\bar{x}_2$ = the mean of the second set of samples

SD_1 = the standard deviation of the first set of samples

SD_2 = the standard deviation of the second set of samples.

n_1 = the number of samples in the first set

n_2 = the number of samples in the second set.

The computer was programmed with standard statistical tables to convert the value of t into a value for the probability, p, that the two sets of results may arise by chance, by the "null hypothesis". The convention was adopted that a value of p less than 0.05 allows rejection of the null hypothesis (Swinscow, 1978) and allowed the results to be considered significantly different from each other. Where results are quoted in the text, (Section 4) or in legend to figures, the value of the mean, standard deviation, and the number of observations are quoted; where two sets of results are to be compared, these values are quoted for each set of results, together with the value of p.

Section 4

RESULTS AND DISCUSSION

4.1 PRELIMINARY EXPERIMENTS

4.1.1 CONFIRMATORY STUDIES: THE EFFECT OF NITROGEN MUSTARD ON MEMBRANE TRANSPORT PROCESSES OF PC6A MURINE PLASMACYTOMA CELLS

4.1.1.1 The effect of nitrogen mustard on ^{86}rubidium uptake into PC6A cells

4.1.1.1.1 Introduction

An earlier investigation (Baxter et al 1982; Spurgin, 1981) showed that the incubation of these PC6A plasmacytoma cells with nitrogen mustard (HN2) in vitro resulted in the inhibition of net ^{86}rubidium uptake compared to controls. The same cell line was used for these confirmatory experiments.

4.1.1.1.2 Results

Figure 3 shows the effect of 10^{-5}M HN2 on the net fractional uptake (methods 3.4.1) of $^{86}Rb^{+}$ into 5 x $10^{6}ml^{-1}$ PC6A cells after a 4h incubation in RPMI at 37°. The fractional uptake of $^{86}Rb^{+}$ was intially linear with time for at least fifteen minutes; thereafter, net uptake was curvilinear with time, presumably as $^{86}Rb^{+}$ efflux became significant (Brown and Lamb, 1978; Sirotnak et al, 1979). The initial rate of $^{86}Rb^{+}$ influx was estimated from the linear portion of the uptake. Nitrogen mustard (10^{-5}M) caused 51% inhibition of the initial rate of $^{86}Rb^{+}$ influx into PC6A cells.

Figure 4 shows the effect of simultaneous addition of 10^{-3}M or 10^{-2}M HN2 and $^{86}Rb^{+}$ on the net fractional uptake of $^{86}Rb^{+}$ into $5x10^{6}ml^{-1}$ PC6A cells incubated in RPMI at 37°; 10^{-3}M HN2 reduced the initial rate of $^{86}Rb^{+}$ uptake by 12%, and 10^{-2}M HN2 caused 44% reduction.

4.1.1.1.3 Discussion

^{86}Rubidium was used as a potassium congener (Beauge and Ortiz, 1970; Brown and Lamb, 1978; Baxter et al, 1982; introduction 1.3).

In the earlier study by Baxter et al (1982), the initial rate of $^{86}Rb^{+}$ uptake into PC6A cells was inhibited by 45% by 10^{-5}M HN2 after a 4h incubation, and by 13% and 40% after simultaneous addition of 10^{-3}M or 10^{-2}M HN2 respectively. Thus the results from the present study were in good agreement.

The cells were incubated with a concentration of HN2 (10^{-5}M) which produced >99% cell kill (Baxter et al, 1982) in an in vitro-in vivo bioassay (Gescher et al, 1981) and which was estimated to represent approximately 2.5 times the pharmacologically achieved concentration in vivo (Baxter et al, 1982; see also introduction 1.3).

Baxter et al (1982) suggested that the locus for the action of HN2 to inhibit $^{86}Rb^{+}$ transport into PC6A cells might be the membrane-bound $Na^{+}K^{+}$ATPase (introduction 1.3). Grunicke and his colleages reported that HN2 inhibited the $Na^{+}K^{+}$ATPase of Ehrlich ascites cells in vivo and that this inhibition was related to the cytotoxicity of the drug (Grunicke et al, 1982; 1983). However, in later accounts, they noted that ouabain-sensitive $^{86}Rb^{+}$ uptake was inhibited by HN2 only at concentrations greater than 10^{-5}M, and that this action could not be correlated with the extent of inhibition of cell growth (Grunicke et al, 1985; Doppler et al, 1985). These authors concluded that the inhibition of $Na^{+}K^{+}$ATPase had no role in the cytotoxic action of HN2, and that the $Na^{+}K^{+}Cl^{-}$cotransporter of Ehrlich ascites cells might be susceptible to HN2 (see also discussion, section 5). The effects of HN2 on $^{86}Rb^{+}$ transport will be discussed in more detail with reference to K^{+} transport in L1210 cells (results 4.2).

Figure 3

The effect of preincubation for 4h with 10^{-5}M nitrogen mustard on fractional uptake of $^{86}Rb^{+}$ into PC6A cells.

Key: 1 ○ control

2 ● 10^{-5}M HN2 (Mean ± S.D.; n=6)

Figure 4

The effect of simultaneous addition of 10^{-3}M or 10^{-2}M nitrogen mustard on fractional uptake of $^{86}Rb^{+}$ into PC6A cells.

Key: 1 ○ control

2 ▲ 10^{-3}M HN2

3 △ 10^{-2}M HN2 (Mean ± S.D.; n=3)

Figure 5

The effect of preincubation for 4h with 10^{-5}M nitrogen mustard on fractional uptake of [^{14}C]-α-aminoisobutyric acid into PC6A cells.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 6

The effect of preincubation for 4h with 10^{-5}M nitrogen mustard on fractional uptake of [^{14}C]-cycloleucine into PC6A cells.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=4)

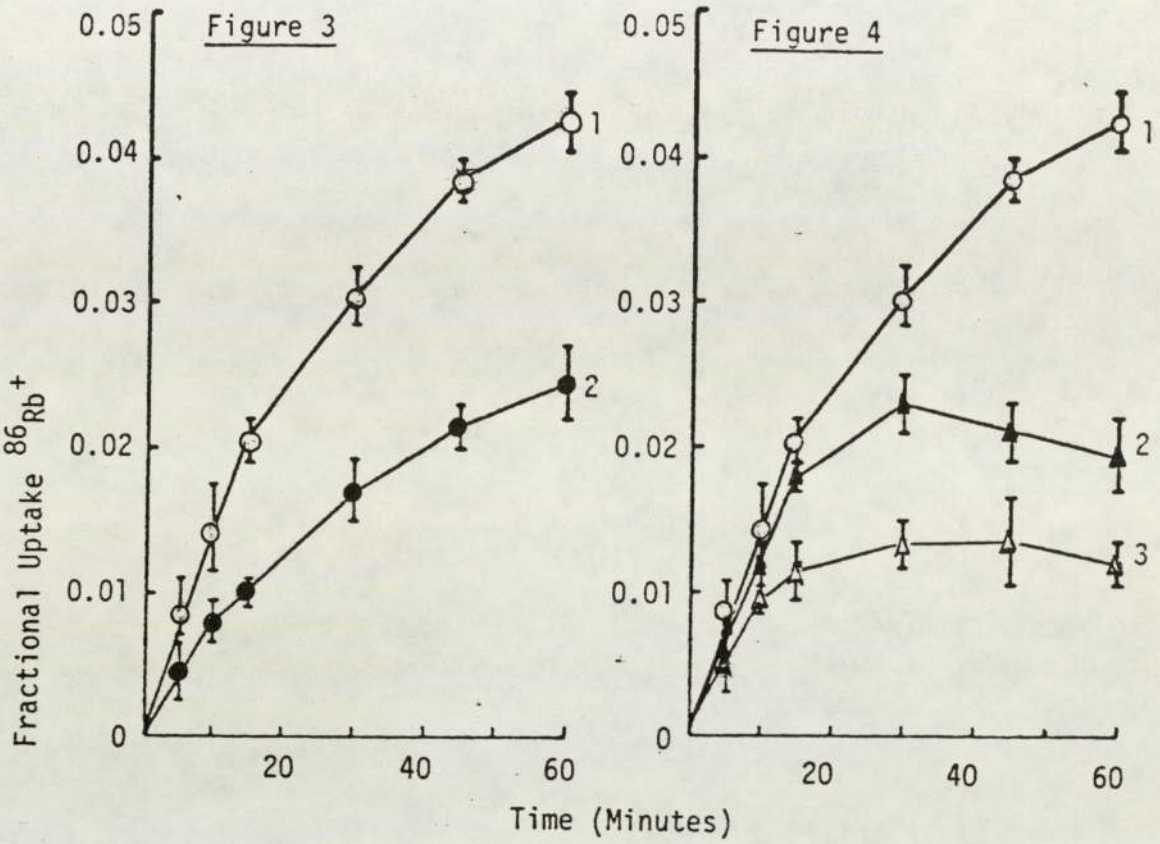


Figure 3
Figure 4
0.05
0.04
0.03
0.02
0.01
0
20
40
60
1
2
3
Fractional Uptake $^{86}Rb^{+}$
Time (Minutes)

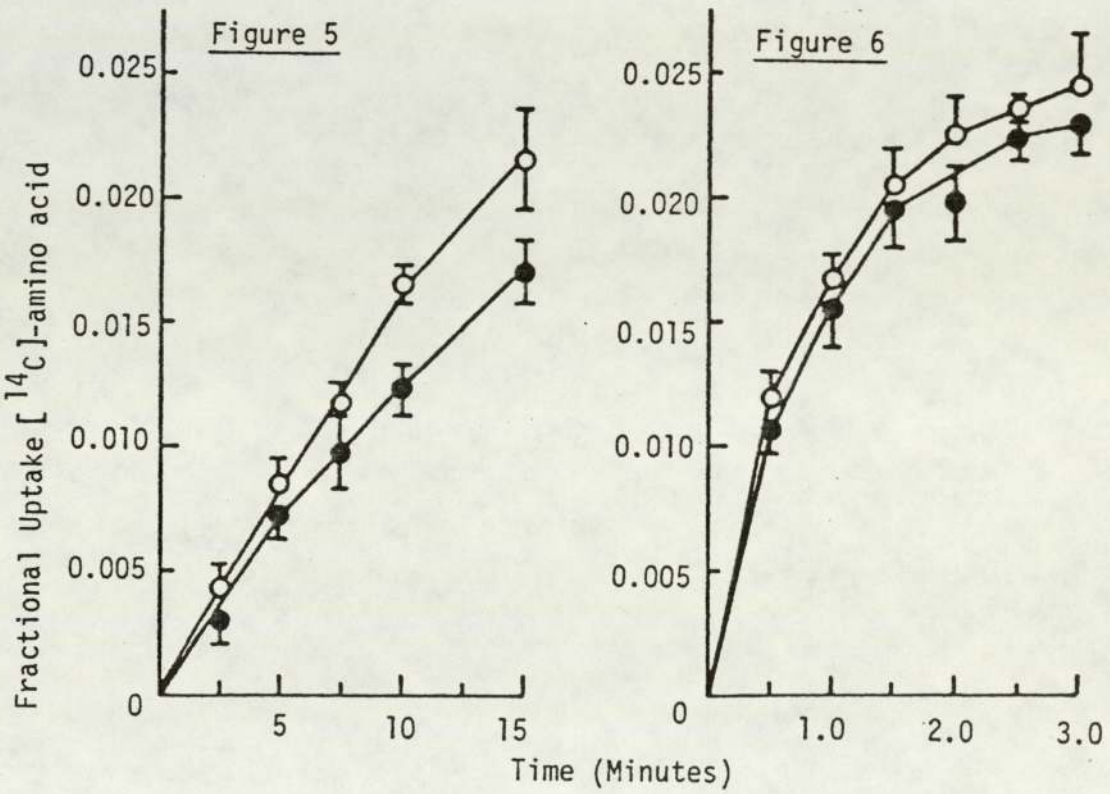


Figure 5
Figure 6
0.025
0.020
0.015
0.010
0.005
0
5
10
15
1.0
2.0
3.0
Fractional Uptake [^{14}C]-amino acid
Time (Minutes)

4.1.1.2 The effect of nitrogen mustard on amino acid transport into PC6A cells.

4.1.1.2.1 Introduction

Baxter et al (1982) showed that incubation of PC6A cells with HN2 caused an inhibition of amino acid transport. The transport of [^{14}C]-α-aminoisobutyric acid ([^{14}C]-α-AIB) was inhibited to a greater extent than was the transport of [^{14}C]-cycloleucine. Confirmatory experiments were repeated under identical conditions.

4.1.1.2.2 Results

Figures 5 and 6 show the uptake of [^{14}C]-α-AIB and [^{14}C]-cycloleucine, respectively, into $5x10^6$ PC6A cells per ml after a 4h incubation in RPMI with and without 10^{-5}M HN2. The fractional uptake (methods 3.4.2) of [^{14}C]-α-AIB was linear with time for at least 7.5 minutes, and the initial rate of uptake was calculated over this time interval. Nitrogen mustard reduced by 25% the initial rate of [^{14}C]-α-AIB uptake (figure 5). The fractional uptake (methods 3.4.2) of [^{14}C]-cycloleucine was not linear, but curvilinear with time, and was unaffected by HN2 (figure 6).

4.1.1.2.3 Discussion

The results of an earlier study (Baxter et al, 1982) showed that the transport of [^{14}C]-α-AIB and [^{14}C]-cycloleucine were reduced by 19% and 5% respectively, after a 4h incubation with 10^{-5}M HN2; therefore the present results were in good agreement.

[^{14}C]-α-AIB and [^{14}C]-cycloleucine are radiolabelled, non-metabolisable amino acid analogues (Noall et al, 1957; Christensen and Jones, 1962) which have been used as markers of the activities of the Na^+-dependent "A" (Oxender and Christensen, 1963) and Na^+-independent "L" (van den Berg and Betel, 1973) systems for amino acid transport respectively (introduction 1.2.2; but see results 4.14).

It appeared that HN2 selectively inhibited the Na^+-dependent transport of amino acids into PC6A cells.

The "A" system simultaneously transports the amino acid substrate and Na^+ ions into the cell (introduction 1.2.2) and depends upon the maintenance of a Na^+ gradient (outside>inside) across the cell membrane, by the action of the Na^+K^+ATPase (Schultz and Curran, 1970). Baxter et al (1982) suggested that the inhibition of [^{14}C]-α-AIB transport by HN2 may arise from inhibition of the Na^+K^+ATPase, although they also considered the possibility of a direct action of HN2 upon the transport system.

The effects of HN2 on amino acid transport will be discussed more fully with reference to L1210 cells (results 4.14).

4.1.1.3 The effect of nitrogen mustard on the exclusion of trypan blue from PC6A cells

4.1.1.3.1 Introduction

Impermeability to trypan blue was used a marker of PC6A cell membrane integrity during incubation with and without HN2, to investigate whether the perturbations of membrane function (results 4.1.1.1; 4.1.1.2) arose from generalised membrane damage or immediate cell death.

4.1.1.3.2 Results

Table 3 shows the percentage of PC6A cells impermeable to trypan blue throughout a 4h incubation in RPMI with and without 10^{-5}M HN2. The permeability to trypan blue did not differ significantly between cells incubated with and without HN2.

Table 3

The effect of 10^{-5}M HN2 on the exclusion of trypan blue from PC6A cells incubated in RPMI.

Time	Percentage of cells impermeable to trypan blue	
(h)	control	10^{-5}M HN2
0	77 ± 7	77 ± 7
0.5	67 ± 3	68 ± 2
1.0	73 ± 7	75 ± 9
2.0	73 ± 6	72 ± 6
2.5	73 ± 6	71 ± 9
3.0	68 ± 7	71 ± 5
3.5	72 ± 9	67 ± 8
4.0	64 ± 4	65 ± 9*

All values are mean ± S.D.; n=4

*p value for comparison with control; p>0.5.

4.1.1.3.3 Discussion.

Although there is evidence to question the reliability of trypan blue exclusion as a marker of cell viability (Tennant, 1964), it may be of use to indicate the general integrity of the cell membrane.

From the results above (table 3) it may be concluded that the action of HN2 to inhibit $^{86}Rb^{+}$ uptake (results 4.1.1.1) and [^{14}C]-α-AIB transport (results 4.1.1.2) into PC6A cells was not preceeded by widespread damage to the membrane. Although >99% of PC6A cells incubated with 10^{-5}M HN2 were destined to die (results 4.1.1.1.3), their permeability to trypan blue was not significantly different from untreated cells over the time course of the experiments. It was concluded that the interference of HN2 with

membrane transport processes did not arise from immediate cell death, nor from non-specific damage to the membrane which might prevent influx of tracer or allow efflux of tracer; rather, it appeared to arise from the interaction of HN2 with some specific cellular target(s). This conclusion was supported by the observation that HN2 had no effect on the accumulation of [^{14}C]-cycloleucine by PC6A cells (results 4.1.1.2).

Further evidence for the specific interaction of HN2 with target(s) in the cell membrane will be discussed with reference to L1210 cells (results 4.2).

4.1.2 PROLIFERATION OF L1210 MURINE LEUKAEMIA CELLS *in vitro*

4.1.2.1 Introduction

The routine subculture of cells requires a knowledge of the minimum initial density, the maximum density achieved, and the mean generation time of the culture. It is necessary to determine the cell number which define the "exponential" (or "log-linear") and "stationary" (or "confluent") phases of the growth of the culture (see section 4.1.2.3 below).

4.1.2.2 Results

Figure 7 shows the increase in the logarithm of the number of L1210 cells with time in culture. A similar result was obtained after each new culture was established. After a short lag period, the cell number increased exponentially with time until a growth plateau was reached. The mean generation time of the culture was 12h, estimated from this portion of the curve.

The minimum cell number required to initiate growth of the culture was found to be 10^4 cells per ml; at cell densities below this value, the lag phase was prolonged and cell death ensued. All cultures reached a maximum cell number of 1-2 x 10^6 cells per ml at the stationary phase.

A regime was established for the routine maintenance and harvest of L1210 cells for experiments (table 4).

Table 4

The regime for the routine maintenance and harvest for experiments of L1210 cells *in vitro*.

Initial cell number per ml	Time of incubation (h)	Harvest cell number per ml
10^4	72	"EXPONENTIAL"
3×10^4	48	$4\text{-}7 \times 10^5$
2×10^5	24	
10^4	96	"STATIONARY"
3×10^4	72	$1\text{-}2 \times 10^6$
5×10^5	48	
10^4	72	"MAINTENANCE"
		$4\text{-}7 \times 10^5$

The "exponential" phase of the growth of the culture was defined by a cell density of 4 to 7 x 10^5 cells per ml, and stationary phase was defined by a cell density of 1 to 2 x 10^6 cells per ml. Maintenance cultures were established at 10^4 cells per ml, 2 to 3 times per week, a procedure which was found to minimise the lag phase.

Upon harvest, the percentage of L1210 cell which was impermeable to trypan blue (methods 3.3.1) was greater than 98%. Cultures were discarded if this value was reduced, or if there was evidence of microbial contamination.

Figure 7

The proliferation of L1210 cells *in vitro*.

Cultures were initiated at different cell numbers. A typical result is shown. Points are the mean of duplicate determinations.

Figure 8

The effect of continuous incubation with nitrogen mustard on the proliferation of L1210 cells during 72h *in vitro*.

(Mean ± S.D.; n=4)

Figure 9

The effect of preincubation for short periods with 10^{-5}M or 10^{-7}M nitrogen mustard on the proliferation of L1210 cells during 72h *in vitro*.

Key: ● 10^{-5}M HN2

○ 10^{-7}M HN2 (Mean ± S.D.; n=4)

Hatched area: effect of continuous incubation

(see figure 8)

Figure 10

The effect of incubation for 1h with nitrogen mustard on the formation of L1210 cell colonies in soft agar.

Results are the mean of 4 experiments.

Figure 7

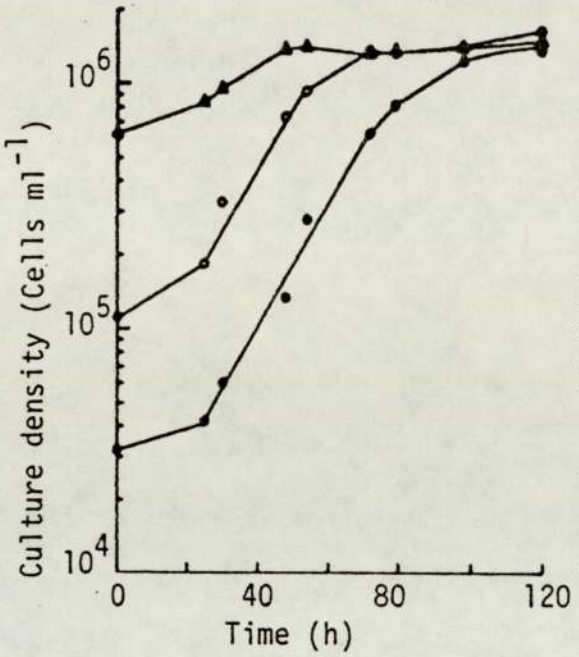


Figure 8

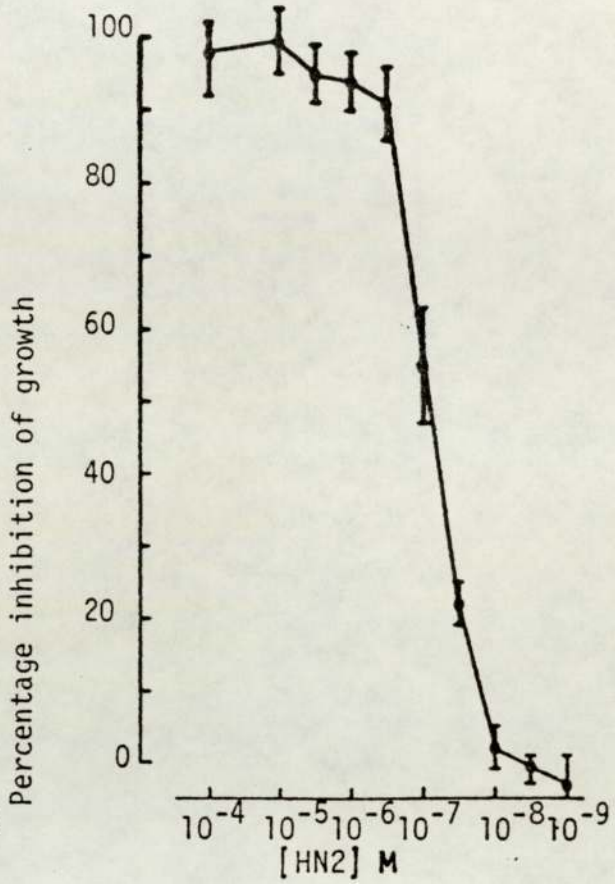


Figure 9

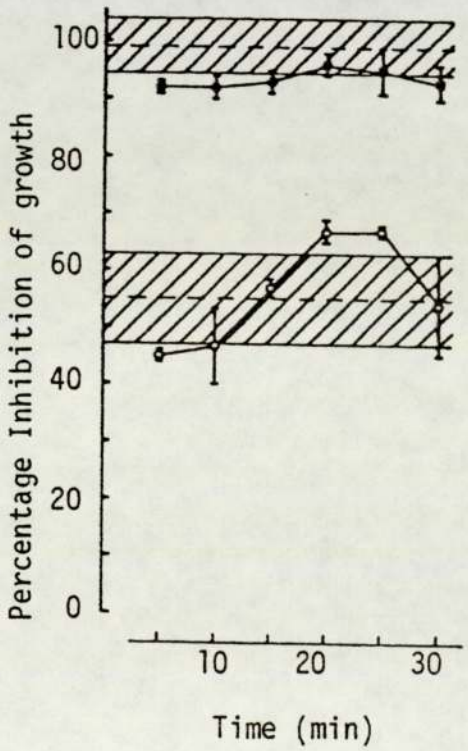
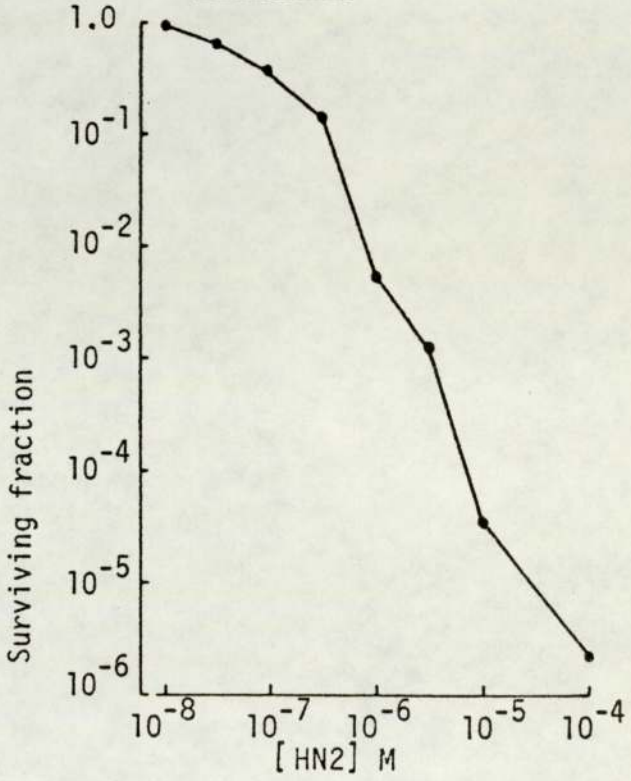


Figure 10



4.1.2.3 Discussion

A regime was established for the routine maintenance and harvest of L1210 cells in vitro. The cell numbers which defined exponential and stationary phases of growth were estimated. The different phases of growth in culture are reflected by changes in the distribution of the cell population in different phases of the cell cycle (results 4.1.3.4). The culture contains the greatest number of cycling cells, in phases S,G_2 and M, during the "exponential" phase of growth. The mean generation time of the culture (i.e., the time taken for a doubling in cell number) measured during this phase is a measure of the time taken for completion of the cell cycle. The stationary culture contains a greater proportion of the cell population in the G_1 and G_0 phases of the cell cycle.

The information derived from these measurements was necessary to allow experiments with populations of L1210 cells distributed differently in the phases of the cell cycle. Such experiments were of interest since cells in different phases of the cell cycle are differently sensitive to the cytotoxic effects of HN2 (introduction 1.1.1).

4.1.3 ESTIMATION OF THE CYTOTOXICITY OF NITROGEN MUSTARD TO L1210 MURINE LEUKAEMIA CELLS in vitro

4.1.3.1 The effect of nitrogen mustard on L1210 cell proliferation during 72 hours in culture.

4.1.3.1.1 Introduction

The effect of a range of concentrations of HN2 upon the increase in L1210 cell number during 72h in culture was examined (methods 3.3.3) to obtain an estimate of which concentrations of HN2 were cytotoxic to L1210 cells. In addition, L1210 cells were incubated for various times with HN2, then washed and incubated for 72h

(methods 3.1.2.2). The effect of drug incubation on the increase in cell number was estimated by comparison with untreated cells.

4.1.3.1.2 Results

The inhibition of cell growth, I, was calculated from the formula:-

$$I = [1 - \frac{T_f - C_i}{C_f - C_i}] \times 100\%$$

where C_i = initial cell number per ml.

C_f = final number per ml of control cells

T_f = final number per ml of treated cells.

Figure 8 shows the inhibition of L1210 cell growth after 72h in culture in the presence of various concentrations of HN2. The minimum concentration of HN2 which caused >99% inhibition of cell growth was 10^{-5}M. The concentration which caused 50% inhibition of cell growth (IC_{50}) was approximately 10^{-7}M HN2.

Figure 9 shows the effect of preincubation for various times with 10^{-5}M or 10^{-7}M HN2 on the growth of L1210 cells in culture. The cells were incubated in RPMI with or without HN2 for the required time, washed three times, finally resuspended in 10%HS in RPMI, and incubated for 72h (methods 3.1.2.2). The percentage inhibition of cell growth was calculated as described above. The inhibition of cell growth by HN2 was not dependent upon the length of time of exposure to the drug; inhibition of cell proliferation was similar after short periods of incubation and continuous incubation (figure 9).

4.1.3.1.3 Discussion

Incubation of L1210 cells with 10^{-5}M HN2 caused >99% inhibition of cell growth <u>in vitro</u>. For experiments to examine the toxic

effects of HN2, the L1210 cells were incubated with 10^{-5}M HN2 so that almost all of the cells were destined to die. Within five minutes of exposure to 10^{-5}M HN2, the cells suffered sufficient damage to ultimately result in death: this observation suggests that HN2 bound rapidly and irreversibly with some susceptible cellular component, or, that interaction with some such component for a short time only was sufficient to effect cytotoxicity. These findings are in contrast to those of Walker and Helleiner (1962) who reported that an incubation period of 3h was required for the maximum killing effect of HN2 on mouse fibroblasts; the incubation medium was supplemented with serum, the proteins of which may have interacted with the drug (results 4.1.3.3.3) to reduce its reaction with the cells.

Kessel et al (1969) showed that HN2 was rapidly accumulated by L1210 cells in vitro, largely in a non-diffusible form. After incubation for 10 minutes with 10^{-5}M HN2 the cell:medium ratio for HN2 distribution was approximately 10. Irreversibility of the reaction of HN2 with cells may be expected from the ability of HN2 to form covalent bonds with a variety of cellular components (introduction 1.1.4.1).

Nitrogen mustard readily hydrolyses in aqueous medium; Spurgin (1981) estimated the half-life of intact drug to be 87 seconds in water at 37^{o}. The presence of chloride ions in RPMI medium would be expected to stabilise the intact molecule, although the half life of HN2 in Fischers medium , which has similar chloride content to RPMI, was still less than 30 minutes (Alexander and Mikulski, 1961). Therefore, the nominal concentration of 10^{-5}M HN2 represents only the initial concentration of the intact molecule.

The cytotoxicity of HN2 to L1210 cells is probably due to the intact HN2 molecule (Brewer et al, 1961) rather than to the product

of hydrolysis, which is not an alkylating agent (Brockman, 1974, Goldenberg et al, 1971). The period of exposure to intact HN2 is limited by its short half-life, so the intact molecule must react rapidly with a sensitive cellular target to inhibit cell growth. If this is true, the observation that the inhibition of L1210 cell growth by HN2 was independent of incubation period may not be unexpected.

Thus, a rapid interaction of 10^{-5}M HN2 with L1210 cells incubated in RPMI left >99% of cells destined to die. However, for periods of incubation of up to 4h, the permeability of HN2-treated L1210 cells to trypan blue was not significantly different to that of cells incubated in HN2-free RPMI (results 4.1.3.3).

The measurement of inhibition of cell growth in vitro to estimate drug cytotoxicity (methods 3.3.3) has two main disadvantages which limit its use for screening for cytotoxic drugs. Firstly, during the incubation for 72h, the number of cells increases by only approximately 2 logarithms; this is therefore the maximum detectable cell kill. The colony forming assay (methods 3.3.4) must be used to obtain a more accurate estimate of the logarithm of the number of cells killed by a given concentration of drug. Secondly, the data is collected at a single (72h) time point, to calculate inhibition of cell growth, but no distinction may be made between cell kill and delayed cell growth due to a temporary lesion which may be repaired at a later time. Such information is of particular importance for the consideration of chemotherapeutic value of a new drug; the drug must kill, not merely delay the growth of, sufficient numbers of cells to reduce the tumour and it must do so at concentrations which may be achieved pharmacologically in vivo. However, the technique provides a rapid result to identify cytotoxic concentrations for

further investigations. The concentration of 10^{-5}M HN2, as the minimum concentration to inhibit >99% L1210 cell growth *in vitro*, is the same as the concentration which inhibited >99% PC6A cell growth in a *in vivo-in vitro* bioassay (Baxter *et al*, 1982; introduction 1.3). Rutman *et al* (1961) calculated that a therapeutic dose of 10^{-5} moles kg^{-1} HN2 corresponded to a maximum concentration of 10^{-5}M HN2 in the body water of a mouse bearing the Ehrlich ascites tumour. The growth of the Ehrlich ascites tumour *in vitro* was inhibited by 90% after incubation with 10^{-5}M HN2 (Grunicke *et al*, 1985; Doppler *et al*, 1985); the authors concluded that 10^{-5}M HN2 was a cytotoxic and pharmacologically achievable concentration.

L1210 cells were incubated with 10^{-5}M HN2 in further experiments to examine the cytotoxic effects of the drug.

4.1.3.2 The effect of nitrogen mustard on colony formation by L1210 cells

4.1.3.2.1 Introduction

The colony forming assay was used to measure more accurately the cytotoxicity of nitrogen mustard to L1210 cells *in vitro*, expressed as the logarithm of the number of cells killed. This technique has several advantages over the measurement of inhibition of growth in culture (results 4.1.3.1.3). Three modifications of the technique were attempted (methods 3.3.4) to establish optimum plating efficiency for L1210 cells.

4.1.3.2.2 Results

Methods 1 (section 3.3.4.1) and 2 (section 3.3.4.2) usually failed to yield colonies from exponentially-growing L1210 cells. When $>2x10^5$ L1210 cells were plated, method 2 produced colonies with a plating efficiency (methods 3.3.4) of approximately 15%. Method 3 (section 3.3.4.3) produced colonies in the presence of 15% FCS with

an optimum plating efficiency of 82 ± 8% (Mean ± S.D.; n=3) which was reduced by substitution with 10% FCS, or 10, 15 or 20% HS. Plating efficiency was optimum for cells from exponentially growing cultures initiated at 2 x 10^5 cells per ml 24h earlier. Plating efficiency of cells from stationary cultures was 45 ± 7% (Mean ± SD; n=3). Method 3 (section 3.3.4.3) was adopted for all further colony forming assays.

Figure 10 shows the logarithm of the surviving fraction of L1210 cells after incubation of 5 x 10^6 ml^{-1} L1210 cells in RPMI for 1h with various concentrations of HN2. The mean plating efficiency of cells incubated in the absence of HN2 was 48±17% (Mean ± S.D.; n=4). Cells which were exposed to the highest concentrations of HN2 were plated at increasingly higher numbers until surviving colonies were observed. Nitrogen mustard at a concentration of 10^{-5}M killed between 4 and 5 logarithms of cells.

4.1.3.2.3 Discussion

The colony forming assay exploits the principle that each viable cell may divide to form a single discrete colony. In practice, the plating efficiency is always less than 100% and the surviving fraction of cells after drug incubation must be calculated as a fraction of the number of colonies formed by untreated cells, and not as a fraction of the number of cells plated.

The number of cells plated was increased to obtain at least 5 surviving colonies after treatment with high concentrations of drug. Ideally, the plating efficiency should have been shown to be constant, but it proved impractical to count colonies on plates where >10^4 untreated cells were present initially.

There are conflicting observations on whether cells incubated with HN2 may divide before death occurs (introduction 1.1.4.2).

Cells which divided once or twice before death do not form colonies of 50 cells or more and therefore do not influence the results of the colony forming assay; this is in contrast to the growth inhibition assay (results 4.1.3.1).

The range of concentrations of HN2 used for the colony forming assay was based on the results of the growth inhibition assay (results, 4.1.3.1). Incubation with 10^{-5}M HN2 produced a surviving fraction of 3.65×10^{-5} which would correspond to a 99.99635% inhibition of cell growth; this would be impossible to detect accurately by use of the growth inhibition assay. The colony forming assay therefore produces a more accurate estimate of the extent of cell death.

For further experiments to examine the toxic effects of HN2, L1210 cells were incubated with 10^{-5}M HN2 so that the majority of cells were destined to die. Although greater concentrations of drug produced greater cell death, 10^{-5}M HN2 was used since it approximated to a pharmacologically achievable concentration (results 4.1.3.1.3).

The mean plating efficency of L1210 cells was reduced to 48% after a 1h incubation in serum-free RPMI in the absence of HN2; this observation suggests that exponentially-growing L1210 cells are sensitive to serum deprivation, presumably to the removal of growth factors in particular. However, serum was not included in the medium in which cells were exposed to HN2, to preclude the possible interaction of HN2 with serum proteins (results 4.1.3.3.3).

4.1.3.3 The effect of nitrogen mustard on the exclusion of trypan blue by L1210 cells

4.1.3.3.1 Introduction

The permeability of L1210 cells to trypan blue was monitored during incubation with and without 10^{-5}M HN2. The cells were

incubated for 4h at 37^{o} in either the complex nutrient medium RPMI 1640 (RPMI) or the simple salt solutions, Krebs-Ringer bicarbonate buffer (KR) or Earle's balanced salt solution (EBSS). The buffer KR was also supplemented with amino acids and glutathione to examine whether these constituents of RPMI had any effect on the permeability of L1210 cells incubated with 10^{-5}M HN2.

4.1.3.3.2 Results

Table 5 shows the exclusion of trypan blue from L1210 cells during incubation for 4h in RPMI, KR or EBSS with and without 10^{-5}M HN2. L1210 cells remained impermeable to trypan blue for shortest times in KR and for longest times in RPMI. After incubation for 3h with 10^{-5}M HN2 in KR, the fraction of cells impermeable to trypan blue was 88% of control; after incubation with 10^{-5}M HN2 in RPMI, this value was 94% of control. There was a rapid increase in the number of cells permeable to trypan blue between 3 and 4h of incubation with 10^{-5}M HN2 in KR; so that after 4h, the fraction of cells impermeable to trypan blue was only 47% of control. When RPMI was used as the incubation medium, this value was 90% of control. The permeability to trypan blue of L1210 cells incubated in EBSS was similar to that of cells incubated in KR.

From these results it was considered that some constituent(s) of the complex medium RPMI supported cell survival for longer periods and afforded some protection from short term (t<4h) damage to the cells by nitrogen mustard. KR buffer was supplemented with 970 mgl^{-1} arginine, 85mgl^{-1} methionine or 1mgl^{-1} reduced glutathione, the concentrations of which were based on the concentrations of non-sulphur-containing amino acids, sulphur-containing amino acids, and glutathione in RPMI medium (Moore et al, 1967). The addition of each of these supplements increased the number of cells which remained impermeable to trypan blue, in comparison with cells incubated in KR alone (table 6).

Table 5

The effect of 10^{-5}M HN2 on trypan blue exclusion by L1210 cells incubated for 0-4h in EBSS, KR or RPMI.

Time	Percentage of cells impermeable to trypan blue					
	EBSS		KR		RPMI	
(h)	control	HN2	control	HN2	control	HN2
0			96 ± 2			
0.5	91±3	92±4	93±4	91±6	94±6	93±3
1.0	92±1	92±3	93±4	90±1	95±1	94±3
1.5	92±5	90±2	89±4	86±13	94±4	91±8
2.5	90±4	78±5	90±4	80±9	92±1	89±1
3.0	89±3	67±7[1]	86±6	76±6[2]	94±4	88±6[3]
3.5	89±7	68±9	83±5	70±3	89±4	86±2
4.0	81±1	59±7	77±4	36±6[5]	90±3	81±7[6]

Mean ± S.D.; n=4

p values for comparison with control

(i) at 3h

[1]p <0.01

[2]p >0.05

[3]p >0.1

(ii) at 4h

[4]p <0.001

[5]p <0.001

[6]p >0.05

Table 6

The effect of addition of arginine (A), methionine (M) and glutathione (G) on the exclusion of trypan blue from L1210 cells incubated in KR with and without 10^{-5}M HN2.

		Percentage of cells impermeable to trypan blue				
Time (h)		0	1	2	3	4
Medium	HN2					
RPMI	-	98±2	95±1	97±6	94±4	90±3
	+	"	94±3	97±4	88±6	81±7
KR	-	"	93±4	96±4	86±6	77±4
	+	"	90±7	95±5	76±6	36±6
KR+A	-	"	97±4	95±4	93±6	93±0
	+	"	94±4	95±0	94±6	80±3
KR+M	-	"	95±4	94±6	97±0	95±2
	+	"	96±7	94±4	94±5	89±8
KR+G	-	"	93±3	94±4	97±1	91±3
	+	"	97±2	97±2	96±4	83±1
KR+A+M+G	-	"	96±4	96±2	96±1	95±4
	+	"	96±4	97±4	95±3	81±7

All values mean ± SD ; n=3

HN2, 10^{-5}M; absent- , present +.

Arginine: 970mgl^{-1}

Methionine: 85mgl^{-1}

Glutathione: 1mgl^{-1}

4.1.3.3.3 Discussion

The impermeability of cells to trypan blue may be useful as a marker of the integrity of the plasma membrane (results 4.1.1.3.3). There was no significant difference ($p>0.05$) in the trypan blue permeability of L1210 cells incubated for 4h with and without 10^{-5}M HN2 in RPMI; yet the large majority of these cells were destined to die (results 4.1.3.1; 4.1.3.2) and certain of their membrane functions were impaired (results 4.1.4). Thus, trypan blue exclusion may indicate only the absence of widespread membrane damage and integrity of cells in the short term. Nitrogen mustard produced a greater increase in trypan blue permeability of L1210 cells incubated in the simple salt solutions (KR, EBSS) in comparison to those incubated in the complex medium RPMI. The addition of amino acids and glutathione to KR allowed trypan blue exclusion for longer periods of incubation. It appeared that the amino acids and glutathione present in RPMI afforded for short periods some protection from the effects of HN2 on the integrity of the cell membrane. It would be of interest to perform a colony forming assay (results 4.1.3.2) to determine whether L1210 cells are more susceptible to the killing effect of HN2 when incubated in KR in comparison to RPMI. Thiol-containing compounds have been shown to protect cells from the cytotoxic effects of HN2, and have been implicated in the mechanism of detoxification of alkylating agents (introduction 1.1.3). HN2 may react covalently with amino, carboxyl and sulphydryl groups of the amino acids of RPMI medium, and effectively reduce the concentration acting upon L1210 cells. For further experiments, L1210 cells were incubated, with and without 10^{-5}M HN2 in KR buffer, for a maximum period of 3h because of the limited period for which the cells remained impermeable to trypan

blue (table 5). The use of KR buffer facilitated the manipulation of external concentrations of specific ions (results 4.2.3).

Later experiments to measure intracellular K^+ concentrations, revealed that K^+ was lost from L1210 cells incubated in KR (results, 4.7); this observation may account for their increased permeability to trypan blue during incubation in this buffer in comparison to RPMI.

Survival of exponentially-growing L1210 cells may be reduced in serum-free medium (results 4.1.3.2.3). However, serum was ommitted from the incubation medium to preclude the possibility that interactions with serum proteins may reduce the effective concentration of HN2. Nitrogen mustard may react covalently with nucleophilic groups in protein molecules (introduction 1.1.4.1; 1.1.4.3). In addition, a structural analogue of HN2, chlorambucil, has been shown to bind reversibly to plasma proteins by van der Vaals binding (Linford, 1961; Linford et al, 1969). Such interactions may protect the cells against the effects of HN2. For example: HN2 caused greater inhibition of $^{32}PO_4^{3-}$ incorporation into Ehrlich ascites cells incubated in KR buffer rather than ascitic plasma (Drysdale et al, 1958); chicken erythrocyte ghosts suffered less damage from sulphur mustard when haemoglobin was present in the incubation medium (Levy, 1965); finally, in contrast to the results obtained for the present study (results 4.1.3.1), incubation for 3h was required for HN2 to effect maximum inhibition of growth of mouse fibroblasts when horse serum was present in the incubation medium (Walker and Helleiner, 1962).

Further experiments to examine the effects of HN2 on membrane transport processes were performed with L1210 cells incubated in serum-free buffers or media. When the results obtained from such

experiments are used to consider the effects on HN2 on tumour cells in vivo, allowance must be made for the possible interaction of HN2 with serum proteins.

4.1.3.4 The effect of nitrogen mustard on the cell cycle distribution of L1210 cells.

4.1.3.4.1 Introduction

Exponentially-growing and stationary cultures of L1210 cells were sampled for flow cytofluorimetry (methods 3.3.2) to examine the distribution of the cell population in different phases of the cell cycle. This distribution was re-examined after a 3h incubation with 10^{-5}M HN2, and after serum deprivation of cells from stationary cultures.

4.1.3.4.2 Results

Figure 11 shows the plots of fluoresence and forward scatter of populations of cells obtained from the FACS flow cytofluorimeter (methods 3.3.2) for the samples described above. Each experiment was repeated three times; a typical result is shown for each experiment. Certain traces have been superimposed for visualisation of differences between them (figure 11).

4.1.3.4.3 Discussion

Dividing cells progress through a series of phases of the cell cycle described as mitosis (M), gap 1 (G_1), synthesis of DNA (S), and gap 2 (G_2). In a proliferating population of cells there is a random distribution in each phase, and therefore the fraction of cells in each phase depends upon its duration (Lamerton, 1974). A portion of cells may be quiescent, non-dividing cells which may be stimulated to divide by growth factors; these cells are usually said to be in G_0 phase.

Figure 11

Forward-scattered light and propidium iodide fluorescence of L1210 cells subjected to FACS analysis.

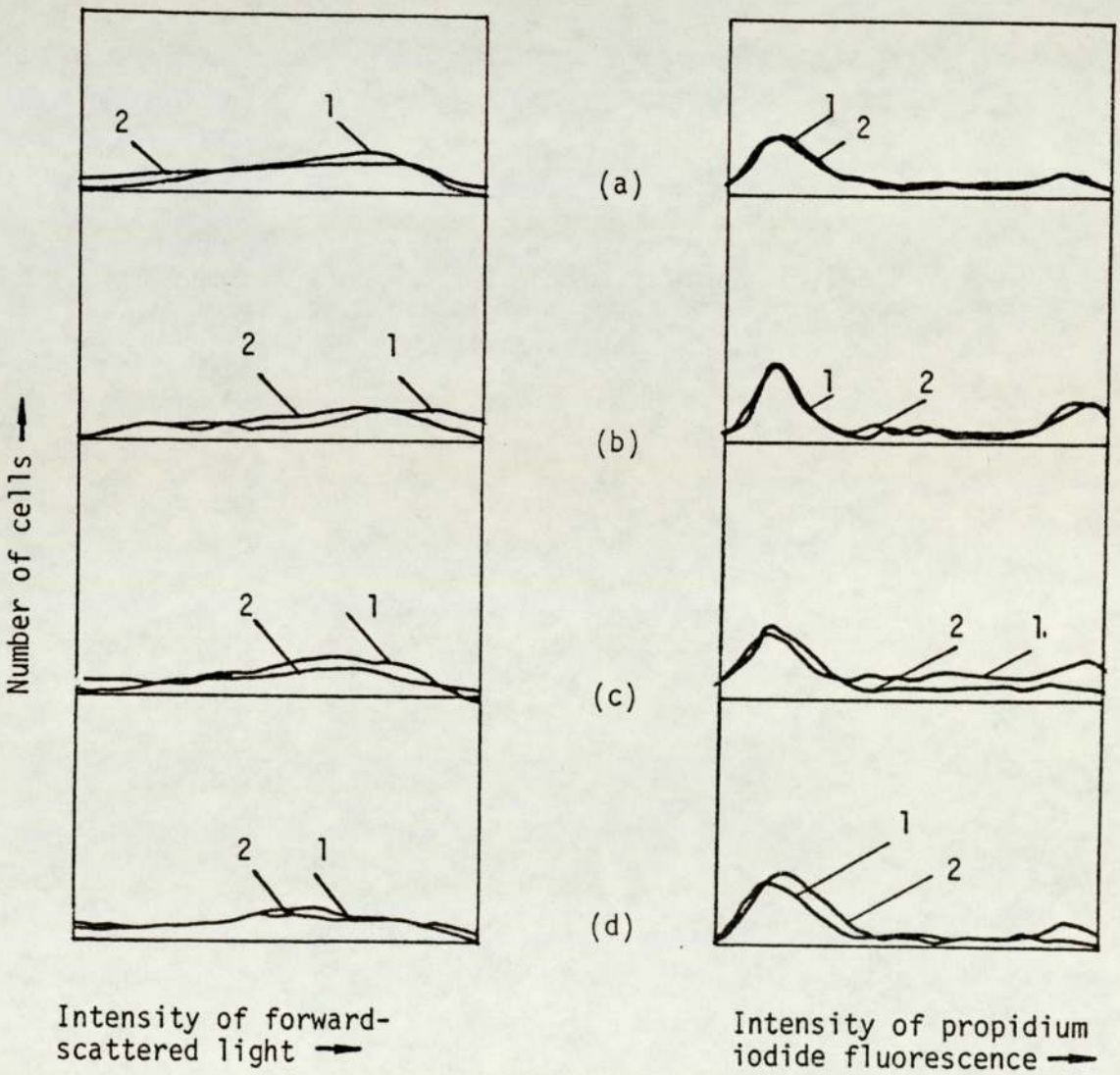
Typical results are shown.

Key: (a) L1210 cells from stationary cultures incubated for 3h in (1) absence and (2) presence of 10^{-5}M HN2.

(b) L1210 cells from exponentially-growing cultures incubated for 3h in (1) absence and (2) presence of 10^{-5}M HN2.

(c) L1210 cells from (1) exponentially growing and (2) stationary cultures.

(d) L1210 cells from (1) stationary cultures and (2) serum-deprived cultures.



The technique of flow cytofluorimetry (van Dilla et al, 1969); Gray and Coffino, 1979; Melamed et al, 1979; Bohmer, 1982) subjects cells stained with a fluorescent dye, to a dye excitation in a flow-through transport system. The fluorescence of individual cells is recorded.

Changes in the cellular complement of DNA throughout the cell cycle are the feature which is most commonly exploited for the analysis of cells cycle distribution. Cells in G_1 have a normal complement of double-stranded DNA; this doubles during S phase and remains at this level until mitosis occurs, and the two daughter cells enter G_1.

The dye propidium iodide binds quantitatively to DNA in vitro and the fluorescence emitted upon excitation by the laser beam is assumed to be proportional to cellular DNA content, although this may not be true for aspherical cells (Gledhill et al, 1976).

It is also assumed that the fraction of DNA accessible to the dye is independent of the cell cycle, and that only dye which is bound to DNA contributes to the measured fluorescence. The light collected from each individual cell as it passes through the exciting laser beam is the sum of fluorescence of the dye and scattered light, which are separated by the use of filters.

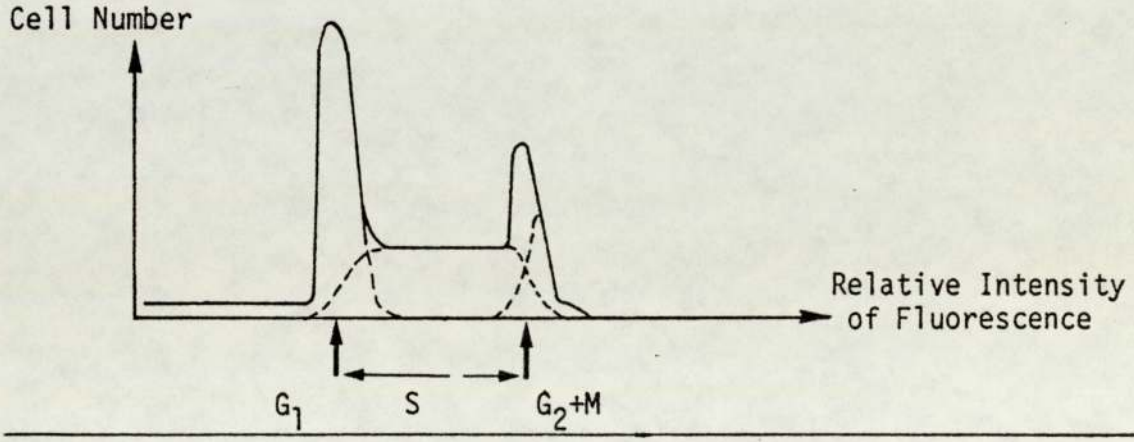
The amount of forward-scattered light depends upon the light collection, angle and aperture, refractive index of suspending medium, and other parameters, and is usually proportional to the volume of the cells (Mullaney et al, 1969; Steinkamp et al, 1976; Gray and Coffino, 1979; Hickman et al, 1984). The flow-through transport system allows rapid analysis of a large number of cells and a profile of DNA fluorescence is obtained by computer (figure 12).

The trace obtained for the fluorescence of individual cells in a population is the sum of three incompletely separated peaks (shown as broken lines in figure 12); the areas under these peaks are proportional to the fraction of cells in each phase. The number of cells in each phase of the cycle may then be determined. For the present study, the same number of cells were counted for each sample and the traces obtained were superimposed (figure 11) to examine differences in distribution of cells in different phases of the cell cycle. The percentage of cells in G_1, S and G_2+M was estimated (figure 12).

Figure 12

THE FLUORESCENCE OF PROPIDIUM IODIDE IN THE DNA OF A POPULATION OF PROLIFERATING CELLS.

(Gray and Coffino, 1979)



L1210 cells from cultures in the exponential phase of growth were more likely to be found in the S,G_2 + M phases of the cell cycle than those taken from cultures in stationary phase; the latter were more likely to be in G_1 phase. L1210 cells which had been deprived of serum for 18h were also less likely to be in the S, G_2 + M phases. However, it should be noted that cells which lack stimulation by serum-derived growth factors are probably quiescent

and thus in the G_0 phase of the cell cycle, although in some tissues cells may rest in G_2 phase (Lamerton, 1974). Cells in G_0 contain only the normal amount of DNA and therefore emit fluorescence at the same intensity as cells in G_1 when subjected to flow cytofluorimety; this technique therefore does not distinguish between the G_0 and G_1 phases. However, the results justify the use of cells from exponential and stationary phases of growth in culture to examine cell cycle-dependent changes in transmembrane transport processes and the effects of HN2 on these processes. Such experiments were of interest since cells in different phases of the cell cycle are differently sensitive to the cytotoxicity of nitrogen mustard (introduction 1.1.2). The increased sensitivity of cells in a particular phase of the cell cycle to a cytotoxic agent produces changes in the distribution of the population in the different phases; these changes are readily detectable by flow cytofluorimetric techniques (Gray and Coffino, 1979). A 3h incubation with 10^{-5}M HN2 did not change the profiles of fluorescence of L1210 cells from cultures in exponential or stationary phases of growth (figure 11). This result shows that interference with the transmembrane transport processes of L1210 cells by HN2 (results, 4.1.4; 4.3; 4.14) was not a reflection of an accumulation of cells in a particular phase of the cell cycle and the different activities of these processes which normally vary as cells progress through the cell cycle (Boonstra et al, 1984).

If the profiles of the intensity of forward scattered light are taken to indicate distributions of cell volume in the population, then the L1210 cells from stationary or serum-deprived cultures were of a smaller average size than those from exponentially-growing cultures. This difference was also detected by use of the Coulter

Channelizer (methods 3.1.6; see figure 61) which records cell sizes according to charge differences. The volume of L1210 cells from exponentially-growing cultures was reduced after incubation for 3h with 10^{-5}M HN2; this observation was confirmed by the measurement of cell volume by distribution of radiolabelled probes (methods 3.11) and is discussed in detail in results, 4.8.

4.1.4 THE EFFECT OF NITROGEN MUSTARD ON ^{86} RUBIDIUM INFLUX INTO L1210 CELLS INCUBATED IN RPMI 1640

4.1.4.1 Introduction

The growth of the L1210 murine leukaemia cell line in vitro was shown to be sensitive to 10^{-5}M HN2 (results 4.1.3.1, 4.1.3.2). As a preliminary study the experiments performed with PC6A cells (results 4.1.1) were repeated with L1210 cells to investigate whether this toxic concentration of HN2 interfered with the accumulation of $^{86}Rb^{+}$.

4.1.4.2 Results

Figure 13 shows the fractional uptake (methods 3.4.1) of $^{86}Rb^{+}$ into $5x10^{6}$ ml^{-1} L1210 cells after incubation for 4h in RPMI with and without 10^{-5}M HN2. Net uptake of $^{86}Rb^{+}$ into control and treated cells initially was linear with time, for at least 15 minutes, and the initial rate of $^{86}Rb^{+}$ influx (results 4.1.1) was estimated over this interval. Nitrogen mustard reduced the initial rate of $^{86}Rb^{+}$ influx into L1210 cells by 49%.

Figure 14 shows the effect of simultaneous addition of greater concentrations of HN2 on the fractional uptake of $^{86}Rb^{+}$ into L1210 cells incubated in RPMI; the addition of 10^{-3}M or 10^{-2}M HN2 inhibited the initial rate of $^{86}Rb^{+}$ influx by 18% and 30% respectively.

Figure 15 shows the fractional uptake of $^{86}Rb^{+}$ into L1210 cells incubated for 30 minutes in RPMI, with and without 10^{-5}M HN2; under these conditions, $^{86}Rb^{+}$ transport was unaffected by nitrogen mustard.

Figure 13

The effect of preincubation for 4h with 10^{-5}M nitrogen mustard on $^{86}Rb^{+}$ & K^{+} influx into L1210 cells incubated in RPMI.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=6)

Figure 14

The effect of simultaneous addition of 10^{-3}M or 10^{-2}M nitrogen mustard on $^{86}Rb^{+}$ & K^{+} influx into L1210 cells incubated in RPMI.

Key: 1 ○ control

2 ● 10^{-3}M HN2

3 ▲ 10^{-2}M HN2 (Mean ± S.D.; n=3)

Figure 15

The effect of preincubation for 30 minutes on $^{86}Rb^{+}$ & K^{+} influx into L1210 cells incubated in RPMI.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 13

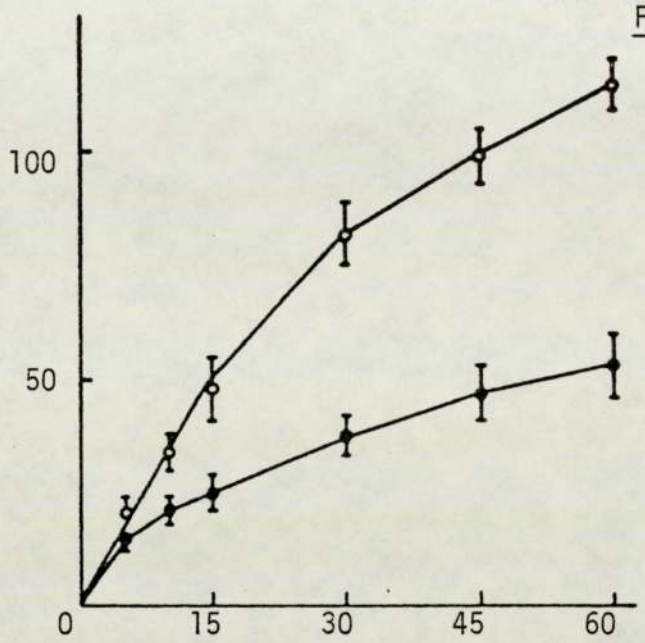


Figure 14

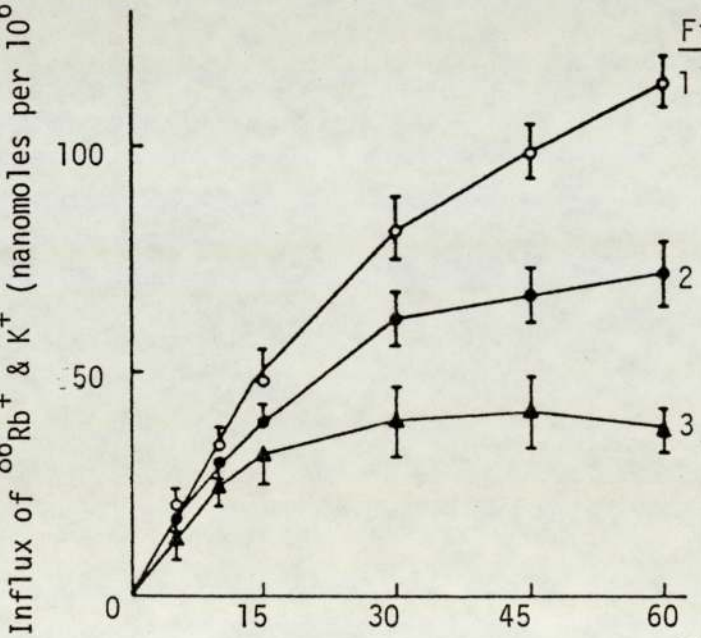
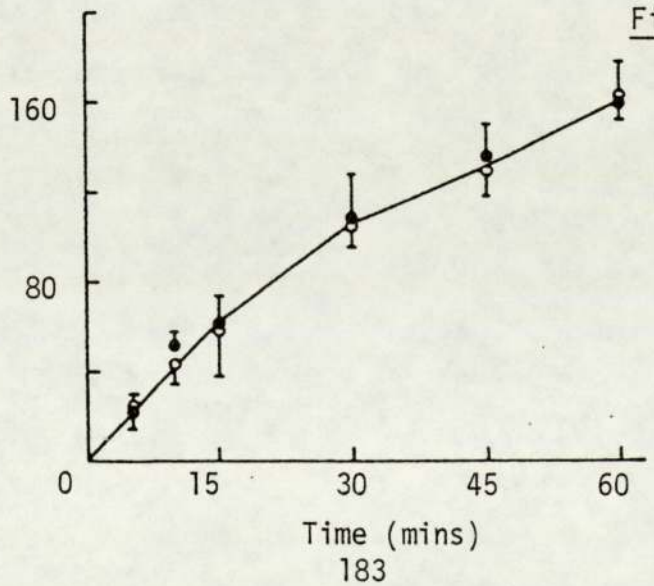


Figure 15



4.1.4.3 Discussion

Cytotoxic concentrations of nitrogen mustard substantially impaired the transport of $^{86}Rb^+$ into L1210 cells. This cell line was used for further experiments to examine the effect of HN2 on ion transport across the plasma membrane; the advantages of the use of L1210 cells, rather than PC6A cells, for such studies are discussed in the introduction (1.3). Nitrogen mustard has also been shown to inhibit the accumulation of $^{86}Rb^+$ by Ehrlich ascites cells *in vivo* (Grunicke *et al*, 1982; 1983) and *in vitro* (Grunicke *et al*, 1985; Doppler *et al*, 1985; see discussion, section 5).

Incubation for 1h with 10^{-5}M HN2 killed between 4 and 5 logarithms of L1210 cells, as measured by a colony forming assay (results 4.1.3.2), and produced >99% inhibition of L1210 cell growth in suspension culture which was irreversible after only short periods of incubation (results 4.1.3.1). However, 10^{-5}M HN2 had no immediate effect on $^{86}Rb^+$ transport into L1210 cells after a 30 minute preincubation despite the rapid, and ultimately cytotoxic, interaction of 10^{-5}M HN2 with L1210 cells during this time. After preincubation for 4h with 10^{-5}M HN2, a substantial inhibition of $^{86}Rb^+$ influx was observed. The differences observed in $^{86}Rb^+$ influx in treated and control cells did not arise from differences in the numbers of cells passing through the oil barriers in the microfuge tubes (methods 3.4.1; appendix 6:1); neither were they likely to be due to an increased efflux of transported $^{86}Rb^+$ from leaky membranes, since the portion of cells permeable to trypan blue did not differ significantly beween treated and control incubations (results 4.1.3.3) implying that the membranes were intact barriers. Inhibition of $^{86}Rb^+$ transport did not arise from loss of active transport due to ATP depletion (results 4.12), and other membrane

transport processes were not affected: nitrogen mustard had no effect on the accumulation of [^{14}C]-cycloleucine by L1210 cells (results 4.14.2), and cell membrane potential was unchanged (results 4.10).

Thus the effect of HN2 upon the transport of $^{86}Rb^{+}$ by L1210 cell membranes appeared to be a specific and selective effect at a particular locus. The nature of this locus, and the pharmacological relevance of the inhibition of $^{86}Rb^{+}$ (K^{+}) transport for the mechanism of action of HN2 were made the subject of further studies.

4.2 ^{86}RUBIDIUM INFLUX INTO L1210 MURINE LEUKAEMIA CELLS

4.2.1 ^{86} RUBIDIUM INFLUX INTO L1210 CELLS INCUBATED IN RPMI, KREBS-RINGER BUFFER AND POTASSIUM-DEPLETED KREBS-RINGER BUFFER.

4.2.1.1 Introduction

Some component of RPMI may reduce the effective concentration of HN2 to which the L1210 cells are exposed, and thus afford a degree of protection against its cytotoxic effects. The putative protective factors (results 4.1.3.3.3) are absent from Krebs-Ringer buffer, and therefore it was desirable to measure accumulation of $^{86}Rb^{+}$ by cells incubated in this buffer. In an attempt to increase the "fractional uptake" of $^{86}Rb^{+}$ and thereby reduce error, the accumulation of $^{86}Rb^{+}$ into cells incubated in a K^{+}-depleted buffer was also examined.

4.2.1.2 Results

Figure 16 shows the "fractional uptake" of $^{86}Rb^{+}$ into $5x10^{6}$ ml^{-1} L1210 cells incubated in RPMI, KR, or K^{+}-depleted (2mM K^{+}) KR. "Fractional uptake" of $^{86}Rb^{+}$ was calculated as the number of counts in cells as a fraction of the total counts in cells and medium. It was assumed that the specific activity of the medium was proportional to the total K^{+} and $^{86}Rb^{+}$ content of the medium; this assumption allowed the calculation from the specific activity of the cells of the amount of total $^{86}Rb^{+}$ and K^{+} which had been transported. Figure 17 shows the same results as figure 16, with the transport of $^{86}Rb^{+}$ and K^{+} expressed as nanomoles K^{+} & $^{86}Rb^{+}$ transported per 10^{6} cells.

4.2.1.3 Discussion

There were similar rates of fractional uptake of $^{86}Rb^{+}$ into L1210 cells incubated in RPMI or KR. A greater rate of fractional uptake of $^{86}Rb^{+}$ was observed when L1210 cells were incubated in K^{+}-depleted KR, which reflected the increased ratio of

radioactive $^{86}Rb^+$ to non-radioactive K^+ in the medium. In order to obtain results which more precisely reflected the absolute rate of transport, and which might be more readily compared with those of other workers, it was preferable to express net K^+ & $^{86}Rb^+$ transport in nanomoles per 10^6 cells. The total K^+ & Rb^+ transport rates were similar for L1210 cells incubated in KR or RPMI; however, in contrast to the measurement of fractional uptake of $^{86}Rb^+$, the rate of K^+ & $^{86}Rb^+$ uptake was lower for L1210 cells incubated in K^+-depleted KR. Therefore, whilst the use of K^+-depleted KR maximised the fractional uptake, and minimised the error in such measurements, it was unsuitable for absolute measurements of K^+ & $^{86}Rb^+$ transport. In addition, K^+-depleted KR may be unsuitable for measurements of K^+ & Rb^+ transport in the presence of the diuretics, since the efficacy of the binding of these compounds with the cotransporter may be reduced (Forbush & Palfrey, 1983).

The calculation of total K^+ & Rb^+ transport in nanomoles per 10^6 cells required the assumption that $^{86}Rb^+$ is a perfect congener for K^+. Whilst this approach has received some criticism (Meares et al, 1978), this assumption was supported by the observation that the K^+ transport processes of L1210 cells did not appear to discriminate between $^{86}Rb^+$ & Rb^+ and $^{42}K^+$ & K^+ (results 4.2.2.2). The advantages of the use of KR, rather than RPMI, as a medium for experiments to examine the effects of HN2 on K^+ & Rb^+ transport, have been discussed in results 4.1.3.3.3. Serum was omitted from the medium so that the effective concentration of HN2 was not reduced (results 4.1.3.3.3). However, it must be remembered that RPMI supplemented with serum is the normal growth medium of the L1210 cells used in this study, and more closely approximates to a physiological medium than the simple salt solution KR. In addition, during the course of this study,

Figure 16

The fractional uptake of $^{86}Rb^+$ into L1210 cells incubated in RPMI, Krebs-Ringer buffer, and K^+-depleted Krebs-Ringer buffer.

Key: 1 ■ RPMI

2 ○ KR

3 ● K^+-depleted KR (Mean ± S.D.; n=3)

Figure 17

The influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in RPMI, Krebs-Ringer buffer, and K^+-depleted Krebs-Ringer buffer.

Key: 1 ■ RPMI

2 ○ KR

3 ● K^+-depleted KR (Mean ± S.D., n=3)

Figure 18

The initial rate of influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in the presence of inhibitors of K^+ transport.

Key: 1 control

2 10^{-3}M ouabain

3 10^{-4}M bumetanide

4 10^{-3}M ouabain +10^{-4}M bumetanide (Mean + S.D.; n=3)

Figure 16

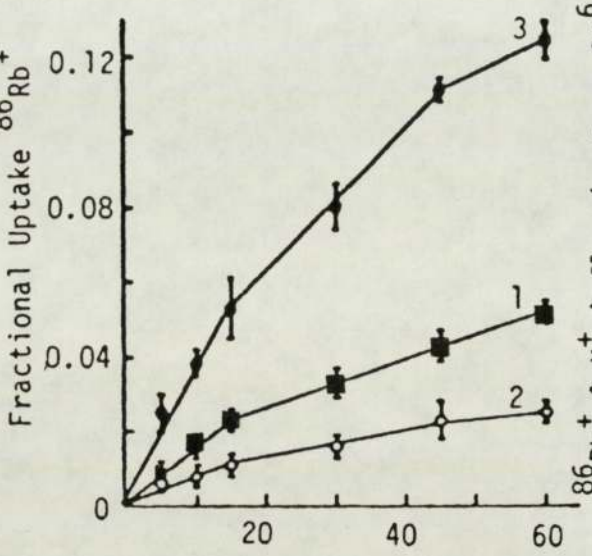
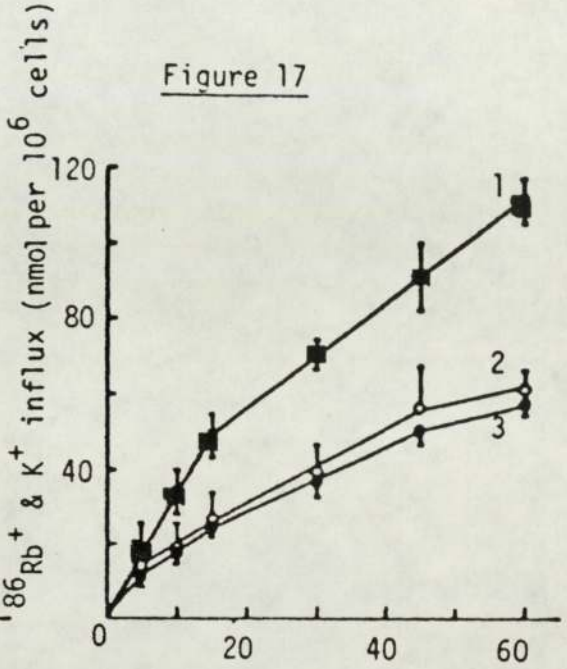
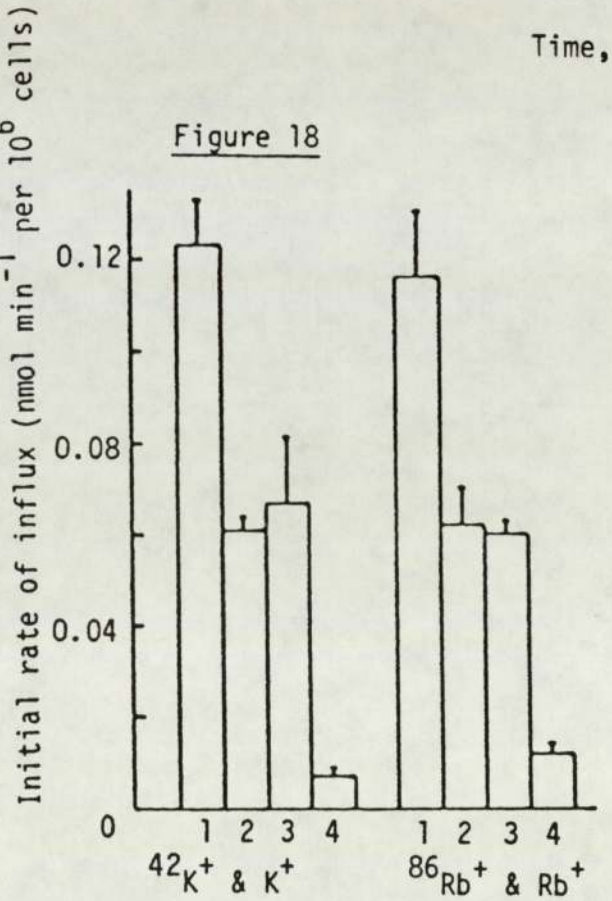


Figure 17



Time, minutes

Figure 18



Amsler et al (1985) reported that removal of serum from the uptake medium immediately reduced $^{86}Rb^+$ uptake by Swiss 3T3 cells. Several other workers have examined K^+ transport processes in cells incubated in serum-free medium (Palfrey et al, 1980; Owen and Prastein, 1985; Boonstra et al, 1984; Bourrit et al, 1985; Atlan et al, 1984); however, it may be of value in the future to investigate the effects of HN2 on the transport of K^+ into L1210 cells incubated in the presence of dialyzed serum.

4.2.2. ^{86}RUBIDIUM INFLUX INTO L1210 CELLS INCUBATED IN THE PRESENCE OF INHIBITORS OF POTASSIUM TRANSPORT

4.2.2.1. General Introduction

Selective inhibitors are widely exploited tools for the dissection of biochemical pathways; for the present study they were used to examine the pathways of K^+ & $^{86}Rb^+$ influx into L1210 murine leukaemia cells. The interaction of ouabain with the membrane-bound Na^+K^+ATPase has been described in detail (introduction 1.2.1); it was presumed that the ouabain-sensitive K^+ and $^{86}Rb^+$ fluxes provided a measure of the activity of the Na^+K^+ATPase. Similary, it was expected that fluxes sensitive to furosemide or bumetanide were mediated by the activity of a $Na^+K^+Cl^-$-cotransporter similar to that described in other systems (introduction 1.2.1). Since both the structural and functional natures of the cellular component(s) responsible for cotransport have yet to be precisely defined, the diuretic-sensitive fluxes of K^+ & $^{86}Rb^+$ were investigated in some detail (results 4.2.2). In the presence of both ouabain and furosemide, the residual K^+ and $^{86}Rb^+$ uptake was presumed to be due to the pathways which mediate "passive" exchange.

4.2.2.2 RUBIDIUM AND POTASSIUM INFLUX INTO L1210 CELLS

4.2.2.2.1 Introduction

The radioisotope ^{86}rubidium has been reported to be a congener for potassium in several systems (Beauge and Ortiz, 1970; Brown and Lamb, 1978; Baxter et al, 1982): its longer half-life allows its use as a more convenient tracer for the movement of K^+ ions than $^{42}K^+$. It was important, for the present study, to establish whether the K^+ transport processes of L1210 cells were able to distinguish between $^{86}Rb^+$ and K^+. Accordingly, the accumulation of $^{86}Rb^+$ & Rb^+ and $^{42}K^+$ & K^+ by L1210 cells was investigated.

4.2.2.2.2 Results

Figure 18 shows the initial rates of influx of ^{42} K^+ & K^+ and $^{86}Rb^+$ & Rb^+ into exponentially growing L1210 cells incubated in KR in the absence and presence of inhibitors of K^+ transport. The rate of transport is expressed as nanomoles K^+ or Rb^+ per 10^6 cells per minute, and was calculated in the manner described in section 4.2.1. There was found to be no significant difference ($p>0.05$) in the rates of accumulation of $^{42}K^+$ & K^+ and $^{86}Rb^+$ & Rb^+ by L1210 cells in the presence of any of the inhibitors. The accumulation of potassium or rubidium by exponentially-growing L1210 cells appeared to be the sum of the following components; 35-45% ouabain-sensitive; 35-45% bumetanide-sensitive, and 5-10% ouabain and bumetanide-insensitive.

4.2.2.2.3 Discussion

The radioisotope $^{86}Rb^+$ has been used as a potassium congener by several workers (Beauge and Ortiz, 1970; Brown and Lamb, 1978; Baxter et al, 1982; and others) and was used in preliminary experiments to trace the movements of the K^+ ion (results 4.1.1; 4.1.4). The calculation of the molar rates of K^+ and Rb^+ movements relied upon

the assumption that the cellular transport processes were unable to distinguish between the two ions (results 4.2.1.2). This approach also required the assumption that the ions are thermodynamically and kinetically identical with regard to mobility and rates of reaction. However, whilst different isotopes may undergo the same reactions, these reactions may proceed at different rates dependent upon the differences in mass of the isotopes. This "isotope" effect is generally only significant for the lighter isotopes (e.g., ^{1}H and ^{2}H), rather than for the heavier isotopes with small differences in mass. However, since the mass of the tracer ion, $^{86}Rb^{+}$, is more than twice that of the predominant K^{+} ion, it was important to investigate whether the difference in mass of the two ions conferred differences in the rates at which they entered cells.

Within the limits of accuracy of the assay technique (methods 3.4.1; appendix 6.1) there were no detectable significant differences in the rates of transport of $^{86}Rb^{+}$ & Rb^{+} and $^{42}K^{+}$ & K^{+} into L1210 cells. The radioisotope $^{86}Rb^{+}$ was therefore used for further experiments to examine K^{+} transport, since its half-life of 18.7 days was more convenient than that of $^{42}K^{+}$ (12.36h). ^{86}Rubidium was used in tracer amounts only, as a marker for the predominant K^{+} ion, and the molar amounts of transport were calculated as before (results 4.2.1.2). However, it has been pointed out by Meares et al, (1970), that transmembrane fluxes of tracer and non-tracer particles may have significant influence upon each other. For this reason, the radioisotope $^{86}Rb^{+}$ was present at minimal molar concentrations.

In common with other systems (introduction 1.2.1) the K^{+} influx into exponentially-growing L1210 cells depends upon a ouabain-sensitive component, a bumetanide-sensitive component, and a component insensitive to both ouabain and bumetanide. The ouabain-

sensitive component is presumably due to the action of Na^+K^+ATPase, which has been very well documented in numerous cell types, including tumour cells (introduction 1.2.1), and will not be investigated further for the present study. In contrast, the diuretic-sensitive component of the K^+ influx may be due to the action of a $Na^+K^+Cl^-$ cotransport system, in common with the system described in other cell types, but which exhibits considerable heterogeneity of activity and regulation (introduction 1.2.1). The $Na^+K^+Cl^-$ cotransport systems of several cell types have been extensively investigated but their structures and mechanisms of action and regulation have not been elucidated. The functional characteristics of the bumetanide-sensitive K^+ transport system of L1210 cells was investigated in further detail.

4.2.2.3 ^{86}RUBIDIUM INFLUX INTO L1210 CELLS FROM EXPONENTIALLY-GROWING, STATIONARY AND SERUM-DEPRIVED CULTURES

4.2.2.3.1 Introduction

Changes in transmembrane ion fluxes as cells progress through the cell cycle may have a role in the events which control cell proliferation (introduction 1.2.2). The use of L1210 cells taken from cultures in either exponential or stationary phases of growth allowed the examination of populations exhibiting different distributions of cells throughout the cell cycle (results 4.1.3.4). The activities of the K^+ transport systems in these two populations of cells were investigated by the use of inhibitors.

The proliferation of cultured cells *in vitro* relies upon the presence of growth factors provided by serum. Deprivation of serum, or attainment of confluency, leads to cessation of multiplication, with cells usually arrested in G_1 or G_0 phases of the cell cycle (Lamerton, 1974; Rozengurt, 1979; Pardee *et al*, 1978). Addition of

serum to these quiescent cultures results in the re-entry of cells into the cell cycle. The nature of the intracellular biochemical signals which control this process has been the subject of much investigation, and the observation that mitogenesis is accompanied by changes in transmembrane ion fluxes has led to consideration of the role of ions as secondary messengers in the process (Kaplan, 1978; Leffert, 1980; Boonstra et al, 1982; Boynton et al, 1982; introduction 1.2.2).

L1210 cells fron stationary cultures (results 4.1.2) were deprived of serum and $^{86}Rb^+$ & K^+ fluxes were examined in the presence of inhibitors of K^+ transport. The aims of this procedure were, firstly, to produce a population consisting mostly of quiescent cells, and to examine the activities of the K^+ transport systems in this population; and, secondly, to examine the effect of the addition of serum on the K^+ transport systems in quiescent L1210 cells. Molar rates of $^{86}Rb^+$ & K^+ influx were calculated in the manner described in results 4.2.1.2.

4.2.2.3.2 Results

Figure 11 shows the approximate distribution throughout the cell cycle of populations of L1210 cells taken from exponentially-growing, stationary, or serum-deprived cultures. The percentage of cells in each phase was calculated from the distribution of fluorescence of individual cells, measured by flow cytofluorimetric equipment (results 4.1.3.4). Only 51% of exponentially-growing cells, but 74% of stationary cells, and 80% of serum-deprived cells, were found in G_1 or G_0 phases. Therefore, exponentially-growing cultures contained the greatest proportion of dividing cells; this portion of the population declined as confluency was achieved, and was further reduced by serum starvation.

Figure 19

The initial rate of influx of $^{86}Rb^+$ & K^+ into exponentially-growing L1210 cells measured in the presence of inhibitors of K^+ transport, after preincubation for 3h with and without 10^{-5}M nitrogen mustard. (Mean + S.D.; n=11)

Figure 20

The initial rate of influx of $^{86}Rb^+$ & K^+ into L1210 cells from stationary cultures, measured in the presence of inhibitors of K^+ transport, after preincubation for 3h with and without 10^{-5}M nitrogen mustard.

(Mean + S.D.; n=4)

Figure 21

The effect of preincubation for 30 minutes with 10% horse serum on the initial rate of influx of $^{86}Rb^+$ & K^+ into serum-deprived L1210 cells.

(Mean + S.D.; n=3)

Figure 22

The effect of simultaneous addition of 10% horse serum on the initial rate of influx of $^{86}Rb^+$ & K^+ into serum-deprived L1210 cells.

(Mean + S.D.; n=3)

Key to figures 19-22

1. no inhibitor
2. 10^{-3}M furosemide
3. 10^{-4}M bumetanide
4. 10^{-3}M ouabain
5. 10^{-3}M ouabain + 10^{-3}M furosemide

Figure 19

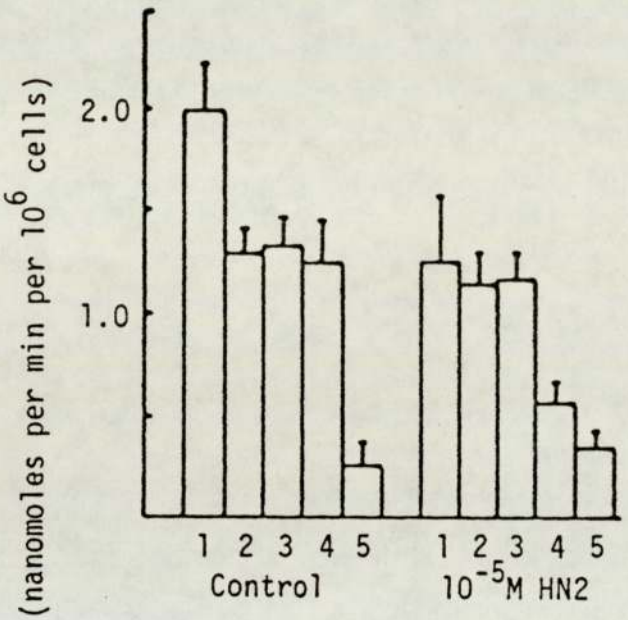
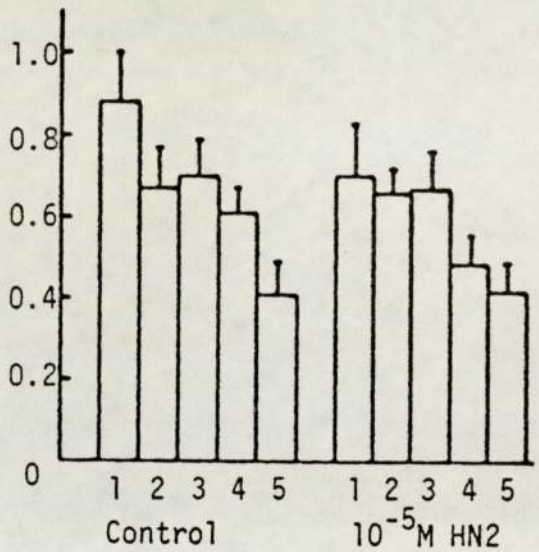


Figure 20



Initial rate of $^{86}Rb^{+}$ & K^{+} influx (nanomoles per min per 10^{6} cells)

Figure 21

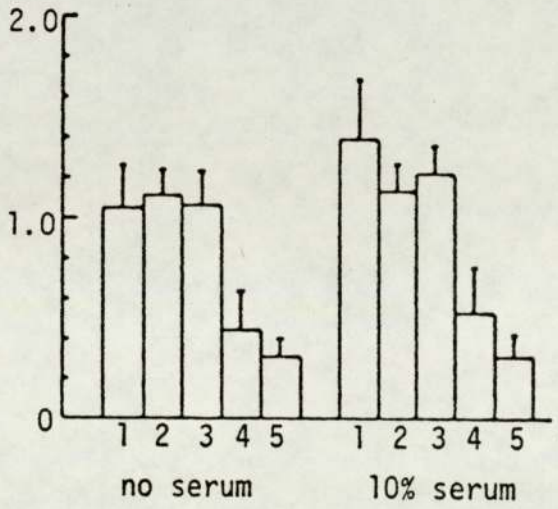
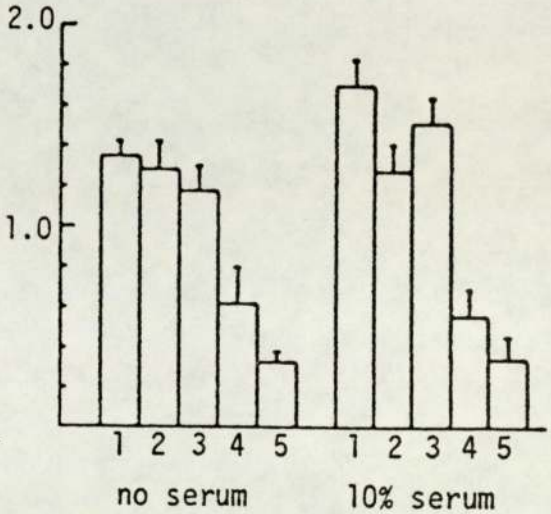


Figure 22



Figures 19 and 20 show the initial rates of $^{86}Rb^+$ & K^+ influx into L1210 cells from exponential and stationary cultures, respectively, after incubation for 3h in KR. Influx was measured in the absence and presence of inhibitors of K^+ transport. Total $^{86}Rb^+$ & K^+ influx into exponentially-growing cells was 1.99±0.22 nanomoles min^{-1} per 10^6 cells; the total flux into stationary cells was 0.88 ± 0.13 nanomoles min^{-1} per 10^6 cells, or, 44% of the flux into exponential cells. This difference was statistically significant ($p<0.05$). Stationary L1210 cells showed statistically-significant ($p<0.05$) reductions in the activities of both ouabain-sensitive and diuretic-sensitive transport systems, but a statistically-significant increase in the fluxes which were insensitive to either inhibitor; the latter fluxes accounted for 46 ± 9% of total $^{86}Rb^+$ & K^+ influx into stationary cells. The diuretic-sensitive fluxes declined from approximately 35% of the total flux in exponentially-growing cells, to approximately 23% of the total flux in stationary cells, although this decrease was not statistically significant ($p>0.05$); in contrast, the ouabain-sensitive flux declined only from 38% of total flux in exponential cells to 30% of total flux in stationary cells. (The components of total fluxes into exponentially-growing and stationary L1210 cells are shown as percentages of the total flux in table 7).

After incubation of stationary phase L1210 cells for 18h in serum-free RPMI, trypan blue was excluded by 90±3% of the cells (mean ± S.D.; n=9). Serum deprivation for 24h resulted in further loss of cell viability, so that only 60±9% of cells were impermeable to trypan blue (mean ± S.D.; n=3). Therefore, although the population of serum-deprived cells appeared to contain some cells which were not in G_1/G_0 phases, the period of incubation in the absence of serum was restricted to 18h.

Figures 21 and 22 show the initial rates of $^{86}Rb^+$ & K^+ influx into serum-deprived L1210 cells incubated in RPMI. In these cells, >90% of total $^{86}Rb^+$ & K^+ influx was due to diuretic-insensitive pathways. Figure 22 shows the effect of a simultaneous addition of 10% HS and $^{86}Rb^+$ upon the initial rate of $^{86}Rb^+$ & K^+ uptake into serum-deprived L1210 cells; the total influx of $^{86}Rb^+$ & K^+ increased significantly ($p<0.05$) from 1.33±0.09 to 1.70±0.12 nanomoles min^{-1} per 10^6 cells. This effect was due to significant ($p<0.05$) increases in the activity of ouabain-sensitive transport from 0.71±0.18 to 1.14 ± 0.14 nanomoles min^{-1} per 10^6 cells, and in the activity of the furosemide-sensitive transport from 0.05±0.14 to 0.42±0.14 nanomoles min^{-1} per 10^6 cells. In contrast, the increase in activity of the bumetanide-sensitive transport from 0.15±0.12 to 0.18±0.12 nanomoles min^{-1} per 10^6 cells was not statistically significant ($p>0.05$).

After preincubation for 30 minutes in the presence of serum, the total $^{86}Rb^+$ & K^+ influx increased from 1.05±0.21 to 1.39±0.30 nanomoles min^{-1} per 10^6 cells, but this was not statistically significant ($p>0.05$); the increase in furosemide-sensitive influx from 0±0.11 to 0.26±0.13 nanomoles min^{-1} per 10^6 cells was statistically significant ($p<0.05$). However, this observation was not matched by statistically-significant increases in bumetanide-sensitive influx. Ouabain-sensitive influx was not significantly increased (figure 21).

The contribution of ouabain-sensitive and diuretic-sensitive components to total fluxes into exponentially-growing, confluent and serum-deprived L1210 cells are summarised in table below.

Table 7

The activities of the potassium transport systems of L1210 cells from exponentially-growing, stationary, and serum-deprived cultures.

	Component				
	Percentage of total $^{86}Rb^+$ & K^+ influx				
Cells	Furosemide -sensitive	Bumetanide -sensitive	Ouabain -sensitive	Ouabain & Furosemide -insensitive	n
Exponential	35 ± 6	33 ± 7	38 ± 10	13 ± 6	11
Stationary	26 ± 12	21 ± 6	30 ± 10	46 ± 9	4
Serum-deprived	1 ± 10	6 ± 11	55 ± 16	27 ± 6	6
Serum-deprived +10% HS at t=0	25 ± 8 ($p<0.01$)*	11 ± 7 ($p>0.05$)	67 ± 8 ($p>0.05$)	21 ± 6 ($p>0.05$)	3
Serum-deprived +10% HS at t=30	19 ± 9 ($p<0.05$)*	12 ± 10 ($p>0.05$)	62 ± 16 ($p>0.05$)	23 ± 6 ($p>0.05$)	3

All values are mean ± S.D.

p values are the probability of differences of fluxes of serum-deprived cells in the presence and absence of serum.

* denotes statistically significant differences.

4.2.2.3.3. Discussion

It is well recognised that the cell membrane plays a crucial role in the control of cell division (Kaplan, 1978), but the molecular events which mediate this control remain obscure. The observation that transmembrane ion fluxes vary as cells progress through the cell cycle has led to the consideration of the role of ions in cell proliferation (Kaplan, 1978; Boynton et al, 1982; Rozengurt, 1981; Leffert, 1980; introduction 1.2.2). In particular, attention has been directed towards the changes in transmembrane ion

fluxes which preceed DNA synthesis in quiescent cells stimulated to divide. Accordingly, the activities of the potassium transport systems in exponentially-growing, stationary, serum-deprived and serum-stimulated L1210 cells were examined.

The total $^{86}Rb^+$ & K^+ influx was greatest in rapidly-dividing L1210 cells, and lowest as stationary phase was entered; after deprivation of serum for 18h, the total $^{86}Rb^+$ & K^+ influx was greater than in stationary cells. This result is surprising, since the serum-deprived population contained fewest dividing cells, and it has been widely reported that quiescent cells exhibit reduced K^+ influx activities (Kaplan, 1978; Boonstra et al, 1982; Panet et al, 1982; 1983; Rozengurt and Heppel, 1975; Rozengurt, 1981; Amsler et al, 1985).

The activity of the ouabain-sensitive Na^+K^+ATPase was maximum in rapidly-dividing L1210 cells, and reduced as stationary phase was entered. This result is consistent with observations of others that Na^+K^+ATPase activity is lowest during G_1 phase, and increases through S, G_2 and M phases (Boonstra et al, 1982; Kaplan, 1978). The contribution of ouabain-sensitive K^+ influx to total K^+ flux did not differ significantly between rapidly-dividing and stationary cells. Again at variance with the reports mentioned above (Kaplan, 1978; Boonstra et al, 1982), the ouabain-sensitive flux in serum-deprived cells was greater than that in stationary cells, and represented a greater portion of the total $^{86}Rb^+$ & K^+ flux. The differences may provide evidence that attainment of confluence and serum-starvation arrest cells at slightly different points in the cell cycle, which may be reflected in differences in K^+ transport (Panet et al, 1983; Amsler et al, 1985).

Ouabain- and diuretic-resistant K^+ fluxes are believed to represent diffusion pathways, and changes in these fluxes may represent changes in the permeability of the membrane to K^+ (Panet et al, 1983; Tupper et al, 1977, Bakker-Grunwald, 1978; Bakker-Grunwald et al, 1980; Geck et al, 1980; Panet and Atlan, 1980). Changes in K^+ permeability throughout the cell cycle are reflected in changes in the transmembrane potential (Kaplan, 1978; Boonstra et al, 1982). Ouabain- and diuretic-resistant $^{86}Rb^+$ & K^+ flux was increased as stationary phase was entered, and represented a greater percentage of total flux in stationary cells. After incubation in the absence of serum, the ouabain- and diuretic-resistant $^{86}Rb^+$ & K^+ influx was reduced, and represented a smaller proportion of the total flux, in comparison to stationary cells. It would be of interest to examine the transmembrane K^+ potential (results 4.10) of L1210 cells from rapidly-dividing, stationary and serum-deprived cultures, to examine the dependence of the potential upon 'passive' K^+ fluxes.

Furosemide-sensitive and bumetanide-sensitive K^+ influx activities declined as stationary phase was entered, and accounted for a smaller portion of the total influx into stationary cells, although the difference was not statistically significant. In contrast, furosemide- and bumetanide-sensitive flux was almost absent in serum-deprived cells, and accounted for a significantly lower percentage of the total flux. These results agree well with previous reports that cells brought to quiescence in the presence of serum, by increased culture density, have substantial diuretic-sensitive K^+ transport activity (O'Brien and Krzeminski, 1983; Amsler et al, 1985; Tupper and Zografos, 1978; Spaggiare et al, 1976), but cells brought to confluence by prolonged serum starvation show reduced activity of this pathway (Amsler et al, 1985; Panet et al, 1982; 1983; Rozengurt

and Heppel, 1975). However, the serum-deprived populations of L1210 cells used in the present study were derived from stationary cultures; whilst it would be of interest to examine diuretic-sensitive K^+ fluxes in rapidly-dividing cells arrested by prolonged serum starvation, it was found that there was a large (approximately 40%) decline of cell viability after incubation in serum free medium for 12h (data not shown).

Within five minutes of addition of serum to serum-deprived L1210 cells there was a 27% stimulation in the total $^{86}Rb^+$ & K^+ influx. The stimulation was less than that shown previously for Swiss 3T3 fibroblasts (Amsler et al, 1985; Rozengurt and Heppel, 1975; Smith and Rozengurt, 1978) and NIH 3T3 cells (Panet et al, 1982; 1983). Stimulation of $^{86}Rb^+$ & K^+ influx occurred upon addition of serum to serum-deprived L1210 cells incubated in Krebs-Ringer medium indicating that the stimulation was independent of Na^+ and amino acid cotransport (data not shown). Addition of serum produced a 60% stimulation in the ouabain-sensitive flux; this is consistent with the observations of others (Rozengurt, 1981). The addition of serum to quiescent cells results in a rapid increase in amiloride-sensitive Na^+ influx (Glaser et al, 1985; Smith and Rozengurt, 1978; Schuldiner and Rozengurt, 1982) and the subsequent elevation in intracellular Na^+ concentration activates Na^+K^+ATPase (Rozengurt and Heppel, 1975; Smith and Rozengurt, 1978, Glaser et al, 1985). Rozengurt has proposed that the subsequent elevation of intracellular K^+ (Rozengurt and Heppel, 1975; Burns and Rozengurt, 1984; Tupper et al, 1977) may play a major role in the initiation of DNA synthesis in stimulated cells (Rozengurt, 1981; Burns and Rozengurt, 1984; Lopez-Rivas et al, 1982; Ledbetter and Lubin, 1977). However, there is some evidence against the close control of DNA synthesis by changes in

intracellular K^+ (Boonstra et al, 1984; Amsler et al, 1985) and the precise role of K^+ in mitogenesis remains unclear (introduction 1.2.2).

Addition of serum to serum-deprived L1210 cells caused a stimulation in the activity of the diuretic-sensitive K^+ transport system, and increased the contribution of this activity to total flux. The increase in diuretic-sensitive K^+ flux was less than that reported by others. Panet et al (1983) showed that addition of serum to quiescent NIH 3T3 mouse fibroblasts produced a 10-20 fold stimulation in ouabain-resistant flux and a 3-4 fold stimulation in ouabain-sensitive flux. Amsler et al (1985) showed a 2-3 fold stimulation in bumetanide-sensitive flux upon addition of serum to quiescent Swiss 3T3 cells. The small response of serum-deprived L1210 cells to addition of serum may be due to the production of these cells from cultures brought to quiesence in the presence of serum (see Amsler et al, 1985). However, addition of serum to serum-deprived cultured human fibroblasts had no effect on the cotransport activity (Owen and Prastein, 1985); it was suggested that this was a net effect of the antagonistic stimuli of elevated cAMP (Boynton and Whitfield, 1983) which inhibited the system, and the response to hormones, which activated it.

The use of two distinct inhibitors of potassum flux, furosemide and bumetanide, revealed unexpected differences in the results: furosemide-sensitive K^+ influx was significantly ($p<0.05$) increased, and contributed a significantly larger fraction to the total K^+ flux, upon the addition of serum. In contrast, bumetanide-sensitive fluxes did not increase significantly, and did not contribute a significantly larger fraction to total flux. It has been reported by others that bumetanide has a greater affinity than furosemide for the

$Na^+K^+Cl^-$ cotransporter (Palfrey et al, 1980) and that furosemide, but not bumetanide, may inhibit a number of other transport pathways (introduction 1.2.1; Palfrey et al, 1980; Ellory et al, 1980). This result may suggest that the addition of serum to serum-deprived cells resulted in the stimulation of K^+ transport via some pathway distinct from the $Na^+K^+Cl^-$ cotransporter. The nature of this pathway is unknown. However, there may be a furosemide-sensitive component of K^+ influx into L1210 cells, which is dependent on external HCO_3^- (results 4.2.3) and which may involve an anion exchanger. The anion exchanger may be activated in response to the changes in intracellular pH which occur upon mitogenic stimulation (introduction 1.2.2; Moolenaar et al, 1983; L'Allemain et al,1984; Glaser et al, 1985). A key future experiment to investigate the role of K^+ transport in the proliferation of L1210 cells will be to examine the dependence of serum-stimulated K^+ fluxes upon changes in intracellular pH (see Paris and Pouyssegur, 1986).

Amsler et al (1985) reported that removal of dialysed serum from mouse fibroblasts caused an immediate fall in K^+ influx, and particularly in the activity of the bumetanide-sensitive pathway. The bumetanide-sensitive influx into rapidly-dividing cells was less sensitive to serum-deprivation that that of quiescent cells. In contrast, bumetanide-insensitive K^+ fluxes were unaffected by removal of serum. Therefore, in future investigations it will be important to examine K^+ transport into L1210 cells incubated in the presence of dialysed serum. However, if K^+ influx into rapidly-dividing L1210 cells declines in the absence of serum, it must do so within 15 minutes of removal of serum, because prolonged incubation in serum-free medium did not reveal a progressive decline in K^+ transport (see figures 30 and 32).

4.2.3 DEPENDENCE OF ^{86}RUBIDIUM TRANSPORT UPON EXTERNAL IONS

4.2.3.1 Introduction

Whilst the protein(s) responsible for $Na^+K^+Cl^-$cotransport have yet to be identified, the cotransporters of many cells have been defined by their functional characteristics (introduction 1.2.1). It is well established that cotransport is sensitive to the "loop" diuretics (Palfrey et al, 1980), which are useful tools for the separation of fluxes which are characteristic of cotransport. In addition, there is a high degree of coupling of the ions for cotransport, accompanied by a high degree of specificity for each ion (introduction, 1.2.1). The function of cotransport in several cells has been defined by this specificity (see section 4.2.3.3). It was of interest to examine the co-ion specificity for bumetanide-sensitive $^{86}Rb^+$ & K^+ transport into L1210 cells.

No detectable differences were found in the rates of bumetanide-sensitive tranpsort of $^{86}Rb^+$ & Rb^+ and $^{42}K^+$ & K^+ (results 4.2.2.2), which suggests that Rb^+ substituted completely for K^+ for cotransport into L1210 cells. The radioisotope $^{86}Rb^+$ was therefore used throughout as a tracer for the movement of K^+ ions, and the molar rates of $^{86}Rb^+$ & K^+ transport were calculated in the manner described in section 4.2.1.2. The effects of replacement of external Na^+ and external Cl^- on bumetanide-sensitive $^{86}Rb^+$ fluxes were investigated.

A K^+/H^+ exchanger, functionally coupled to Cl^-/HCO_3^- or Cl^-/OH^- exchange, has been proposed as a model to account for the net loss of K^+ and Cl^- during regulatory volume decrease in Amphiuma red blood cells in hypotonic medium (Cala, 1980, 1985; Kregenow, 1981; MacKnight and Leaf, 1985). It was important to establish whether the reduction in $^{86}Rb^+$ & K^+ transport in HN2-treated cells was independent of external HCO_3^-. External bicarbonate was replaced by nitrate and $^{86}Rb^+$ & K^+ uptake was calculated as before (results 4.2.1.2).

Figure 23

The effect of substitution of external chloride ($[Cl^-]_o$) with bromide, nitrate or gluconate on the initial rate of bumetanide-sensitive influx of $^{86}Rb^+$ & K^+ into L1210 cells.

Key: 1 ○ Bromide

2 ● Nitrate

3 ▲ Gluconate (Mean ± S.D.; n=3)

Figure 24

The effect of substitution of external sodium ($[Na^+]_o$) with lithium, choline or tetramethylammonium on the initial rate of bumetanide-sensitive influx of $^{86}Rb^+$ & K^+ into L1210 cells.

Key: 1 ○ Lithium

2 ● Choline

3 ▲ Tetramethylammonium (Mean ± S.D.; n=3)

Figure 25

The effect of nitrogen mustard and furosemide on the influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in the presence and absence of bicarbonate.

Key:

$+HCO_3^-$		$-HCO_3^-$
1 ●	control	4 ○
2 ■	3h 10^{-5}M HN2	5 □
3 ▲	15 min 10^{-3}M furosemide	6 △

(Mean ± S.D.; n=3)

Figure 23

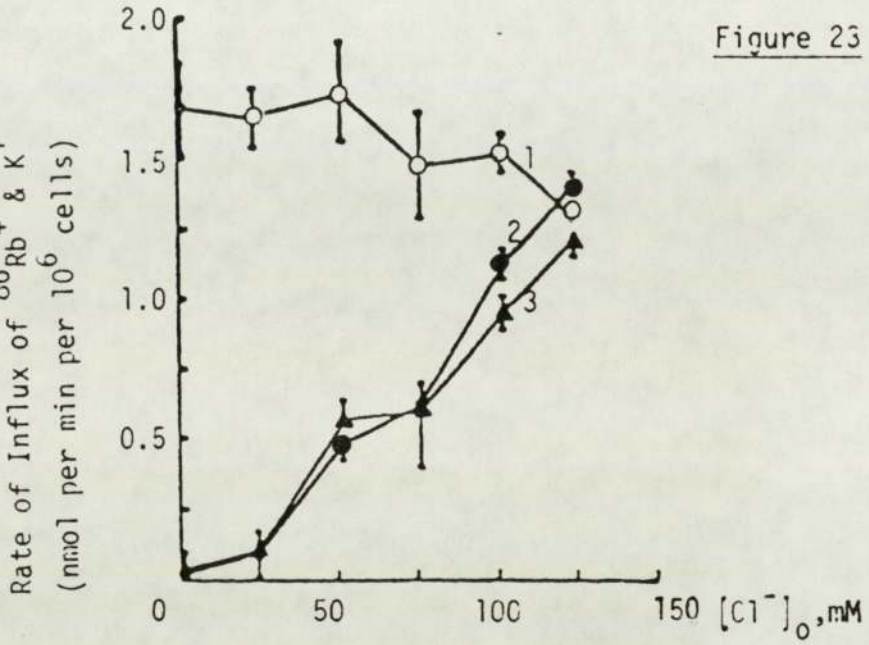


Figure 24

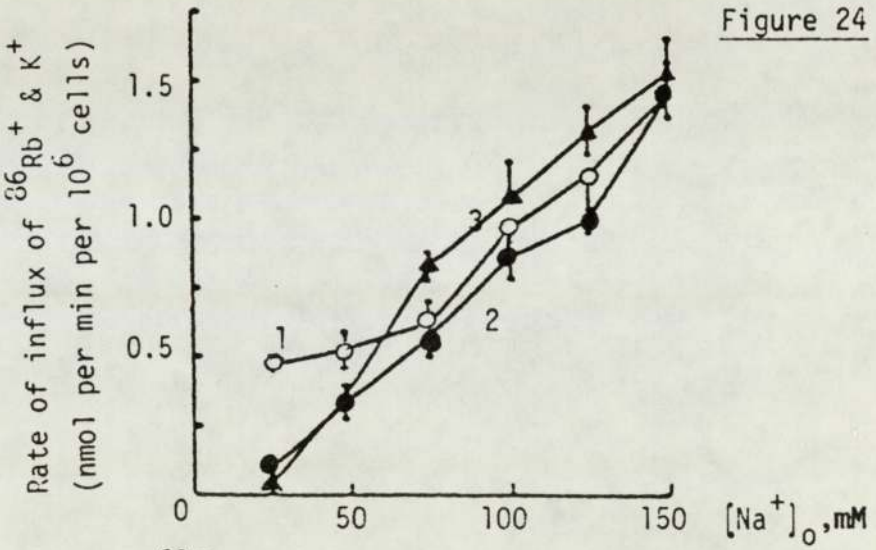
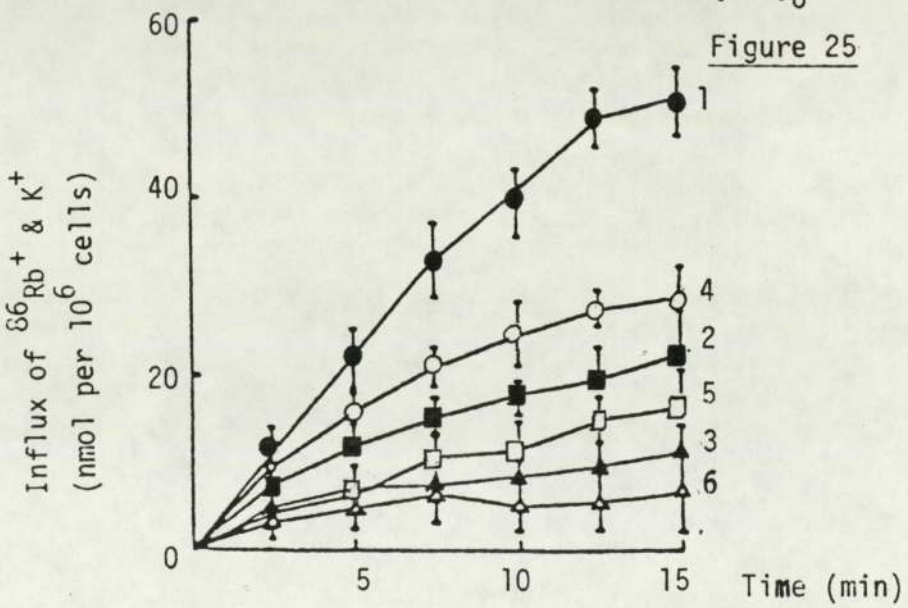


Figure 25



4.2.3.2 Results

Figure 23 shows the effect of substitution of external Cl^- ions with Br^-, NO_3^- or gluconate$^-$ on the initial rate of bumetanide-sensitive $^{86}Rb^+$ & K^+ uptake into L1210 cells. In the presence of the substituting ions NO_3^- or gluconate$^-$ the rate of $^{86}Rb^+$ & K^+ uptake increased with increasing Cl^- concentration. Dependence of $^{86}Rb^+$ & K^+ uptake on Cl^- concentration was lost when Br^- was the substituting ion; with complete replacement of Cl^- by Br^- the rate of $^{86}Rb^+$ & K^+ transport was 29 ± 12% greater than in unsubstituted Cl^- medium.

Figure 24 shows the effect of substitution of external Na^+ ions with Li^+, choline$^+$ or tetramethylammonium$^+$, on the initial rate of bumetanide-sensitive $^{86}Rb^+$ & K^+ uptake into L1210 cells from exponentially-growing cultures. The rates of $^{86}Rb^+$ & K^+ transport are expressed as percentages of the rate of transport in media in which Na^+ had not been substituted. The rate of $^{86}Rb^+$ & K^+ transport was proportional to the external concentration of Na^+ when the substituting ion was choline$^+$ or TMA^+. When Li^+ was the substituting ion, the rate of $^{86}Rb^+$ & K^+ uptake was proportional to external Na^+ concentration only at low (<75mM) concentrations of Li^+. At higher concentrations of Li^+ (>75mM), the uptake of $^{86}Rb^+$ & K^+ did not depend upon external Na^+.

Figure 25 shows the effect of complete substitution of external HCO^-_3 by NO_3^- on the transport of $^{86}Rb^+$ & K^+ into exponentially-growing L1210 cells in the presence and absence of 10^{-3}M furosemide. Complete replacement of HCO_3^- by NO_3^- inhibited the initial rate of $^{86}Rb^+$ & K^+ transport by 28% in the absence of furosemide, but by an insignificant amount in the presence of furosemide.

4.2.3.3 Discussion

The use of the simple salt solution, Krebs-Ringer bicarbonate buffer, readily allowed the manipulation of the extracellular concentrations of ionic species. Earlier results (results 4.2.2.2) showed that the Rb^+ ion quantitatively substituted for K^+ for transport by the diuretic-sensitive pathway, in agreement with the findings of other studies of cultured human fibroblasts (Owen and Prastein, 1985), avian erythrocytes (Gardner et al, 1975; Palfrey et al, 1980; McManus and Schmidt, 1978; Ueberschar and Bakker-Grunwald, 1983), mouse 3T3 cells (Sussman and O'Brien, 1985; O'Brien and Krzeminski, 1983), mouse macrophages (Panet and Atlan, 1980), Ehrlich ascites cells (Geck et al, 1980), vascular smooth muscle cells (Owen, 1984), human erythrocytes (Palfrey et al, 1980) and others (Aiton et al, 1981, 1982; Aiton and Simmons, 1983).

It was not possible to substitute $choline^+$ or TMA^+ for Na^+ so as to allow diuretic-sensitive $^{86}Rb^+$ & K^+ uptake, but partial replacement of Na^+ by Li^+ was found. Lithium has been reported to partially replace Na^+ for diuretic-sensitive $^{86}Rb^+$ or K^+ transport into cultured human fibroblasts (Owen and Prastein, 1985), HeLa cells (Aiton et al, 1980), and avian red cells (Gardner et al, 1975), and to almost completely replace Na^+ for cotransport into Ehrlich ascites cells (Geck et al, 1980). $Choline^+$ did not substitute for Na^+ to allow diuretic-sensitive $^{86}Rb^+$ & K^+ transport into HeLa cells (Aiton et al, 1980), fibroblasts (Owen and Prastein, 1985) or NIH 3T3 cells (Atlan et al, 1984), but partly substituted for Na^+ in Swiss 3T3 cells (Amsler et al, 1985); TMA^+ did not substitute for Na^+ for cotransport into Ehrlich ascites cells (Geck et al, 1980).

Cotransport activity in a variety of cells was not supported by replacement of extracellular Cl^- by NO_3^- (Geck et al, 1980; Aiton et

al, 1981; Aiton and Simmons, 1983), NO_2^-, PO_4^{3-}, Amsler et al, 1985), SO_4^{2-} (Amsler et al, 1985), I^-, acetate (Aiton et al 1981), methylsulphate, thiocyanate and gluconate (Owen and Prastein, 1985; McManus and Schmidt, 1978); however, Br^- has been shown to substitute, in part, for Cl^- to allow cotransport in several cell types (Gardner et al, 1975; Haas et al, 1982; Palfrey and Greengard, 1981; Bakker-Grunwald, 1981; Aiton and Simmons, 1983; Owen and Prastein, 1985; McManus and Schmidt, 1978; Geck et al, 1980). Thus, the results of the present study are in good agreement with the characteristics of diuretic-sensitive Rb^+ or K^+ transport of other cell types, with the exception that Br^- appeared to completely substitute for Cl^- for $^{86}Rb^+$ & K^+ transport into L1210 cells; indeed, Br^- appeared to stimulate cotransport activity. This result is also at variance with the findings of Aiton et al (1981) who reported that replacement of extracellular Cl^- by Br^- stimulated furosemide-insensitive $^{86}Rb^+$ fluxes, and inhibited furosemide-sensitive fluxes, into HeLa cells.

From the results shown in figure 23, it cannot be determined whether the curves for replacement of Cl^- by NO_3^- or gluconate display a linear, hyperbolic or sigmoidal relationship between the rate of bumetanide-sensitive $^{86}Rb^+$ & K^+ transport and the Cl^- concentration. Therefore, it is impossible to draw any conclusions regarding the stoichiometry of transport. In addition, it appears that elucidation of the stoichiometric relationship among the ions for cotransport cannot reliably be based on such data, since the shape of the curve may vary depending upon the substituting ion (Owen and Prastein, 1985; Aiton et al, 1981).

Figures 23 and 24 show that the influx of $^{86}Rb^+$ & K^+ was not saturated over the ranges of $[Na^+]_o$ and $[Cl^-]_o$ up to the

concentrations of these ions in Krebs-Ringer medium; the values of K_m for Na^+ and Cl^- which may be calculated from this data would therefore have little physiological relevance (Owen and Prastein, 1985). In addition, the apparent K_m of the transport system for Cl^- will depend upon the substituting ion, and is therefore of spurious value (Owen and Prastein, 1985). For this reason, the data was not shown as Lineweaver-Burke plots.

The total influx of $^{86}Rb^+$ & K^+ into L1210 cells was reduced in the absence of external HCO_3^-, in NO_3^--containing medium at the same pH. In the presence of furosemide, substitution of HCO_3^- by NO_3^- had no significant effect upon $^{86}Rb^+$ & K^+ uptake. This result suggests that either a significant portion of $^{86}Rb^+$ & K^+ uptake into L1210 cells is influenced by external HCO_3^- and sensitive to furosemide, or, that NO_3^- inhibits furosemide-sensitive, cotransporter-mediated $^{86}Rb^+$ & K^+ influx. Whilst NO_3^- is thought to inhibit cotransporter activity in avian and human erythrocytes (M. Haas, personal communication), this possibility is not supported by the data shown in figure 23; if bumetanide-sensitive $^{86}Rb^+$ & K^+ uptake into L1210 cells was inhibited by NO_3^-, it would expected that NO_3^- would depress cotransport even as the concentration of Cl^- was increased, thus abolishing the steady increase in cotransport with increasing Cl^- concentration (Owen and Prastein, 1985). No such effect of NO_3^- was observed.

If there is a HCO_3^--dependent K^+ influx into L1210 cells, it may be expected to be sensitive to furosemide, which has been shown to inhibit the stilbene-sensitive anion exchanger of avian erythrocytes (Palfrey et al, 1980); the possibility may be investigated by examination of the effect of DIDS (diisothiocyanatostilbene disulphonic acid) on $^{86}Rb^+$ & K^+ transport into L1210 cells.

The inability of NO_3^- to replace Cl^- for bumetanide-sensitive $^{86}Rb^+$ & K^+ transport distinguishes this process from the anion exchange system, which will accept NO_3^- (Aull, 1972; Levenson and Villereal, 1976). Forbush and Palfrey (1983) reported that NO_3^- reduced the binding of [^{3}H]-bumetanide to membranes of dog kidney medulla, which may suggest that NO_3^- binds to the cotransporter proteins but is not transported into the cell. Indeed, the results presented here do not confirm that Na^+ and Cl^- are transported into the cell with $^{86}Rb^+$ & K^+, merely that they are required for the transport of $^{86}Rb^+$ & K^+ *via* a diuretic-sensitive process. Attempts to measure diuretic-sensitive fluxes of Na^+ and Cl^- in L1210 cells proved to be unsuccessful (results 4.5), and improved methodology is needed to confirm whether these ions are cotransported with $^{86}Rb^+$ & K^+. However, if, as suggested by Geck *et al* (1980), the cotransport of Na^+,K^+ and Cl^- depends upon the transmembrane gradients of all three ions, it is likely that all three ions are cotransported into L1210 cells, since the diuretic-sensitive transport of $^{86}Rb^+$ & K^+ was not saturated over the physiological ranges of concentrations of Na^+ and Cl^-.

4.2.4 THE EFFECT OF THE SODIUM IONOPHORE MONENSIN ON ^{86}RUBIDIUM INFLUX

4.2.4.1 Introduction

It was suggested by Baxter *et al* (1982) that the observed inhibition of amino acid transport into PC6A cells treated with nitrogen mustard might arise from dissipation of the transmembrane sodium concentration gradient (see introduction 1.3). If, as suggested by other investigators, the activity of the diuretic-sensitive cotransport system depends upon the sum of the electrochemical gradients of all three ions (introduction 1.2.1), it

may be expected that perturbation of the Na^+ gradient may have consequent effects upon the transport $^{86}Rb^+$ & K^+. It was therefore important to investigate whether changes in intracellular Na^+ concentration may affect $^{86}Rb^+$ fluxes into L1210 cells.

The Na^+ ionophore monensin was used to elevate the intracellular Na^+ concentration of exponentially-growing L1210 cells, as measured by the equilibrium distribution of $^{22}Na^+$. The effect of monensin on $^{86}Rb^+$ & K^+ uptake was examined as described previously.

4.2.4.2 Results

Table 23 shows the effect of incubation for 15 minutes with $2x10^{-5}M$ monensin on the intracellular Na^+ concentration of L1210 cells in Krebs-Ringer medium in the presence and absence of $10^{-3}M$ ouabain. Monensin alone elevated $[Na^+]_i$ from 23.1±3.7mM to 191.7±13.6mM; in the presence of ouabain, $[Na^+]_i$ became 221.1±17.1mM.

Figure 26 shows the effect of preincubation for 15 minutes with $2x10^{-5}M$ monensin, in the presence and absence of ouabain, on the influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in Krebs-Ringer medium. Monensin produced a 75% stimulation in the transport of $^{86}Rb^+$ & K^+, measured over the linear portion of the curve; the stimulation was abolished in the presence of ouabain.

4.2.4.3 Discussion

The monocarboxylic acid ionophore monensin catalyses an electroneutral exchange of Na^+ for H^+ across the plasma membrane, thereby allowing equilibration of intracellular and extracellular Na^+ (Pressman et al, 1975; Pressman, 1976). Incubation of L1210 cells with $2x10^{-5}M$ monensin produced elevation of $[Na^+]_i$ above $[Na^+]_o$ (143mM) which was exacerbated in the presence of ouabain. This result suggests that the elevation of $[Na^+]_i$ caused an activation of

Figure 26

The effect of monensin and ouabain on the influx of $^{86}Rb^+$ & K^+ into L1210 cells.

Key: 1○ control

2● 2×10^{-5}M monesin

3■ 2×10^{-5}M monensin + 10^{-3}M ouabain

(Mean ± S.D.; n=3)

Figure 27

The effect of preincubation for 1h with 10^{-4}M dibutyrylcAMP on the influx of $^{86}Rb^+$ & K^+ into L1210 cells.

Key: ○ control

● 10^{-4}M dbc AMP (Mean ± S.D.; n=3)

Figure 28

The effect of continuous incubation with dibutyrylcAMP on the proliferation of L1210 cells during 72h <u>in vitro</u>.

(Mean ± S.D.; n=3)

Figure 26

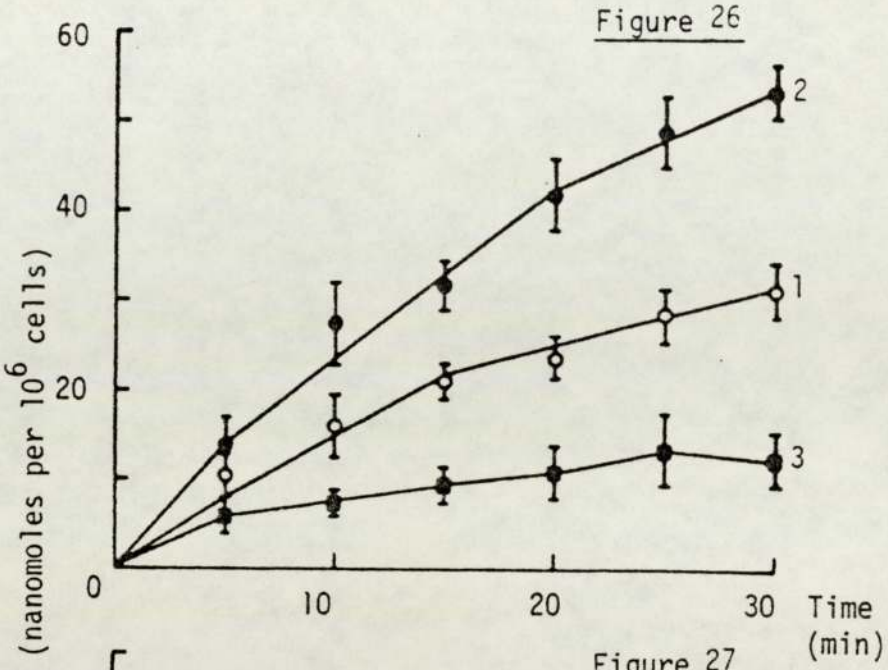


Figure 27

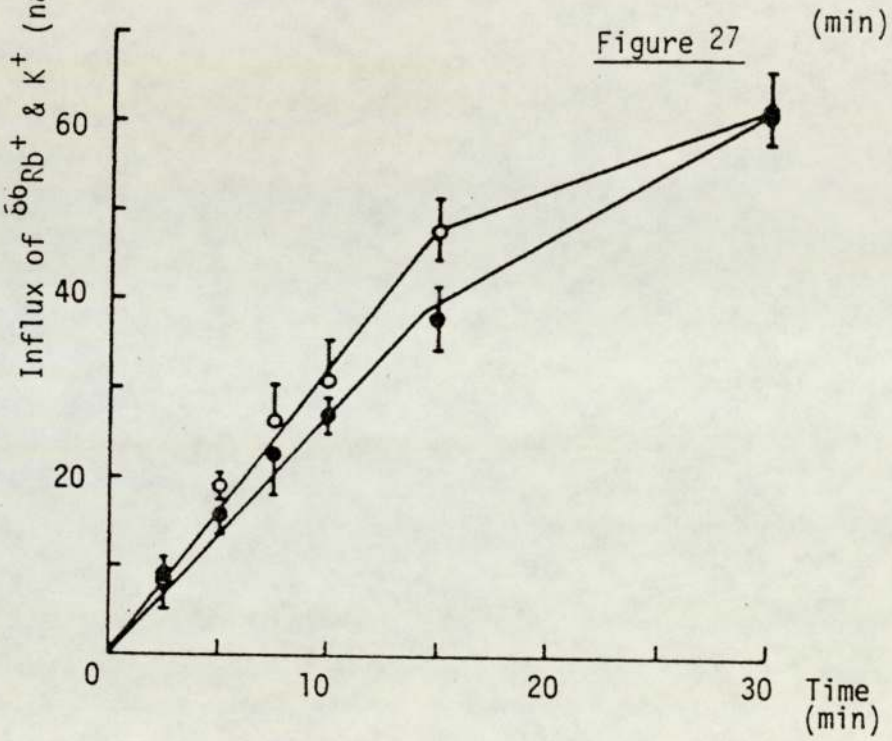
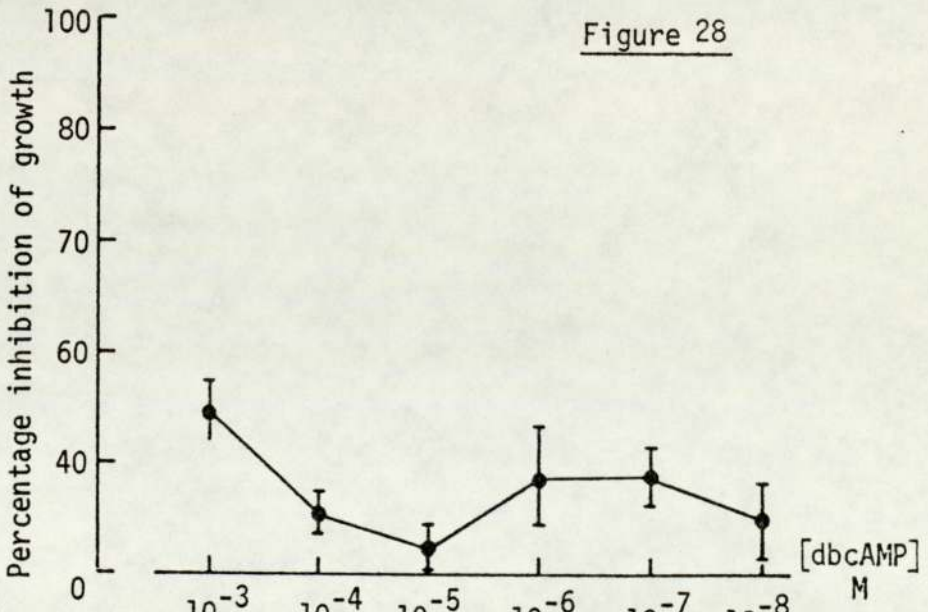


Figure 28



the Na^+K^+ATPase which, in part, removed some of the Na^+ which had entered through the action of monensin. Furthermore, the conclusion is supported by the observation that the flux of $^{86}Rb^+$ & K^+ into L1210 cells was stimulated by monensin, and that the stimulation was abolished in the presence of ouabain. Activation of Na^+K^+ATPase by monensin has been detected in several cell types, including rat hepatoma (Boonstra et al, 1984), mouse neuroblastoma (Van Zoelen et al, 1982), Swiss 3T3 cells (Smith and Rozengurt 1978), Balb/c 3T3 cells (Rozengurt, 1981) and others (Mendoza et al, 1980).

Monensin was shown to inhibit the Na^+-dependent transport of an amino acid analogue (results 4.14.1.2). However, HN2 was subsequently found to have no effect on intracellular Na^+ concentrations of L1210 cells (results 4.7). In addition, HN2 did not stimulate ouabain-sensitive $^{86}Rb^+$ & K^+ uptake (results 4.3.1.2). These observations led to the conclusion that changes in intracellular Na^+ concentrations do not result in, and do not result from, the inhibition of $^{86}Rb^+$ & K^+ transport in HN2-treated L1210 cells. Inhibition of amino acid transport by HN2 must be due to some other mechanism (results 4.14).

4.2.5 THE EFFECT OF DIBUTYRYL ADENOSINE CYCLIC MONOPHOSPHATE ON ^{86}RUBIDIUM INLFUX

4.2.5.1 Introduction

The work of Tisdale and Phillips in the early 1970's (reviewed in introduction 1.1.4.5) showed that nitrogen mustard elevated cellular concentrations of cAMP in rat Walker carcinoma cells. Inhibition of $^{86}Rb^+$ transport may result in the elevation of cAMP concentration, if Na^+K^+ATPase is inhibited (Kaplan, 1978), or it may be the result of elevation of cAMP, if the $Na^+K^+Cl^-$ cotransport system is responsive to changes in cAMP. The cotransport systems of

different cell types exhibit heterogeneity in their responses to cyclic nucleotides (introduction 1.2.1) and thus it was important to establish whether changes in intracellular cAMP might affect the fluxes of $^{86}Rb^+$ & K^+ in L1210 cells.

Dibutyryl adenosine cyclic monophosphate (dbcAMP) was used as an analogue of cAMP, and its effects upon $^{86}Rb^+$ & K^+ transport were examined in the manner described previously.

4.2.4.2 Results

Figure 27 shows the effect of preincubation for 1h with 10^{-4}M dbcAMP on the total $^{86}Rb^+$ & K^+ transport into exponentially-growing L1210 cells. Dibutyryl cAMP had no effect upon $^{86}Rb^+$ & K^+ influx into L1210 cells.

Figure 28 shows the effect of continuous incubation with different concentrations of dbcAMP on the growth of L1210 cells during 72h in culture. Continuous incubation with 10^{-4}M dbcAMP inhibited cell growth by 11±4%.

4.2.5.3 Discussion

The secondary messenger cAMP is involved in the regulation of activity of a variety of cellular enzymes, and thus, cellular cAMP concentrations are tightly regulated, at approximately 10^{-7}M (Robison et al, 1971). It may be expected then, that an agent which elevated cAMP levels beyond the capacity of the regulatory processes of the cell would have widespread toxic effects upon cell metabolism.

Nitrogen mustard and other alkylating agents have been shown to elevate cAMP concentrations of Walker cells; this action was thought to arise from inhibition of the low k_m form of the enzyme cAMP phosphodiesterase (Tisdale 1974, 1977; Tisdale and Phillips 1975a, 1975b, 1975c, 1976a, 1976b, 1976c).

It was of interest to examine the effect of cAMP on $^{86}Rb^+$ & K^+

fluxes for the following reasons; firstly, inhibition of Na^+K^+ATPase may elevate ATP and therefore cAMP levels (Kaplan 1978), and HN2 has been shown to inhibit ouabain-sensitive ATPase activity *in vitro* (Baxter *et al*, 1982). In addition, elevated cAMP concentrations may directly inhibit Na^+K^+ATPase (Moszik, 1969). Secondly, changes in intracellular ion concentrations have been shown to cause changes in cellular cAMP concentrations (Robison *et al*, 1971; Schultz *et al*, 1984). Thirdly, HN2 may interfere with the function of the $Na^+K^+Cl^-$ cotransporter which may be associated with cytoskeletal components (Jorgensen *et al*, 1985). Nitrogen mustard has been shown to affect cytoskeletal structure (introduction 1.1.4.6), and changes in cytoskeletal components may affect the activity of enzymes involved in cAMP metabolism (Howard *et al*, 1980; Margolis and Wilson, 1979). Lastly, and most importantly, the $Na^+K^+Cl^-$ cotransport system in some cell types is sensitive to changes in intracellular cAMP concentrations; therefore it was essential to determine whether the inhibition of $^{86}Rb^+$ & K^+ influx into HN2-treated L1210 cells was the result of an elevation of cellular cAMP due to inhibition of phosphodiesterase.

Phosphorylated nucleotides do not readily enter cells (Posternak, 1971); the derivative dibutyryl cAMP was synthesised in an attempt to produce an analogue of cAMP which would more readily traverse cell membranes and be more resistant to metabolism by cAMP phosphodiesterase. Intracellular cAMP concentrations (total free and bound cAMP) are usually of the order of 10^{-7}M (Robison *et al*, 1971b) and MacManus and Whitfield (1969) have calculated that 10^{-6}M cAMP is the maximum concentration reached in rat lymphocytes. However, preincubation for 1h with 10^{-4}M dbcAMP had no effect upon the $^{86}Rb^+$ & K^+ influx into L1210 cells. ^{86}Rubidium efflux from preloaded L1210

cells was also insensitive to dbcAMP (results 4.4). This finding is in agreement with the observation of Spurgin (1981) that preincubation for 2h with 10^{-6}M dbcAMP had no effect upon $^{86}Rb^{+}$ influx into PC6A murine plasmacytoma cells.

The response of $Na^{+}K^{+}Cl^{-}$ cotransport activity to changes in intracellular cAMP may represent a major regulatory mechanism and displays considerable heterogeneity between cell types. The $Na^{+}K^{+}Cl^{-}$ cotransport system of nucleated avian erythrocytes has received a great deal of attention since it is subject to hormonal regulation although the functional significance of this regulation is unknown. Avian erythrocyte $Na^{+}K^{+}Cl^{-}$ cotransport is stimulated by β-adrenergic agonists, cholera enterotoxin, adenosine, any agents which elevate cAMP, and analogues of cAMP (Palfrey and Greengard, 1981; Rudolf et al, 1977; McManus and Schmidt, 1978; Riddick et al, 1971; Alper et al, 1979; Palfrey and Rao, 1983; McManus and Schmidt, 1978; Haas and McManus, 1981; Kregenow et al, 1976); stimulation by β-adrenergic agents is blocked by β-adrenergic blocking agents such as propranolol (Riddick et al, 1971). It has been suggested that the stimulation of cotransport involves protein phosphorylation (Palfrey and Greengard, 1981; Rudolph and Greengard, 1974; Palfrey et al, 1980) and a model has been proposed for the mechanism (Alper et al, 1979; Rudolph and Greengard, 1974; Palfrey et al, 1980). Catecholamines may react with the surface receptor coupled to an adenylate cyclase, which then generates elevated cAMP concentrations. This may then act upon a cAMP-dependent protein kinase to liberate the catalytic portion of this enzyme, which in turn phosphorylates a substrate that regulates ion transport.

In contrast, elevated levels of cAMP inhibit cotransport in flounder intestine, rabbit ileum, and cultured human fibroblasts

(Owen and Prastein, 1985; Palfrey and Rao, 1983; Frizzel et al, 1975, 1979, Guandelini et al, 1982; Garay, 1982; Rao et al, 1983) and human and mammalian erythrocytes (Garay, 1982; McManus and Schmidt, 1983).

Garay (1982) has shown that isoproterenol has no effect on cotransport in human erythrocytes, which lack β-adrenergic receptors. However, the secondary messengers involved in the response to hormones may also vary between cell types. Bourrit et al (1985) reported that adrenaline and isoproterenol inhibited cotransport in NIH 3T3 cells, but that cAMP did not appear to be involved. In flounder intestine and rabbit ileum, cotransport was more sensitive to inhibition by elevated concentrations of cGMP than cAMP (Rao et al, 1983; Frizzell et al, 1979; Guandalini et al, 1982). In cultured HSWP human foreskin fibroblasts, cotransport was inhibited by isoproteronol and norepinephrine, which act on β-adrenergic receptors; by prostaglandin E_2, which acts on adenylate cyclase, and by 8-bromocyclic AMP, which is an analogue of cAMP (Owen and Prastein 1985). Norepinephrine had no effect on the cotransporter of cultured mouse fibroblast 'L' cells (Gargus et al, 1978) which lack β-adrenergic receptors (Ross and Gilman, 1977).

β-adrenergic receptors are present in the plasma membrane of L1210 cells and binding of hormones at these receptors elevated cAMP levels (Howard et al, 1980). However, from the results presented in this section, the $Na^+K^+Cl^-$ cotransport system of L1210 cells does not appear to be sensitive to changes in cellular cAMP concentration. Tisdale and Phillips reported that chlorambucil and cisplatin also elevated cAMP in Walker cells (see references quoted above) but neither of these compounds inhibited $^{86}Rb^+$ uptake into L1210 cells (results 4.18 and 4.19); this observation supports the conclusion that changes in cellular cAMP concentrations of L1210 cells do not affect diuretic-sensitive $^{86}Rb^+$ & K^+ fluxes.

However, the later observation that HN2 had no effect on the transport of $^{86}Rb^+$ & K^+ and Na^+-dependent amino acids into a diuretic- and HN2-resistant cell line, (results 4.16.3) raised the possibility that HN2 may affect a common regulator of these processes, and forced a reexamination of this conclusion.

4.3 THE EFFECT OF NITROGEN MUSTARD ON ^{86}RUBIDIUM INFLUX INTO L1210 CELLS

4.3.1 THE EFFECT OF NITROGEN MUSTARD ON ^{86}RUBIDIUM INFLUX INTO L1210 CELLS FROM EXPONENTIALLY GROWING CULTURES

4.3.1.1 The effect of various concentrations of nitrogen mustard on ^{86}rubidium influx

4.3.1.1.1 Introduction

Preliminary experiments showed that cytotoxic concentrations (10^{-5}M, 10^{-3}M 10^{-2}M) of nitrogen mustard impaired the transport of $^{86}Rb^{+}$ into L1210 cells (results 4.1.4). If this inhibition is related to the cytotoxicity of HN2, it would be expected that the inhibition is concentration- and time-dependent.

RPMI was used as an incubation medium in the preliminary experiments (results 4.1.4) but it was subsequently found that some components of RPMI may protect the cells from the cytotoxic effect of HN2 (results 4.1.3.3). This protection was absent when cells were incubated in the simple salt solution Krebs-Ringer bicarbonate buffer (results 4.1.3.3). In order that the effective concentration of HN2 was not reduced, and the external ion concentrations could be easily manipulated, it was desirable to examine the effect of HN2 on $^{86}Rb^{+}$ & K^{+} transport into L1210 cells incubated in KR buffer. The use of K^{+}-depleted KR allowed maximum fractional uptake of $^{86}Rb^{+}$ (results 4.2.1) and therefore minimised error. Thus, the effects of HN2 on $^{86}Rb^{+}$ uptake into L1210 cells incubated in KR and K^{+}-depleted both KR were examined.

4.3.1.1.2 Results

Preliminary results showed that preincubation of L1210 cells for 4h in RPMI with 10^{-5}M HN2 reduced the initial rate of $^{86}Rb^{+}$ transport by 49% (results 4.1.4).

Figure 29 shows the effect of preincubation of exponentially-growing L1210 cells for 4h in a K^+-depleted KR medium with 10^{-5}M HN2 on the net uptake of $^{86}Rb^+$ & K^+. Net uptake into control cells was intially linear with time for 15 minutes, but uptake into HN2-treated cells was almost completely abolished and was accompanied by an increase in the fraction of cells permeable to trypan blue; 68±5% of control but 21±7% of treated cells retained the ability to exclude the dye (mean±S.D; n=3). Therefore, K^+-depleted KR medium was considered to be an unsuitable medium for the prolonged incubation of L1210 cells with HN2. Accordingly, L1210 cells were preincubated in normal KR medium with and without HN2, then washed and resuspended in K^+-depleted KR for the measurement of fractional uptake of $^{86}Rb^+$. The processes of washing and resuspending cells had no effect on the viability of control or HN2-treated cells, as judged by permeability to trypan blue (data not shown).

The fractional uptake of $^{86}Rb^+$ into L1210 cells in K^+-depleted KR was allowed to proceed for 15 minutes and used as an estimate of the initial rate of transport of $^{86}Rb^+$. Figure 30 shows the intial rate of $^{86}Rb^+$ transport into L1210 cells incubated in K^+-depleted KR, following preincubation for 0-4h in normal KR in the presence and absence of 10^{-5}M HN2. The rate of $^{86}Rb^+$ influx into L1210 cells did not decline during preincubation in the absence of HN2. In contrast, incubation with 10^{-5}M HN2 caused a progressive decline in the initial rate of $^{86}Rb^+$ influx; and only after preincubation for 4h was this decline accompanied by a significant increase in the number of cells permeable to trypan blue (table 5; results 4.1.3.3). Experiments to examine the effects of HN2 on L1210 membrane function were thereafter limited to a preincubation period of 3h. After incubation of L1210 cells for 3h in KR with 10^{-5}M HN2, the initial rate of Rb^+ influx,

measured in K^+-depleted KR, was inhibited by 81% in comparison with controls.

Figure 31 shows the effect of incubation of L1210 cells in KR with various concentrations of HN2, on the initial rate of $^{86}Rb^+$ influx, measured in K^+-depleted KR. Concentration of HN2 which were inhibitory to the growth of L1210 cells in vitro (results 4.1.2) caused a progressive inhibition of the intial rate of $^{86}Rb^+$ influx.

The influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in KR was also examined. The initial rate of $^{86}Rb^+$ transport was linear for 15 minutes, and the molar rates of $^{86}Rb^+$ & K^+ transport were calculated in the manner described in results 4.2.1.2, from measurements of $^{86}Rb^+$ influx during this period.

Figure 32 shows the effect of preincubation in KR for 3h with 10^{-5}M HN2 on the uptake of $^{86}Rb^+$ & K^+ into L1210 cells incubated in KR. The initial rate of $^{86}Rb^+$ & K^+ uptake was reduced by 32% in the presence of 10^{-5}M HN2 (see also figures 33 (35% inhibition) and 19 (37% inhibition)). This result is in contrast to the results of measurements of flux in K^+-depleted medium. Figure 33 shows the effect of preincubation of L1210 cells for 3h in KR with various concentrations of HN2, on the initial rate of $^{86}Rb^+$ & K^+ influx, measured in KR.

In summary, the results presented in this section show that nitrogen mustard inhibits the influx of $^{86}Rb^+$ & K^+ into exponentially growing L1210 cells in a concentration- and time- dependent manner. A greater proportion of the $^{86}Rb^+$ & K^+ uptake into L1210 cells was sensitive to HN2 when uptake was measured in K^+depleted medium rather than in normal KR.

Figure 29

The effect of preincubation for 4h in K^+-depleted Krebs-Ringer buffer with 10^{-5}M nitrogen mustard on the influx of $^{86}Rb^+$ & K^+ into L1210 cells, measured in K^+-depleted Krebs-Ringer buffer.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 30

The effect of preincubation in Krebs-Ringer buffer with 10^{-5}M nitrogen mustard on the influx of $^{86}Rb^+$ & K^+ into L1210 cells, measured in K^+-depleted Krebs-Ringer buffer.

(Mean + S.D.; n=5)

Figure 31

The effect of preincubation in Krebs-Ringer buffer with various concentrations of nitrogen mustard on the influx of $^{86}Rb^+$ & K^+ into L1210 cells, measured in K^+-depleted Krebs-Ringer buffer.

(Mean + S.D.; n=4)

Figure 29

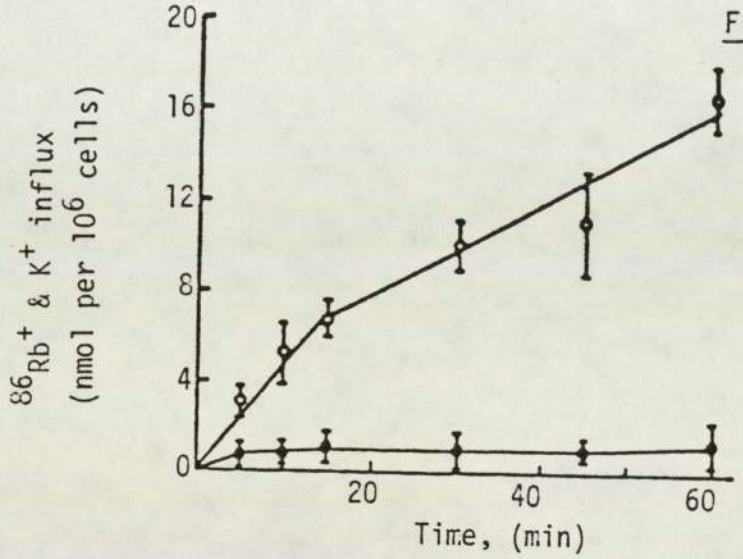


Figure 30

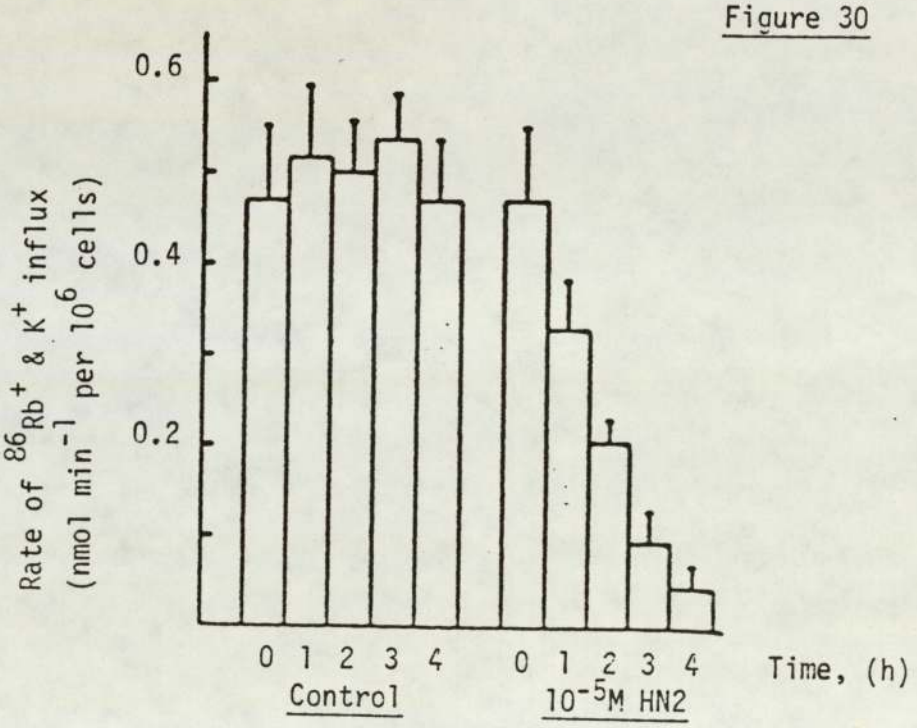


Figure 31

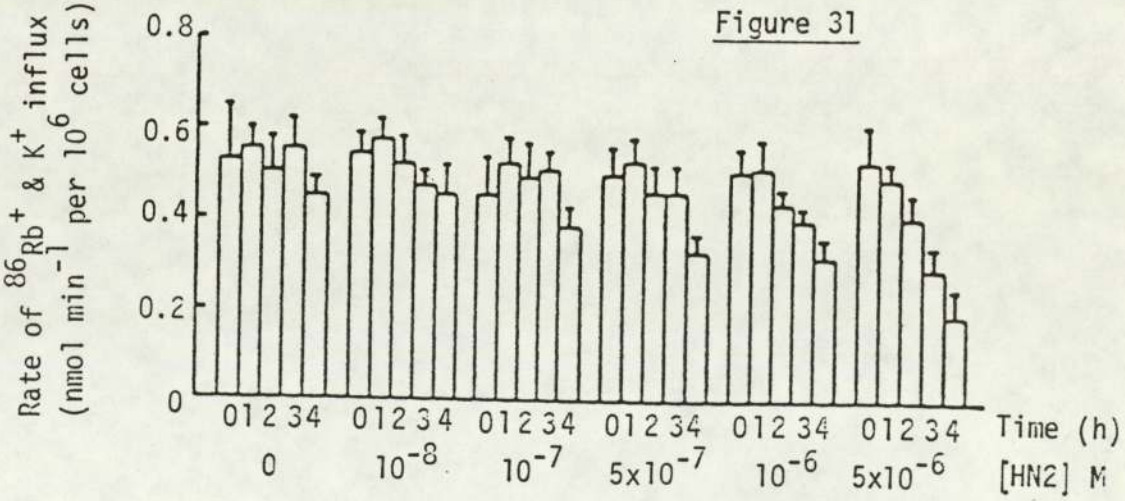


Figure 32

The effect of preincubation for 3h in Krebs-Ringer buffer with 10^{-5}M nitrogen mustard on the influx of $^{86}Rb^{+}$ & K^{+} into L1210 cells, measured in Krebs-Ringer buffer.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=4)

Figure 33

The effect of preincubation for 3h in Krebs-Ringer buffer with various concentrations of nitrogen mustard on the initial rate of influx of $^{86}Rb^{+}$ & K^{+} into L1210 cells, measured in Krebs-Ringer buffer.

(Mean + S.D.; n=5)

Figure 32

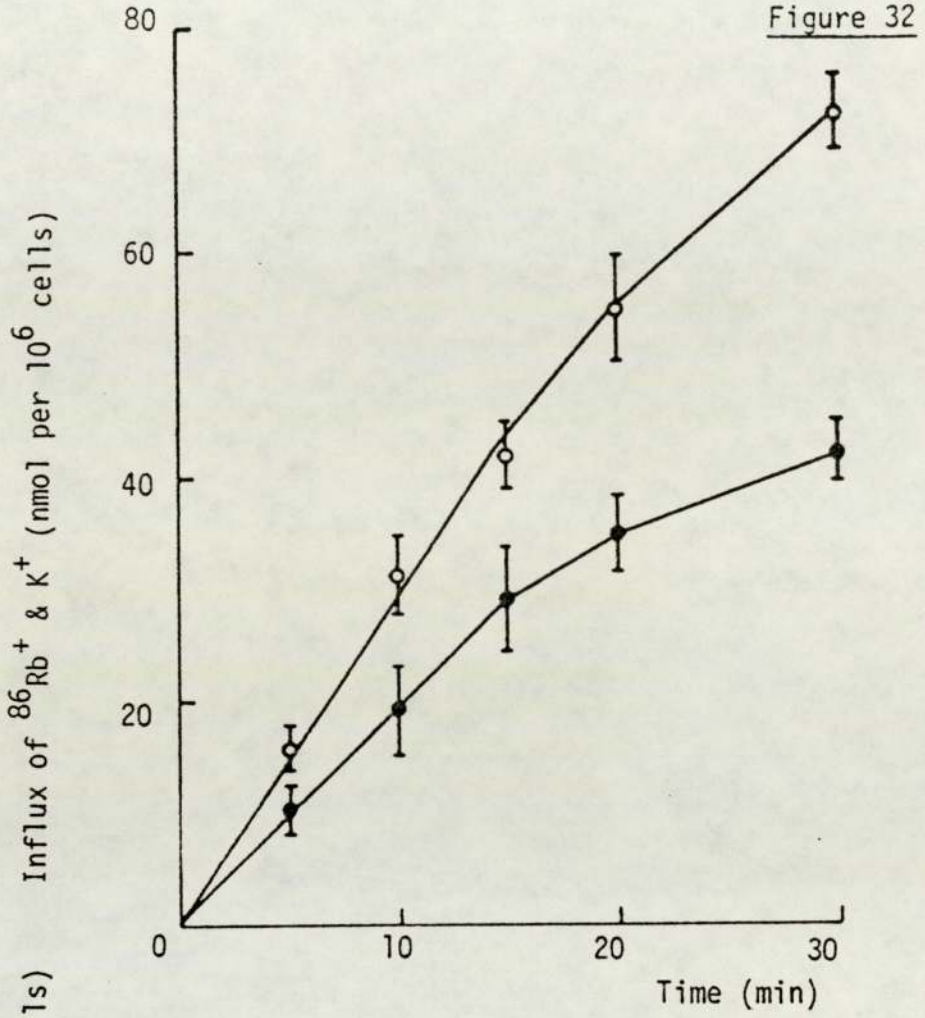
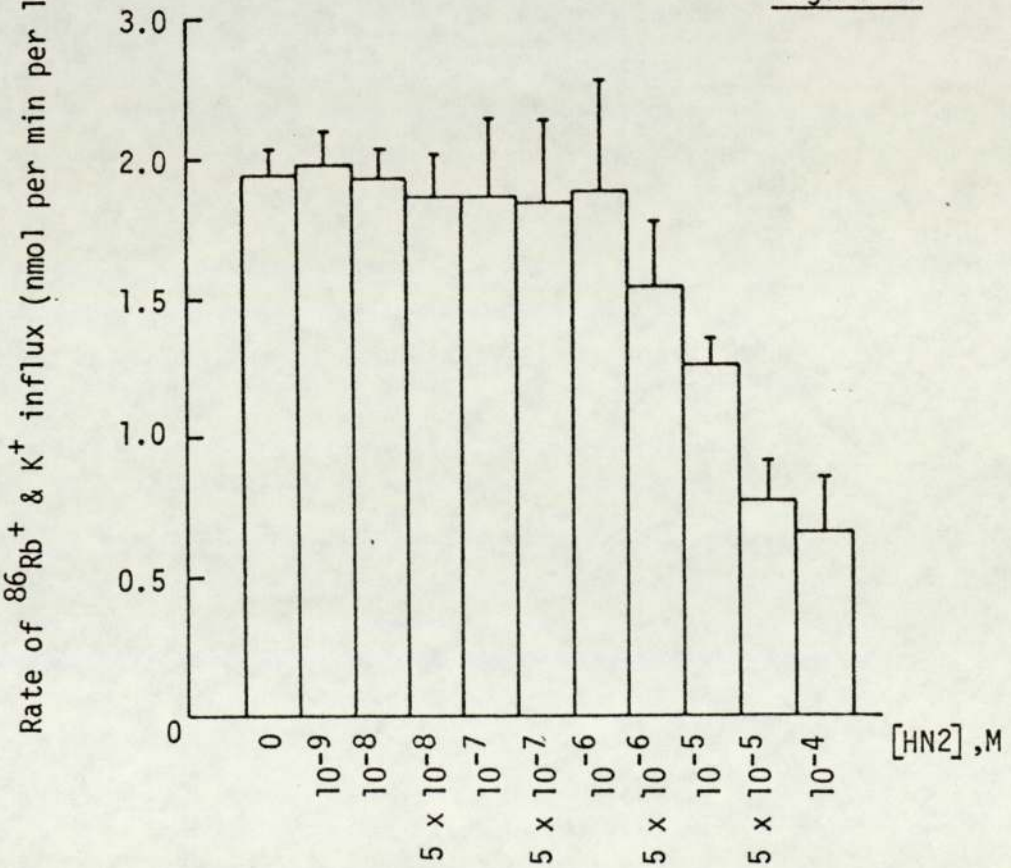


Figure 33



4.3.1.1.3 Discussion

The influx of $^{86}Rb^+$ & K^+ into rapidly-dividing L1210 cells was inhibited in a time-dependent manner by cytotoxic concentrations of nitrogen mustard. Concentrations of nitrogen mustard which were not inhibitory to the multiplication of L1210 cells *in vitro* had no effect upon $^{86}Rb^+$ & K^+ influx after incubation for up to four hours. These observations suggest that the inhibition of K^+ flux may have a role in the pharmacological action of HN2. However, the cytotoxicity of HN2 to the growth of L1210 cells *in vitro* was maximal and irreversible within short periods of incubation (results 4.1.3.1); yet the onset of inhibition of $^{86}Rb^+$ & K^+ influx was evident only after prolonged periods of incubation with pharmacological concentrations of HN2 (see also results 4.1.4). Immediate inhibition of $^{86}Rb^+$ & K^+ influx into L1210 cells was observed only upon addition of higher concentrations of HN2 (results 4.1.4) which are unlikely to be achieved *in vivo*.

The slow onset of inhibition of $^{86}Rb^+$ & K^+ uptake by HN2 was evident from the results of preliminary experiments (results 4.1; 4.1.4), and from the results of a previous study (Spurgin, 1971; Baxter *et al*, 1982). It is unlikely to be due to an effect of HN2 on the synthesis and turnover of the transport proteins; it has been shown by other workers that the synthesis of RNA and protein continues for several hours, or even days, after incubation with HN2. Levis *et al* (1963) measured DNA, RNA and protein contents of RCP guinea pig kidney cells after incubation with 0.75 gml^{-1} ($3.9x10^{-6}M$) HN2; they found that synthesis of RNA and protein proceeded at normal rates for 24h, and continued at only slightly slower rates for a further 48h. DNA synthesis was arrested after approximately 12-18h when the cells reached the G_2 phase, having

synthesised the premitotic amount of DNA. Similar findings were reported by Brewer et al (1961); DNA synthesis in mouse fibroblasts in vitro was halted at the premitotic amount within 24h of incubation with 0.1 gml^{-1} ($5.2x10^{-7}M$) HN2, but synthesis of protein and RNA continued at almost normal rates for several days. These results suggest that the interaction of HN2 with DNA (introduction 1.1.4.2) which occurs quite rapidly (see this section, below) does not interfere with the function of the molecule as a template for transcription of information into RNA. Furthermore, although HN2 has been shown to impair synthesis of proteins in a cell free system (Johnson and Ruddon; 1967), it does not appear to have an immediate effect on translation of RNA into protein by the ribosomes of the intact cell.

The delay in onset of inhibition of $^{86}Rb^{+}$ transport by HN2 was not due to the slow formation of an active product of HN2 by hydrolysis or by reaction with some component of the medium. The hydrolysis product of HN2 (figure 2) is not an alkylating agent (Brockman, 1974; Goldenberg et al, 1971), and is not thought to exhibit anititumoural activity. Spurgin (1981) has also shown that the hydrolysis product of HN2 had no effect on $^{86}Rb^{+}$ influx into PC6A cells. Brewer et al (1961) showed that HN2 incubated for 12h in culture medium was no longer inhibitory to the growth of mouse fibroblasts in vitro and concluded that the intact molecule reacted directly with a sensitive cellular component. The short half-life of the intact HN2 molecule in aqueous solution (introduction 1.1.3; Spurgin 1981) thus must limit the exposure time of cells to active drug.

Baxter et al (1982) suggested that the slow onset of inhibition of $^{86}Rb^{+}$ accummulation by PC6A cells incubated with $10^{-5}M$ HN2 might be due to the slow inactivation of some essential membrane

component. However, in consideration of the rapidity of the ultimately cytotoxic interaction of HN2 with L1210 cells (results 4.1.3.1), it is difficult to propose a mechanism by which this may occur.

Spurgin (1981) suggested that there may be a slow release of intact drug from pools in lipophilic regions of the plasma membrane, since the formation of the reactive aziridinium ion (introduction 1.1.1.3) cannot occur in non-polar solvents (Price, 1975). If the susceptible target for HN2 is a protein on the cytoplasmic side of the membrane, or if the sensitive region of a transmembrane protein is located near the cytoplasmic side, the delay in onset of inhibition may represent the time needed for the HN2 molecules to pass through the membrane. However, the presence of such pools would be difficult to reconcile with the observation that HN2 does not appear to be readily removed from L1210 cells by repeated washing (results 4.1.3.1).

A slow rate of inactivation of a sensitive component might be expected from the requirement of bifunctionality for the biological activity of nitrogen mustard. Tisdale (1974) discussed the possible mechanism to account for the inactivation of the enzyme cAMP phosphodiesterase by chlorambucil, but not by its monofunctional analogue. He suggested that the rate of alkylation is probably not important to the mechanism, since the monofunctional analogue might be expected to react at the same or greater velocity as the bifunctional analogue. The requirement of bifunctionality for activity of nitrogen mustard (introduction 1.1.2) most probably arises from the necessity to cross-link some sensitive component(s). An intramolecular cross-link might stabilise the inactive form of a protein, and prevent a conformational change

required for activity. For a protein which does not require a conformational change for activity, inactivation might arise from intermolecular cross-linking; this might be expected to occur at a slower rate than intramolecular cross-linking, and will depend on the concentration of the protein (Tisdale, 1974). Therefore, since bifunctionality is also required for the inhibition of $^{86}Rb^+$ & K^+ influx (results 4.17; Spurgin (1981); Baxter et al, 1982), indicating that a cross-linking event is essential, the rate-limiting step in the inhibition may be the second alkylating reaction.

It is impossible from the results presented in this thesis to deduce whether the inhibition of $^{86}Rb^+$ influx into L1210 cells by HN2 arises from intra- or inter-molecular cross-linking. Baxter et al (1982) and Spurgin (1981) have previously considered the Na^+K^+ATPase of PC6A cells as a likely target for cross-linking by HN2; the enzyme is sensitive to alkylation, it requires a conformational change for activity, and is sensitive to cross-linking agents (introduction 1.2.1). Grunicke and his colleagues (Grunicke et al, 1982; 1983; introduction 1.3; see also discussion, section 5) have presented evidence that HN2 interferes with $^{86}Rb^+$ influx via the Na^+K^+ATPase of Ehrlich ascites cells. However, no evidence was found in the present study for an interaction of HN2 with the Na^+K^+ATPase of L1210 murine leukaemia cells. A $Na^+K^+Cl^-$ cotransport system, which has similar properties to systems described in other cell types (results 4.2) appears to be the only target for the inhibition of $^{86}Rb^+$ & K^+ influx in HN2-treated L1210 cells (results 4.3.1.2; discussion, section 5), and the major target in HN2-treated Ehrlich ascites cells (Grunicke et al, 1985, Doppler et al, 1985). Precise definition of the interaction of HN2 with the $Na^+K^+Cl^-$ cotransporter awaits the developement of suitable methodology for identification, isolation,

purification and characterisation of the cellular components responsible for the function. This problem has been addressed by several workers (Forbush and Palfrey, 1983; Jorgensen et al, 1985), but with limited success to date.

The possibility must therefore be considered that the inhibition of $^{86}Rb^{+}$ transport arises as an indirect effect of HN2, rather than from direct reaction of the drug with the transport proteins. A molecule such as HN2 is capable of reaction with many cellular components (introduction 1.1.3; 1.1.4.1), and may affect some component which functions in a regulatory capacity with respect to the activity of the transport proteins. However, evidence for a direct interaction of HN2 with the transport protein(s) is provided by the observation that high concentrations (10^{-3} and 10^{-2}M) of HN2 produced immediate inhibition of $^{86}Rb^{+}$ influx into L1210 (results 4.1.4) and PC6A cells (results 4.1.1; Spurgin, 1981; Baxter et al, 1982). The slow onset of inhibition of $^{86}Rb^{+}$ influx into L1210 cells incubated with pharmacological concentrations (10^{-5}M) of HN2 is considered further in section 5.

4.3.1.2 The effect of nitrogen mustard on ^{86}rubidium influx in the presence of inhibitors of potassium transport

4.3.1.2.1 Introduction

The use of selective inhibitors to examine the activities of K^{+} transport systems has been discussed and demonstrated in section 4.2.2. The L1210 cells used in this study were shown to possess a ouabain-sensitive K^{+} & $^{86}Rb^{+}$ influx, presumably due to the activity of a $Na^{+}K^{+}$ATPase. An equal portion of the net K^{+} & $^{86}Rb^{+}$ influx was sensitive to inhibition by the diuretics furosemide and bumetanide; the activity was dependent upon external Na^{+} and Cl^{-} and displayed selectivity for Na^{+}, Li^{+}, K^{+}, Rb^{+}, Cl^{-} and Br^{-} characteristic of the

$Na^+K^+Cl^-$ cotransport systems described in a variety of cell types (introduction 1.2.1). A residual portion of the net $^{86}Rb^+$ & K^+ transport (10-15%) was insensitive to ouabain and furosemide, and was presumably due to passive transport.

Nitrogen mustard was shown to inhibit the influx of $^{86}Rb^+$ & K^+ in a concentration- and time-dependent manner. The experiment described in this section was carried out to determine which of the components of $^{86}Rb^+$ & K^+ influx into L1210 cells were sensitive to inhibition by HN2.

4.3.1.2.2 Results

Figure 19 shows the effect of preincubation for 3h in KR with 10^{-5}M HN2 on the initial rate of $^{86}Rb^+$ & K^+ transport into exponentially-growing L1210 cells, measured in KR in the presence and absence of inhibitors of K^+ transport. Nitrogen mustard inhibited $^{86}Rb^+$ & K^+ influx by 37±15% (mean ± S.D; n=11); this result is similar to those shown in section 4.3.1.1 (figures 32 and 33).

In L1210 cells incubated in the absence of HN2, 35±6% of $^{86}Rb^+$ & K^+ influx was furosemide-sensitive, 33±7% was bumetanide-sensitive, 37±10% was ouabain-sensitive, and 13±6% was ouabain- and furosemide-insensitive. In HN2-treated L1210 cells an insignificant amount of the residual $^{86}Rb^+$ & K^+ influx was sensitive to furosemide (9±12%) or bumetanide (8±10%); in contrast, 52±8% of the residual flux was sensitive to ouabain, and 28±6% was insensitive to ouabain and furosemide (figure 19). These changes were not due to a decline in cell viability (table 5; results 4.1.3.3). This result suggests that a bumetanide- and furosemide-sensitive component of $^{86}Rb^+$ & K^+ influx was absent from HN2-treated L1210 cells.

4.3.1.2.3 Discussion

Ouabain and the diuretics have been widely used as selective inhibitors of the activities of the Na^+K^+ATPase and $Na^+K^+Cl^-$ cotransport systems respectively (introduction 1.2.1), and are useful for the identification of the components of K^+ transport. For the present experiment, the effect of HN2 upon the $Na^+K^+Cl^-$ cotransporter was examined in the presence of ouabain, and its effect upon Na^+K^+ATPase was examined in the presence of diuretics.

Preincubation in KR for 3h with 10^{-5}M HN2 reduced $^{86}Rb^+$ & K^+ transport into L1210 cells by 37% (figure 19), which was similar to the inhibition caused by 10^{-4}M bumetanide (33%) or 10^{-3}M furosemide (35%); at these concentrations, the diuretics completely inhibit cotransport in a variety of cell types (introduction 1.2.1). Furthermore, addition of furosemide or bumetanide to HN2-treated L1210 cells revealed the loss of a diuretic-sensitive component of $^{86}Rb^+$ & K^+ transport. These observations strongly implicate the $Na^+K^+Cl^-$ cotransporter as the target of the inhibition of $^{86}Rb^+$ & K^+ uptake in HN2-treated cells, and also demonstrate that the bumetanide- and furosemide - insensitive components of transport, which are due in the greater part to the action of Na^+K^+ATPase (results 4.2.2.3) were insensitive to HN2. Thus, nitrogen mustard appeared to selectively inhibit the activity of the $Na^+K^+Cl^-$ cotransporter of L1210 cells; this possibility was further investigated by examination of the dependence of the sensitive $^{86}Rb^+$ & K^+ flux upon external ions (results 4.3.1.3).

The selective effect of HN2 on the diuretic-sensitive $^{86}Rb^+$ & K^+ influx was a surprising result, since HN2 may be expected to react with a wide variety of cellular components (introduction 1.1.3; 1.1.4.1; Wheeler, 1966), and has been shown to have widespread effects upon membrane structure (introduction 1.1.4.6).

As early as 1947, Sir Rudolph Peters suggested that sulphur mustard may be cytotoxic through an interaction with cell membranes, producing changes in permeability (Peters, 1947). Nitrogen mustard and its derivatives have been shown to inhibit osmotically- (Levy, 1965; Augsten, 1982) and metabolically- (Wildenauer et al, 1980) induced swelling of cells and plasma membrane vesicles, and changes of cell shape. A trifunctional analogue of HN2 was shown to cross-link spectrin (Wildenauer and Weger, 1979; Wildenauer et al, 1980), a component of the cytoskeleton of erythrocytes and other cells. There is some evidence that nitrogen mustard may cause changes in membrane fluidity, which arise from changes in the mobility of protein, but not lipid, components of the plasma membrane (Grunicke et al, 1982; 1983). However, these widespread changes did not cause changes in the ouabain- and diuretic- resistant $^{86}Rb^{+}$ & K^{+} flux, which are presumed to represent passive K^{+} flux.

The lack of effect of HN2 on the function of $Na^{+}K^{+}$ATPase was a surprising result because of the structure of the $Na^{+}K^{+}$ATPase. Nitrogen mustard was shown to inhibit ouabain-sensitive phosphatase activity in a crude membrane preparation from PC6A cells (Baxter et al, 1982; Spurgin, 1981). The $Na^{+}K^{+}$ATPase was considered to be a likely target for HN2 (Baxter et al, 1982; Spurgin, 1981; introduction 1.3); it is susceptible to alkylation since it contains vital sulphydryl groups (Schoot et al, 1978; 1979; Skou, 1963); it is sensitive to crosslinking agents (Huang and Askari, 1979; de Pont, 1979; Askari et al, 1980; Freedman, 1974), since it is a subunit enzyme (Schwartz et al, 1975) which requires a conformational change for activity (dePont, 1979; introduction 1.2.1). In contrast, little is known of the structural nature of the cellular components which perform $Na^{+}K^{+}Cl^{-}$ cotransport (introduction 1.2.1), and it is

difficult to predict the mechanism by which HN2 may inhibit the function.

The mechanism of inhibition of cotransport by HN2 appears to differ from that of the diuretics, which appear to bind reversibly at one of the anion-binding sites, and require Na^+ , K^+ and Cl^- for binding (Forbush and Palfrey, 1983). In contrast, HN2, which is not a structural analogue of the diuretics, binds irreversibly (results 4.1.3.3), and independently of K^+ ions (results 4.3.1.1). Sublethal concentrations of the diuretics (results 4.6) have immediate effects upon K^+ transport (results 4.2.2), but the inhibition of K^+ transport by HN2 is concentration- and time- dependent (results 4.3.1.1). Studies with a monofunctional analogue of HN2 reveal that two alkylating groups are required to inhibit $Na^+K^+Cl^-$ cotransport and indicate that a cross-linking event is involved in the mechanism (results 4.17; Spurgin, 1981; Baxter et al, 1982). The delay in onset of inhibition of K^+ transport after exposure to pharmacological concentrations of HN2 was evident from preliminary experiments (results 4.1.1 and 4.1.4) and has been noted by others (Spurgin, 1981; Baxter et al 1982; Grunicke et al, 1985). Nitrogen mustard has been shown to inhibit $Na^+K^+Cl^-$ cotransport into Ehrlich ascities tumour cells in vitro (Grunicke et al, 1985; Doppler et al, 1985); the authors of these studies concluded that inhibition of cotransport did not play a causal role in the inhibition of cell growth by HN2. Their conclusion, and the possibility that HN2 may inhibit cotransport via an indirect mechanism, will be considered further in the general discussion (section 5).

A recent report has suggested that a protein of 34,000 Daltons is a component of the $Na^+K^+Cl^-$ contransporter, and is associated with components of the cytoskeleton (Jorgensen et al, 1985). Thus, the

possibility that HN2 inhibited cotransport activity via interaction with cytoskeletal components deserves further consideration.

4.3.1.3 The effect of nitrogen mustard on ^{86}rubidium influx in the absence of external sodium, chloride or bicarbonate

4.3.1.3.1 Introduction

Incubation with nitrogen mustard inhibited the function of bumetanide- and furosemide-sensitive $^{86}Rb^+$ & K^+ influx into L1210 cells (results 4.3.1.2). Results showed that this flux appeared to be due to a $Na^+K^+Cl^-$ cotransport system similar to that described in other cell types (results 4.2), and that it was characteristically dependent upon external Na^+ and Cl^- ions. Accordingly, the dependence upon external Na^+ and Cl^- ions of the HN2-sensitive component of $^{86}Rb^+$ & K^+ influx was examined. Extracellular Na^+ was replaced with choline$^+$ and extracellular Cl^- with NO_3^-, since neither substituting ion was able to support bumetanide-sensitive $^{86}Rb^+$ & K^+ transport; the ouabain-resistant $^{86}Rb^+$ & K^+ fluxes were then examined.

Replacement of extracellular HCO_3^- by NO_3^- suggested that a fraction of the furosemide-sensitive $^{86}Rb^+$ & K^+ movement may be dependent upon external HCO_3^- (results 4.2.3); therefore the effect of HN2 on $^{86}Rb^+$ & K^+ influx into L1210 cells incubated in HCO_3^-free medium was also examined.

4.3.1.3.2 Results

In the presence of external Na^+, addition of furosemide, bumetanide or nitrogen mustard inhibited ouabain-resistant $^{86}Rb^+$ & K^+ influx. The residual flux was similar to that seen in the absence of Na^+ (figure 34). The experiment was repeated in the absence of Na^+ and with an increased amount of $^{86}Rb^+$ (25μCiml^{-1}) to improve the sensitivity of the transport measurement; nitrogen mustard,

Figure 34

The effect of nitrogen mustard, 2-chloroethyldimethylamine, furosemide or bumetanide on the influx of $^{86}Rb^+$ & K^+ into L1210 cells, measured in Krebs-Ringer buffer in the presence of $10^{-3}M$ ouabain.

Key: 1 ○ control, 3h
2 ● $10^{-5}M$ HN2, 3h
3 ◒ $10^{-4}M$ HN2-1, 3h
4 △ control, $-Na^+$, 3h
5 □ $10^{-4}M$ bumetanide, 15 min
6 ■ $10^{-3}M$ furosemide, 15 min (Mean ± S.D.; n=5)

Figure 35

The effect of nitrogen mustard, 2-chloroethyldimethylamine, furosemide or bumetanide on the influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in Na^+-free Krebs-Ringer buffer, in the presence of $10^{-3}M$ ouabain.

Key: 1 ○ control, 3h
2 ● $10^{-5}M$ HN2, 3h
3 ◒ $10^{-4}M$ HN2-1, 3h
4 □ $10^{-4}M$ bumetanide, 15 min
5 ■ $10^{-3}M$ furosemide, 15 min (Mean ± S.D.; n=6)

Figure 36

The effect of nitrogen mustard or furosemide on $^{86}Rb^+$ & K^+ influx into L1210 cells measured in the presence and absence of external chloride, in the presence of $10^{-3}M$ ouabain.

Key:	$+Cl^-$		$-Cl^-$
1	●	control, 3h	4 ○
2	■	$10^{-5}M$ HN2, 3h	5 □
3	▲	$10^{-3}M$ furosemide, 15 min	6 △

(Mean ± S.D.; n=4)

Figure 34

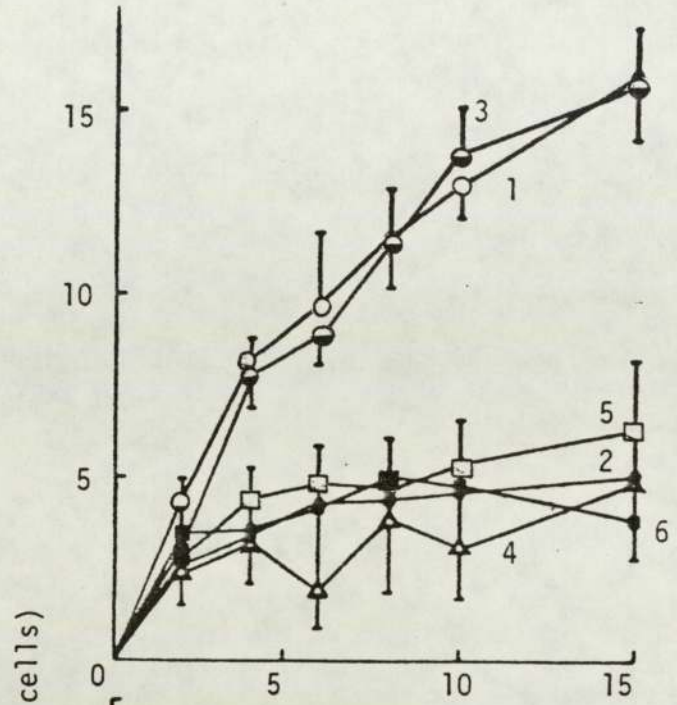


Figure 35

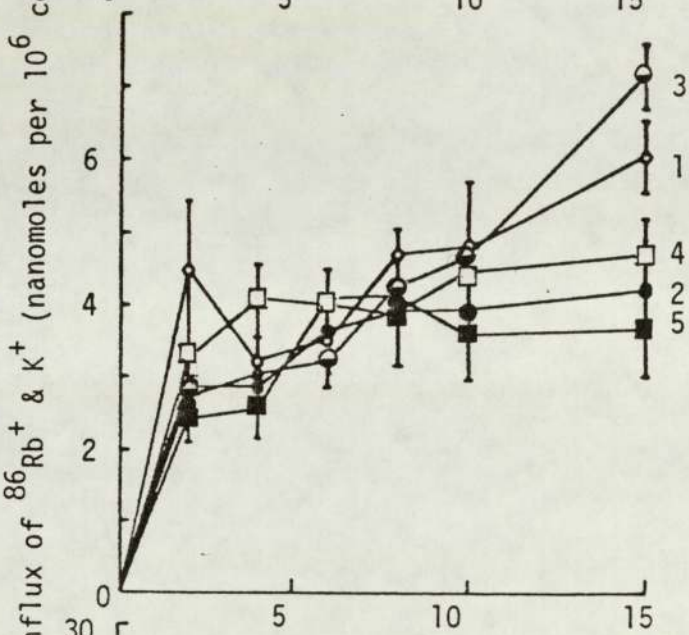
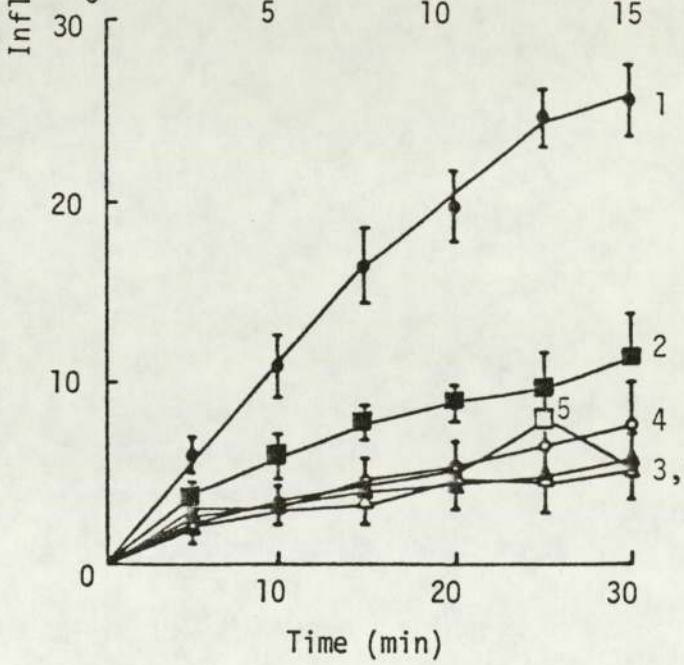


Figure 36



Influx of $^{86}Rb^{+}$ & K^{+} (nanomoles per 10^{6} cells)

Time (min)

furosemide and bumetanide had no effect on ouabain-resistant $^{86}Rb^+$ & K^+ influx into L1210 cells in the absence of external Na^+ (figure 35).

Figure 36 shows the uptake of $^{86}Rb^+$ & K^+ into L1210 cells incubated in the presence of 10^{-3}M ouabain. The cells were incubated for 3h with and without HN2, then transferred to fresh KR or Cl^--free KR, both containing ouabain, and with or without 10^{-3}M furosemide; the molar rate of $^{86}Rb^+$ & K^+ influx was calculated as before (results 4.2.1.2). In the presence of external Cl^-, furosemide and nitrogen mustard both produced substantial inhibition of $^{86}Rb^+$ & K^+ influx. In the absence of external Cl^-, in NO_3^--substituted KR, neither furosemide nor HN2 had any effect upon $^{86}Rb^+$ & K^+ transport (figure 36).

Figure 25 shows the effect of preincubation with 10^{-5}M HN2 on the influx of $^{86}Rb^+$ & K^+ into L1210 cells in the presence and absence of external HCO_3^-. Nitrogen mustard inhibited the influx of $^{86}Rb^+$ & K^+ by 40% in the presence, or absence, of HCO_3^-; in contrast, furosemide appeared to inhibit a HCO_3^- -dependent portion of $^{86}Rb^+$ & K^+ influx (results 4.2.3).

4.3.1.3.3 Discussion

Earlier results (results 4.2.3) showed that the diuretic-sensitive portion of $^{86}Rb^+$ & K^+ influx into L1210 cells was wholly dependent upon external Na^+ and Cl^- ions. Nitrogen mustard inhibited the diuretic-sensitive, ouabain-resistant portion of $^{86}Rb^+$ & K^+ influx into L1210 cells (results 4.3.1.2). The co-ion dependence of the HN2-sensitive $^{86}Rb^+$ & K^+ influx was examined.

In the presence of external Na^+ , the diuretics and nitrogen mustard reduced the ouabain-resistant $^{86}Rb^+$ & K^+ influx to a level not significantly different ($p>0.05$) to that observed in the absence

of Na^+. This result suggested that the diuretic- and HN2-sensitive portion of $^{86}Rb^+$ & K^+ influx into L1210 cells was wholly dependent upon external Na^+ , and provides evidence against the operation, in these cells, of a Na^+-independent K^+Cl^- cotransport system which has been proposed for several cell types (Kregenow, 1981; Parker, 1983; Lauf, 1982; Lauf and Theg, 1980; Dunham and Ellory, 1981).

Further evidence to support this view was provided by the failure of bumetanide, furosemide, or HN2 to inhibit the initial rate of ouabain-resistant $^{86}Rb^+$ & K^+ influx in Na^+-free medium. However, the fraction of $^{86}Rb^+$ loaded, as equilibrium distribution was approached, appeared to be reduced in L1210 cells exposed to HN2 or the diuretics in Na^+-free medium containing ouabain. This result suggested a reduced intracellular K^+ content in comparison to controls exposed to ouabain alone, and may indicate that the $Na^+K^+Cl^-$ cotransporter has an important role in the regulation of K^+ homeostasis in L1210 cells (see also results 4.7).

In the presence of external Cl^-, furosemide caused a greater inhibition of ouabain-resistant $^{86}Rb^+$ & K^+ influx than did HN2; this difference did not appear to be due to the incomplete inhibition of diuretic-sensitive $^{86}Rb^+$ & K^+ flux by HN2, since the same conditions were found to completely and reproducibly inhibit in several other experiments (results 4.3.1.2). Thus the results suggest that a Cl^--dependent portion of $^{86}Rb^+$ & K^+ influx into L1210 cells is sensitive to furosemide but not to HN2. The nature of this component is unknown; however, it may involve HCO_3^- ions, since a portion of furosemide-sensitive, but not HN2-sensitive, $^{86}Rb^+$ & K^+ flux was dependent upon external HCO_3^- (results 4.2.3). If this unknown portion is furosemide-sensitive, it must also be Na^+-dependent (see above), and if the anion exchanger is involved (results 4.2.3), the

role of Na^+ may be through the Na^+/H^+ antiporter to regulate intracellular pH. It would be of interest to examine this possibility, but it would be difficult to obtain definitive results, since amiloride is not totally specific for the Na^+/H^+ antiporter (see introduction 1.2.1). In the absence of external Cl^-, HN2 had no effect on ouabain-resistant $^{86}Rb^+$ & K^+ uptake, providing further evidence that the HN2-sensitive portion of $^{86}Rb^+$ & K^+ uptake is wholly Cl^- dependent.

In the interpretation of the results presented in this section, it should be noted that the data show that external Na^+ and Cl^- are required for the HN2-sensitive influx of $^{86}Rb^+$ & K^+ into L1210 cells; it may be that these ions are merely bound to the transporter and not transported into the cell. Direct evidence for transport of Na^+ and Cl^- into L1210 cells was not found, because of methodological difficulties (results 4.5). In addition, the binding of the diuretics, and therefore their efficiency, may be reduced in the absence of external Na^+ or Cl^- (Forbush and Palfrey, 1983). An analogue of the diuretics which binds irreversibly to the cotransporter would therefore be of much use to confirm the results obtained with the existing diuretics.

4.3.2 THE EFFECT OF NITROGEN MUSTARD ON THE INFLUX OF ^{86}RUBIDIUM INTO L1210 CELLS FROM STATIONARY CULTURES

4.3.2.1 Introduction

A large body of evidence indicates that dividing cells from diverse sources are more sensitive to the cytotoxic effects of nitrogen mustard than are their quiescent counterparts (Ross, 1953; 1962; introduction 1.1.2). If the inhibition of K^+ fluxes into rapidly-dividing L1210 cells has a causal role in the cytotoxic action of HN2, it would be expected that the K^+ fluxes into L1210

cells from stationary cultures would be less sensitive to HN2. Since marked changes in activity of the K^+ transport systems in L1210 cells were found as stationary phase was entered (results 4.2.2.3), it was of prime importance to examine the effects of HN2 on K^+ influx into L1210 cells from stationary cultures.

4.3.2.2 Results

Figure 37 shows the effect of preincubation for 3h with 10^{-5}M HN2 on the influx of $^{86}Rb^+$ & K^+ into $5x10^6$ ml^{-1} stationary phase L1210 cells incubated in KR buffer. The initial rate of $^{86}Rb^+$ & K^+ influx into control and HN2-treated cells was linear for 5 minutes; after this period, the uptake of $^{86}Rb^+$ & K^+ into control cells was 4.35±0.80 nanomoles per 10^6 cells, and into HN2-treated cells was 3.75±1.00 nanomoles per 10^6 cells, representing a mean inhibition of 14%, although the difference in the fluxes was not statistically significant ($p>0.05$). A similar result was found in a second series of experiments, to examine the influx of $^{86}Rb^+$ & K^+ into stationary phase L1210 cells incubated in the presence of inhibitors of potassium transport (results 4.2.2.3; figure 20). The initial rate of $^{86}Rb^+$ & K^+ influx into control cells was 0.88±0.12 nanomoles min^{-1} per 10^6 cells; after incubation for 3h with 10^{-5}M HN2, the initial rate of influx was 0.70±0.13 nanomoles min^{-1} per 10^6 cells. This result represented a mean inhibition of 20%, but again, the difference in the fluxes was not statistically significant ($p>0.05$). The inhibition produced by HN2 was similar to that produced by 10^{-3}M furosemide or 10^{-4}M bumetanide. In the presence of furosemide or bumetanide there was no further reduction of $^{86}Rb^+$ & K^+ flux into HN2-treated cells; in contrast, in the presence of 10^{-3}M ouabain, influx was reduced by 20% in HN2-treated cells.

4.3.2.3 Discussion

L1210 cells were maintained in culture without a change of medium until stationary phase was attained. Cells brought to confluency in the presence of serum possess reduced, but not absent, diuretic-sensitive K^+ influx (results 4.2.2.3; Amsler et al, 1985; Tupper and Zografos, 1978; Spaggiare et al, 1976). This portion alone was inhibited by HN2. It was expected, therefore, that the total $^{86}Rb^+$ & K^+ transport into stationary phase cells was less sensitive to inhibition by HN2 than that of rapidly-dividing cells. The results showed that the diuretic-sensitive portion of $^{86}Rb^+$ & K^+ influx into stationary phase L1210 cells was sensitive to HN2, and that HN2 inhibited a smaller portion of the total flux into these cells than into cells from growing cultures. Flow cytofluorimetic techniques confirmed that stationary cultures contained fewer dividing cells than exponentially-growing cultures (results 4.1.3.4); therefore, the inhibition of $Na^+K^+Cl^-$ cotransport by HN2 may explain the increased susceptibility of rapidly-dividing cells to the cytotoxic effect of HN2 (see general discussion, section 5). These results may be extended, in part, by examining the effect of HN2 on $^{86}Rb^+$ & K^+ fluxes into serum-deprived cultures, which contained very few dividing cells (results 4.1.3.4) and displayed negligible diuretic-sensitive influx (results 4.2.2.3). Attempts to correlate the inhibition of DNA synthesis with the cytotoxicity of HN2 have produced conflicting results on the sensitivity of the different phases of the cell cycle (Walker and Helleiner, 1963; DeCosse and Gelfant, 1970; Mauro and Madoc-Jones, 1970; Clarkson and Mitchell, 1981; Dean and Fox, 1984; introduction 1.1.4.2). Studies of the effect of HN2 on $^{86}Rb^+$ & K^+ fluxes into cells from synchronised cultures will resolve further the role of inhibition of K^+ transport in the cytotoxicity of HN2 to dividing cells.

Figure 37

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the influx of $^{86}Rb^{+}$ & K^{+} into L1210 cells from stationary cultures.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 38

The effect of injection of 2mgkg^{-1} nitrogen mustard on the influx of $^{86}Rb^{+}$ & K^{+} into L1210 murine leukaemia cells isolated from DBA/2 mice.

Key: ○ control, 0.5ml 0.9% (w/v) NaCl

● 2 mgkg^{-1} HN2, in 0.5ml 0.9% (w/v) NaCl

Points represent the mean ± S.D. of triplicate determinations for each of three animals in each group.

Figure 37

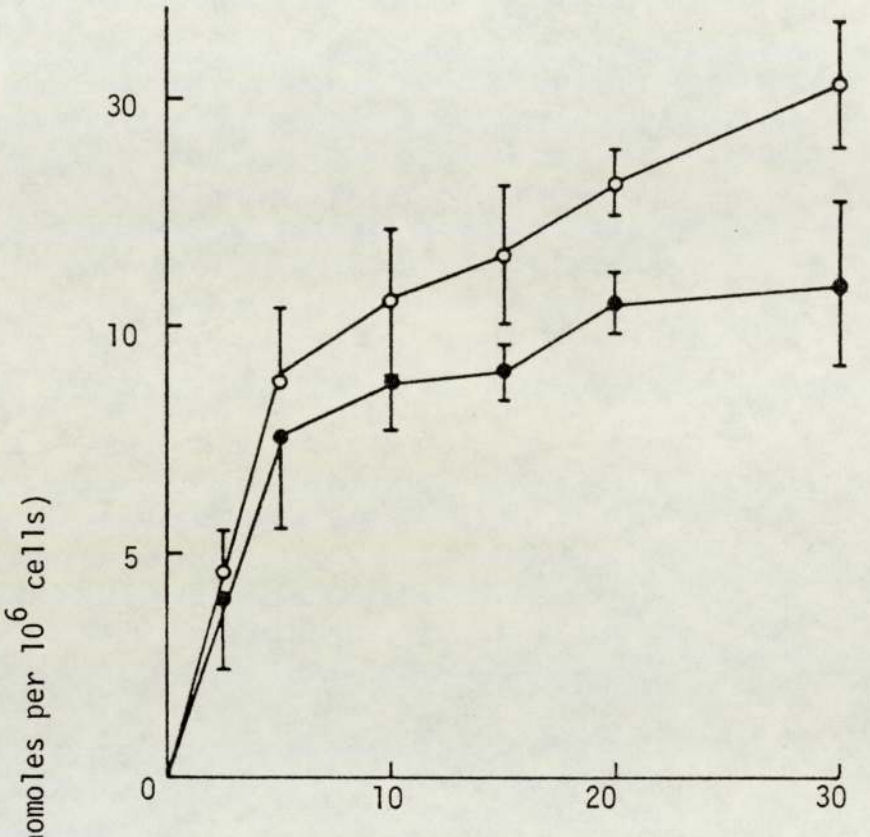
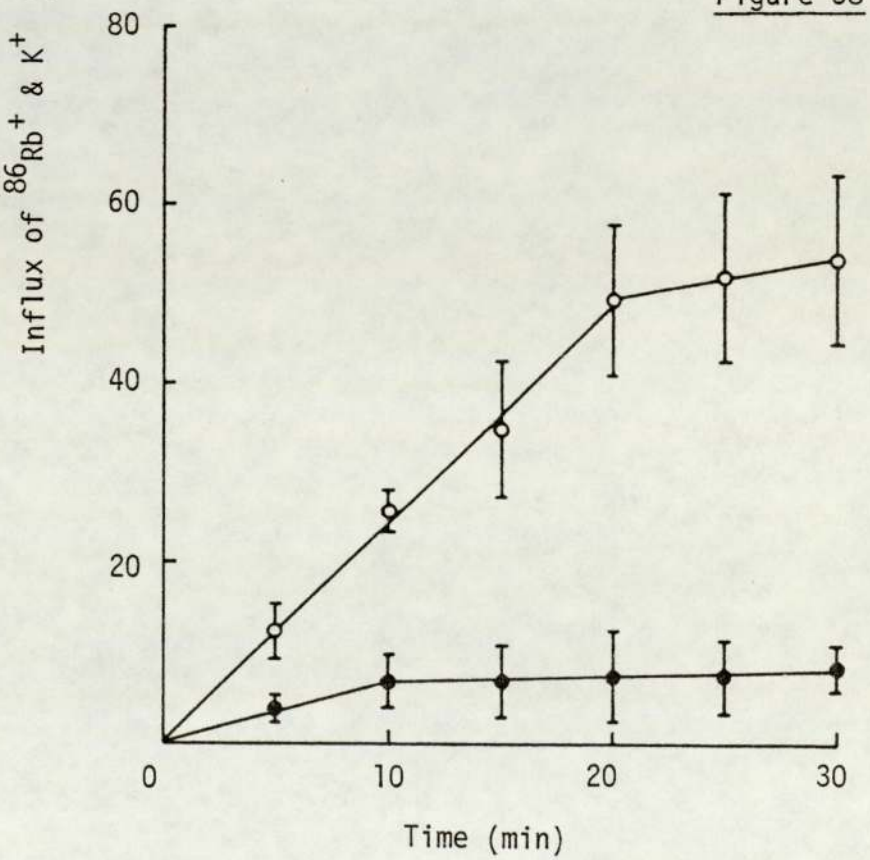


Figure 38



4.3.3 THE EFFECT OF NITROGEN MUSTARD ON ^{86}RUBIDIUM INFLUX INTO L1210 MURINE LEUKAEMIA CELLS *in vivo*

4.3.3.1 Introduction

Nitrogen mustard inhibited the $^{86}Rb^+$ & K^+ influx into rapidly-dividing L1210 murine leukaemia cells maintained *in vitro*. The experiment described in this section was carried out to determine whether the inhibition of K^+ flux had a pharmacological role *in vivo*. In addition, $^{86}Rb^+$ & K^+ fluxes into cultured cells were measured in the absence of serum, which may reduce the effective concentration of HN2 to which the cells were exposed (results 4.1.3.3); thus it was important to establish whether the observed inhibition of $^{86}Rb^+$ & K^+ influx was an artefact of the experimental conditions *in vitro*.

The murine leukaemia line, designated L1210S, was passaged in mice. Mice bearing the tumour were injected with 2mgkg^{-1} HN2, or 0.9% NaCl, and sacrificed 3h later, whereupon the $^{86}Rb^+$ & K^+ flux into ascites cells from each animal were examined.

4.3.3.2 Results

Figure 38 shows the influx of $^{86}Rb^+$ & K^+ into L1210S ascites cells isolated from animals after injection with 2mgkg^{-1} HN2 or 0.9% NaCl. The results show the mean uptake of $^{86}Rb^+$ & K^+ by cells isolated from three animals in each group. The rate of influx of $^{86}Rb^+$ & K^+ into cells isolated from control animals was initially constant, at 2.45±0.45 nanomoles min^{-1} per 10^6 cells, for 20 minutes. In contrast, the initial rate of $^{86}Rb^+$ & K^+ influx into cells from HN2-treated mice was constant, at 0.71±0.23 nanomoles min^{-1} per 10^6 cells, for only 10 minutes, whereafter there was no net influx. There was no significant difference in the viability of ascites cells from animals in control and experimental groups: 85±6%

of control cells and 79±2% (Mean ± S.D., n=3) of experimental cells were impermeable to trypan blue.

4.3.3.3 Discussion

The diuretic-sensitive $^{86}Rb^+$ & K^+ influx into L1210 cells *in vitro* was completely inhibited by preincubation for 3h with 10^{-5}M HN2 (results 4.3.1.2). Assuming equal distribution of HN2 in body fluid, 2mgkg^{-1} HN2 approximates to a concentration of 10^{-5}M. This dose probably represents an overestimate of a therapeutic dose, since the ID_{90} of HN2 against the L1210S leukaemia *in vivo* was found to be 0.80mgkg^{-1} (D. Chubb and S. Langdon; personal communication); but it was used to make possible a direct comparison with *in vitro* results. Three hours after injection of HN2, there was a significant (71%) inhibition of the rate of $^{86}Rb^+$ & K^+ flux into ascites cells. The inhibition of $^{86}Rb^+$ & K^+ flux was not attributable to a decrease in viability of treated cells in comparison to controls. However, equivalent treatment with HN2 inhibited a greater portion of the total $^{86}Rb^+$ & K^+ flux into the L1210S *in vivo* than into the L1210 *in vitro* cell line. This result suggests that either the $Na^+K^+Cl^-$ cotransporter contributes a greater portion to the total $^{86}Rb^+$ & K^+ flux into L1210S *in vivo* than into L1210 *in vitro*; or, that HN2 is capable of inhibiting other K^+ transport systems *in vivo* but not *in vitro*. The first possibility may arise because the tumour cells taken from the animal are deprived of serum only for the duration of the transport measurement, whereas the cultured cells were also exposed to HN2 in serum-free medium; removal of serum has been shown to inhibit Na^+ K^+Cl^- cotransport in rapidly-growing cells (Amsler *et al*, 1985). However, prolonged (<4h) incubation in serum-free medium did not cause progressive inhibition of $^{86}Rb^+$ & K^+ influx into cultured L1210 cells (results 4.3.1.1). The second possibility may

arise if a metabolite of HN2 is formed *in vivo*, and is capable of inhibiting diuretic-resistant K^+ transport; however, this seems unlikely since the hydrolysis products of HN2 were shown to be without effect upon $^{86}Rb^+$ transport into PC6A ascites cells *in vitro* (Spurgin, 1981). These questions may be partly resolved by the use of inhibitors of K^+ transport.

In conclusion, this preliminary experiment suggests that cytotoxic concentrations of HN2 may inhibit K^+ transport into tumour cells *in vivo*, and that the cultured L1210 cell line may be a useful model in which to study the biochemical basis of this inhibition. The determination of the role which perturbation of K^+ fluxes may play in the (partly) selective antitiumour effects of HN2 awaits the characterisation of the $Na^+K^+Cl^-$ cotransport system and its role in K^+ regulation in normal and tumour cells *in vivo*.

4.4. THE EFFECT OF NITROGEN MUSTARD ON ^{86}RUBIDIUM EFFLUX FROM L1210 CELLS

4.4.1. ^{86}RUBIDIUM EFFLUX FROM L1210 CELLS INCUBATED IN POTASSIUM-REPLETE MEDIUM

4.4.1.1 Introduction

Nitrogen mustard selectively inhibited the portion of $^{86}Rb^+$ and K^+ influx into L1210 cells, which was mediated by a $Na^+K^+Cl^-$ cotransport system with properties in common with those described for other cell types (results 4.3.2). It has been proposed that in some cell types the $Na^+K^+Cl^-$ cotransporter may mediate net outward flux (Atlan et al, 1984; Panet and Atlan, 1980) or no net flux (Tupper, 1975; Garay, 1982), allowing K^+/K^+, Na^+/Na^+ (McManus and Schmidt, 1978; Duhm and Gobel, 1984), or Na^+/K^+ exchange (Jayme et al, 1981) under physiological ion concentrations. Thus for the characterisation of the function of the $Na^+K^+Cl^-$ cotransporter in L1210 cells, it was important to determine whether there was a diuretic-sensitive component of $^{86}Rb^+$ (K^+) efflux, and to determine the net flux, if any, mediated by the system. At the same time, it was of interest to investigate the effect of HN2 on $^{86}Rb^+$ and K^+ efflux from L1210 cells.

L1210 cells from rapidly-growing cultures were loaded to isotopic equilibrium with $^{86}Rb^+$, then resuspended in either KR or RPMI to begin efflux.

4.4.1.2. Results

Preliminary experiments to measure $^{86}Rb^+$ uptake into L1210 cells established that isotopic equilibrium was reached within two hours of addition of $^{86}Rb^+$ (data not shown); L1210 cells were incubated in RPMI with $^{86}Rb^+$ for this period prior to measurement of $^{86}Rb^+$ efflux.

Figure 39

The effect of preincubation for fifteen minutes with furosemide or bumetanide on the efflux of $^{86}Rb^{+}$ from preloaded L1210 cells incubated in RPMI in the presence of 10^{-3}M ouabain.

Key: ○ control

▲ 10^{-3}M furosemide

△ 10^{-4}M bumetanide (Mean ± S.D.; n=4)

Figure 40

The effect of preincubation for 3h with nitrogen mustard or 2-chloroethyldimethylamine on the efflux of $^{86}Rb^{+}$ from preloaded L1210 cells incubated in RPMI in the presence of 10^{-3}M ouabain.

Key: ○ control

◒ 10^{-4}M HN2-1

● 10^{-5}M HN2 (Mean ± S.D.; n=4)

Figure 41

The effect of various agents on the initial efflux of $^{86}Rb^{+}$ from preloaded L1210 cells incubated in Krebs-Ringer buffer.

Key: Preincubation for 15 minutes

1. control
2. 10^{-4}M bumetanide
3. 10^{-3}M ouabain
4. 10^{-3}M furosemide
5. 10^{-3}M dibutyrylcAMP
5. 10^{-4}M dibutyrylcAMP
6. 10^{-4}M dibutyrylcAMP

Preincubation for 3 hours

7. control
8. 10^{-5}M HN2
9. 10^{-5}M HN2-1
10. 10^{-4}M HN2-1 (Mean + S.D.; n=7)

Figure 39

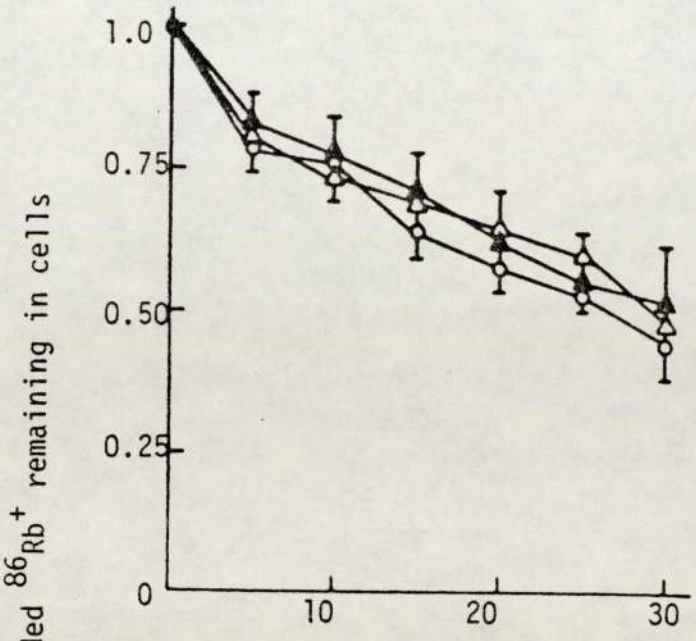


Figure 40

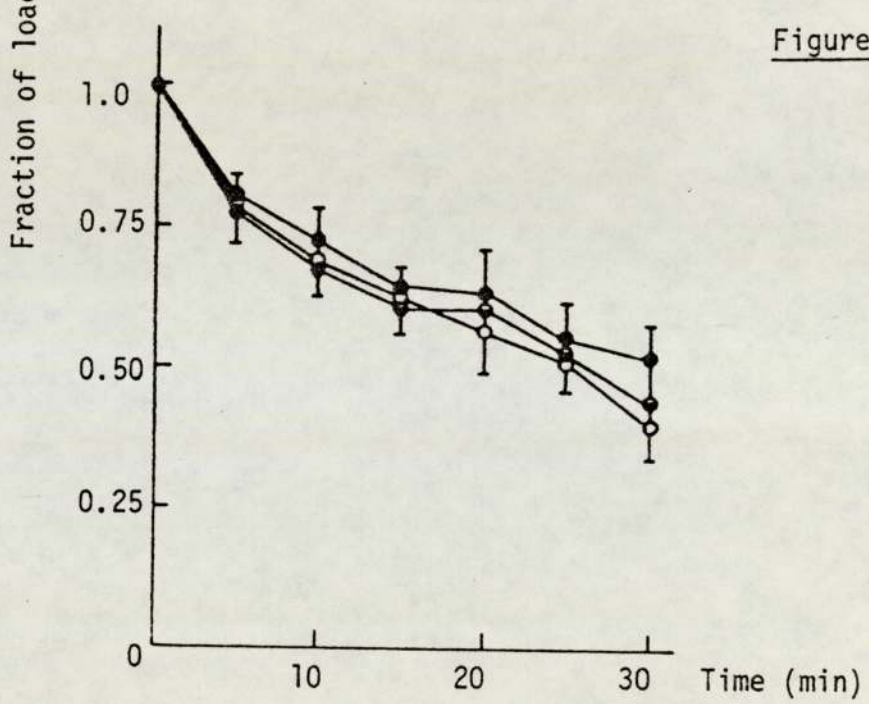
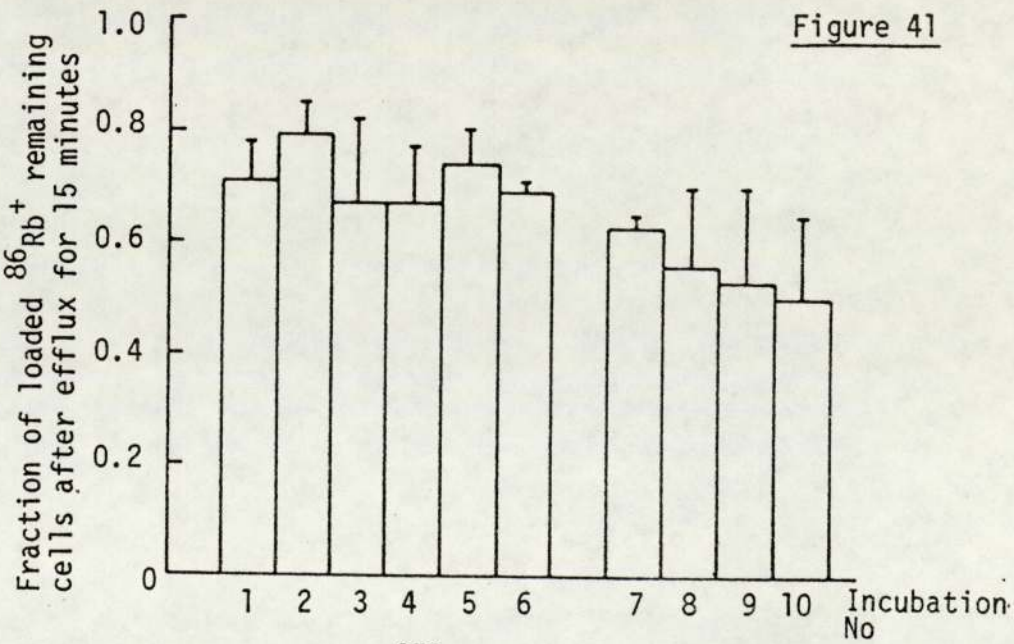


Figure 41



The efflux of $^{86}Rb^+$ was expressed as the radioactivity of the cells at a given time point, as a fraction of the radioactivity of the cells at the beginning of efflux; i.e., the fraction of the loaded $^{86}Rb^+$ which remained in the cells.

Figure 39 shows the effect of addition of 10^{-4}M bumetanide or 10^{-3}M furosemide on the efflux of $^{86}Rb^+$ from preloaded L1210 cells, resuspended in RPMI containing 10^{-3} M ouabain. Ouabain was present to prevent re-entry of effluxed $^{86}Rb^+$ via the Na^+K^+ATPase. Efflux of $^{86}Rb^+$ was slow, with about 50% of the original activity retained in the cells after incubation for 30 minutes in isotope-free RPMI. Within this period, neither furosemide nor bumetanide had a significant effect upon the efflux of $^{86}Rb^+$ from preloaded cells.

Figure 40 shows the effect of preincubation of $^{86}Rb^+$-loaded L1210 cells for 3h with 10^{-5}M HN2, on the $^{86}Rb^+$ efflux into RPMI. Nitrogen mustard had no significant effect upon $^{86}Rb^+$ efflux after incubation for 30 minutes in RPMI. Identical results were obtained when the cells were resuspended in KR buffer (data not shown).

When $^{86}Rb^+$-loaded L1210 cells were resuspended in KR buffer, approximately 30% of the initial activity was lost to the medium within fifteen minutes (figure 41). This value did not alter significantly when 10^{-3}M ouabain was present in the medium, indicating that measurements of $^{86}Rb^+$ efflux made at this time point were not significantly affected by re-entry of $^{86}Rb^+$. The effect of various agents on the efflux of $^{86}Rb^+$ from L1210 cells was measured after incubation of preloaded cells in isotope-free medium for fifteen minutes. Furosemide (10^{-3} M) had no effect on $^{86}Rb^+$ efflux; bumetanide appeared to produce a slight inhibition (11%) of $^{86}Rb^+$ efflux, although this was not statistically significant ($p>0.05$). There was no effect of preincubation for 15 minutes with dibutyryl

cAMP (10^{-3}M, 10^{-4}M) on $^{86}Rb^{+}$ efflux, indicating that the system which mediated efflux was insensitive to cAMP. The $^{86}Rb^{+}$ influx into L1210 cells was also unresponsive to dbcAMP (results 4.2.5).

^{86}Rubidium-loaded L1210 cells were incubated in the loading buffer, containing $^{86}Rb^{+}$, for a further three hours with and without 10^{-5}M HN2. Upon resuspending the cells in isotope-free KR, HN2 was found to produce a small increase (11%) in the amount of activity lost to the medium, but this was not statistically significant ($p>0.05$).

Identical results were obtained when $^{86}Rb^{+}$-loaded L1210 cells were incubated in RPMI for measurement of $^{86}Rb^{+}$ efflux (data not shown).

4.4.1.3 Discussion

A substantial fraction (35-40%) of the total $^{86}Rb^{+}$ and K^{+} influx into rapidly-dividing L1210 cells was found to be sensitive to furosemide (10^{-3}M) or bumetanide (10^{-4}M) (results 4.2.2.3). Under the same conditions, at physiological K^{+} concentrations, neither furosemide nor bumetanide was found to inhibit $^{86}Rb^{+}$ efflux. This result was independent of the presence of ouabain in the medium, indicating that $^{86}Rb^{+}$ efflux was not a manifestation of a separate mode of operation of the $Na^{+}K^{+}$ATPase (McManus and Schmidt, 1978; Beauge and Lew, 1977). Ouabain has been shown to accelerate diuretic-sensitive $^{86}Rb^{+}$ efflux (Aiton and Simmons, 1983; Bourrit et al, 1985; Jayme et al, 1981); the mechanism by which this occurs may be due to an elevation of intracellular Na^{+} concentrations, which activates the transporter (Bourrit et al, 1985), or to the stimulation of K^{+}/K^{+} exchange (Aiton and Simmons, 1983), although the effect may arise from ouabain preventing the re-entry of $^{86}Rb^{+}$ (Jayme et al, 1981). The results described in this section show no effect

of ouabain on $^{86}Rb^+$ efflux; however, prolonged preincubation of $^{86}Rb^+$-loaded L1210 cells with 10^{-3}M ouabain caused a stimulation of $^{86}Rb^+$ efflux into K^+-free medium (results, 4.4.2).

The results described above suggest that, at physiological concentrations of K^+, the $Na^+K^+Cl^-$ cotransporter of L1210 cells does not mediate a K^+ efflux, and there is a net influx of K^+. Therefore, the system does not mediate $Na^+K^+Cl^-$ efflux, K^+Cl^- efflux, Na^+/K^+ exchange or K^+/K^+ exchange. This finding is at variance with the results of studies of diuretic-sensitive K^+ transport systems of rabbit reticulocytes (Panet and Atlan, 1980) and NIH-3T3 cells (Atlan et al, 1984), which mediated net K^+ efflux, and human erythrocytes (Garay, 1982; Dunn, 1970; Lubowitz and Whittam, 1969) and Ehrlich ascites cells (Tupper, 1975; Bakker-Grunwald, 1978), in which there was no net flux under physiological conditions. However, bumetanide was shown to be without effect on $^{86}Rb^+$ efflux from murine macrophages incubated in medium of physiological ion concentrations in the absence of ouabain (Bourrit et al, 1985), suggesting that the $^{86}Rb^+$ efflux under normal conditions was due to a simple diffusion. Similar results have been obtained with ascites cells (Bakker-Grunwald, 1978; Bakker-Grunwald et al, 1980), MDCK and HeLa cells (Aiton and Simmons, 1983), and SV3T3 cells (Bakker-Grunwald et al, 1982).

The diuretic-sensitive Na^+-K^+ cotransport pathway was at first considered to be a second pump (Hoffman and Kregenow, 1966), since ions could be moved against their concentration gradients. There was also evidence that the pathway mediated exchange of ions (Dunn, 1973; Lubowitz and Whittam, 1969). McManus and Schmidt (1978) reviewed the evidence concerning the system in avian erythrocytes. They suggested that the energy driving the transport was derived from the net effect

of the two electrochemical gradients of Na^+ and K^+, and that net movement of either ion could occur only when an imbalance of these gradients was created. The obligatory coupling of each ion with its co-ion allowed the movement of the ion against its gradient; thus the movement of the co-ion down its gradient supplied the energy for the apparently active transport of the other ion. Therefore, the furosemide-sensitive transmembrane fluxes of Na^+ and K^+ depend upon the ionic concentrations in the cells and in the extracellular medium (Palfrey and Greengard, 1981; Panet and Atlan, 1980; Atlan et al, 1984; Garay, 1982).

The cotransporter has been shown to mediate net inward flux into Ehrlich ascites cells (Geck et al, 1980) and mouse fibroblasts (Gargus and Slayman, 1980; Jayme et al, 1984) which have been loaded with Na^+ and depleted of K^+. In addition, net diuretic-sensitive inward flux has been observed during regulatory volume increase after hyperosmotic shock (MacKnight and Leaf, 1985; Hoffmann, 1985; see results 4.8.3). In contrast, flux through the $Na^+K^+Cl^-$ cotransporter does not seem to have a role in regulatory volume decrease. An electroneutral, diuretic-sensitive K^+Cl^- cotransporter has been found in red cells of duck (Kregenow, 1981), dog (Parker, 1983), fish (Lauf, 1982) and genetically low K^+ sheep (Lauf and Theg, 1980; Dunham and Ellory, 1981); however, the loss of KCl during RVD of Ehrlich cells occurred via separate, bumetanide-insensitive K^+ and Cl^- channels (Hoffman, 1985), and RVD of Amphiuma red cells involved net K^+/H^+ exchange (Cala, 1985). Loss of K^+Cl^- from HeLa and MDCK cells also occurred via pathways distinct from the $Na^+K^+Cl^-$ cotransporter (Aiton and Simmons, 1983). The system which transports K^+Cl^- is therefore probably structurally distinct from the system which cotransports $Na^+K^+Cl^-$ and Na^+Cl^- (Ellory et al, 1980).

Similarly, the system which is responsible for Na^+/Na^+ exchange is independent of K^+ and Cl^-, and is unlikely to be related to the structures which mediate $Na^+K^+Cl^-$ transport (Wiater and Dunham, 1983).

Jayme et al (1981) found that there was a net inward movement of Na^+ and outward movement of K^+ (down their individual concentration gradients) in mouse fibroblasts incubated in the presence of ouabain, and suggested that this observation represented Na^+/K^+ exchange. However, in a later study, Jayme and his colleagues found that a portion of the Na^+ influx was amiloride-sensitive; they concluded that their earlier observations represented the initial rapid entry of external Na^+, followed by the coefflux of Na^+ and K^+ out of the cell down the net electrochemical gradient (Jayme et al, 1984). This is in contrast to the conclusions of Atlan et al (1984). These authors observed a net K^+ efflux from NIH3T3 cells under physiological ion concentrations, and suggested that the cotransporter mediated the facilitated diffusion of Na^+ into the cell, and K^+ out of the cell, down their individual concentration gradients. They speculated that the mechanism by which this occurred was a net influx of Na^+ & K^+, but with a larger K^+ efflux, independent of Na^+ efflux; however, in this model, Na^+ would be required to bind to the transporter, but would not itself be transported. Similarly, Cl^- would be required to bind to maintain electroneutrality. It is not clear whether this model would explain the electroneutrality of the net movement of $Na^+K^+2Cl^-$ into K^+-depleted Ehrlich cells (Geck et al, 1980); however, McManus and Schmidt (1978) have suggested that the cotransporter of avian erythrocytes may have a net charge at some point during its active "cycle".

The absence of a diuretic-sensitive $^{86}Rb^+$ efflux from L1210 cells argues against the operation of Na^+/K^+ exchange in these cells under physiological conditions. However, it was not possible to investigate whether a Na^+Cl^- efflux was present because of methodological difficulty in measuring $^{22}Na^+$ and $^{36}Cl^-$ fluxes (results 4.5).

The diuretic-sensitive cotransporter also mediates a K^+/K^+ exchange (McManus and Schmidt, 1978; Spaggiare et al, 1976; Haas and McManus, 1983; Tupper et al, 1977). No evidence was found for the operation of diuretic-sensitive K^+/K^+ exchange in L1210 cells; there was no diuretic-sensitive $^{86}Rb^+$ efflux and the $^{86}Rb^+$ efflux was independent of external K^+. Thus, the major pathway of $^{86}Rb^+$ and K^+ efflux from L1210 cells is not via the $Na^+K^+Cl^-$ cotransporter. This result agrees, in part, with work on Balb/C 3T3 cells, which were shown to possess diuretic-sensitive K^+/K^+ exchange (Spaggiare et al, 1976) which was only a minor component of the total K^+ efflux (O'Brien and Krzeminski, 1983).

In summary, under physiological conditions there is no diuretic-sensitive component of K^+ efflux from L1210 cells. Therefore, the $Na^+K^+Cl^-$ cotransporter mediates only a net influx of K^+, which is sensitive to HN2.

4.4.2 ^{86}RUBIDIUM EFFLUX FROM L1210 CELLS INCUBATED IN POTASSIUM-FREE MEDIUM

4.4.2.1 Introduction

The net diuretic-sensitive transmembrane movement of ions depends upon the ionic concentrations in the cells and extracellular medium (Palfrey and Greengard, 1981; Atlan et al, 1984), and is driven by the sum of the electrochemical gradients of all three ions (Geck et al, 1980). Therefore, the manipulation of external ion concentrations may reverse the net diuretic-sensitive flux.

Under physiological ion concentrations there was no diuretic-sensitive component of $^{86}Rb^+$ efflux from L1210 cells, and the $^{86}Rb^+$ efflux was resistant to inhibition by nitrogen mustard (results 4.4.1). The experiments described in this section were carried out to investigate whether low extracellular concentrations of K^+, Na^+ and Cl^- induced a net diuretic-sensitive $^{86}Rb^+$ efflux from L1210 cells, and to determine whether $^{86}Rb^+$ efflux under these conditions was sensitive to nitrogen mustard.

4.4.2.2 Results

Figure 42 shows the efflux of $^{86}Rb^+$ from preloaded L1210 cells incubated in K^+-free KR buffer (isosmotically substituted with sodium chloride). There was an initially rapid phase of $^{86}Rb^+$ loss (0-5 minutes), followed by a slower component which was exponentially related to time. A single time point of 15 minutes was chosen for further experiments to examine the rate of $^{86}Rb^+$ efflux, since this represented an early point in the second, slower phase of $^{86}Rb^+$ efflux.

Figure 43 shows the effect of preincubation of $^{86}Rb^+$-loaded L1210 cells with 10^{-5}M HN2 or 10^{-3}M ouabain, upon the efflux of $^{86}Rb^+$ into K^+-free KR. ^{86}Rubidium efflux was expressed as activity remaining in the cells fifteen minutes after resuspending in efflux medium, as a fraction of the activity in the cells at the beginning of efflux. Incubation with 10^{-3}M ouabain caused a progressive increase in the rate of $^{86}Rb^+$ efflux. In contrast, 10^{-5}M nitrogen mustard had no effect on the rate of $^{86}Rb^+$ efflux after preincubation for 3h. However, after preincubation for 4h with 10^{-5}M HN2, there was a marked increase in the loss of $^{86}Rb^+$ from HN2-treated cells in comparison with controls; this decline was matched by a decline in the percentage of cells which were impermeable to trypan blue

(results 4.1.3.3), and probably represented a non-specific leak of $^{86}Rb^{+}$ from dying cells.

Figure 44 shows the effect of various agents on the efflux of $^{86}Rb^{+}$ from preloaded L1210 cells resuspended in K^{+}-free KR buffer. Preincubation of L1210 cells with 10^{-3}M furosemide, for fifteen minutes or three hours, had no significant effect on the loss of $^{86}Rb^{+}$ after fifteen minutes in K^{+}-free KR, in comparison to controls at the same time point. Bumetanide (10^{-4}M) appeared to cause a small inhibition of $^{86}Rb^{+}$ efflux after preincubation for 15 minutes or 3 hours; however, the differences from controls were not statistically significant ($p>0.05$). Preincubation for 15 minutes with 10^{-3}M ouabain had no significant effect on the efflux of $^{86}Rb^{+}$; however, after prolonged incubation with ouabain (3h), the stimulation of $^{86}Rb^{+}$ efflux from ouabain-treated cells was again evident. The efflux of $^{86}Rb^{+}$ into K^{+}-free medium was insensitive to nitrogen mustard or its monofunctional analogue.

Comparison of the rates of $^{86}Rb^{+}$ loss from preloaded L1210 cells incubated in KR and K^{+}free KR showed no significant differences ($p>0.05$), indicating that the loss of $^{86}Rb^{+}$ was independent of external K^{+}.

The removal of K^{+} from the external medium failed to produce a diuretic-sensitive $^{86}Rb^{+}$ efflux from L1210 cells; this result was independent of the presence of 10^{-3}M ouabain (data not shown).

In order to produce the maximum transmembrane concentration gradients of all three ions, and thus to make conditions most favourable for efflux, L1210 cells were resuspended in 150mM tetramethylammonium gluconate, containing 10^{-3}M ouabain. The efflux of $^{86}Rb^{+}$ into TMAgluconate was initally very rapid, with 60% of the

Figure 42

The efflux of $^{86}Rb^+$ from preloaded L1210 cells incubated in K^+-free Krebs-Ringer buffer. (Mean ± S.D.; n=3)

Figure 43

The effect of preincubation with 10^{-3}M ouabain or 10^{-5}M nitrogen mustard on the initial efflux of $^{86}Rb^+$ from preloaded L1210 cells incubated in K^+-free Krebs-Ringer buffer.

Key: ○ control
● 10^{-5}M HN2
▲ 10^{-3}M ouabain (Mean ± S.D.; n=3)

Figure 44

The effect of various agents on the initial efflux of $^{86}Rb^+$ from preloaded L1210 cells incubated in K^+-free Krebs-Ringer buffer.

Key:

Preincubation for 15 minutes	Preincubation for 3 hours
1. control	5. control
2. 10^{-3}M furosemide	6. 10^{-3}M furosemide
3. 10^{-4}M bumetanide	7. 10^{-4}M bumetanide
4. 10^{-3}M ouabain	8. 10^{-3}M ouabain
	9. 10^{-5}M HN2-1
	10. 10^{-5}M HN2

(Mean + S.D.; n=4)

Figure 45

The effect of preincubation with bumetanide or nitrogen mustard on the efflux of $^{86}Rb^+$ from preloaded L1210 cells incubated in 150 mM TMA gluconate.

Key: ○ control, 3h
● 10^{-5}M HN2, 3h
▲ 10^{-4}M bumetanide, 15 min.

(Mean of triplicate determinations of one experiment)

Figure 42

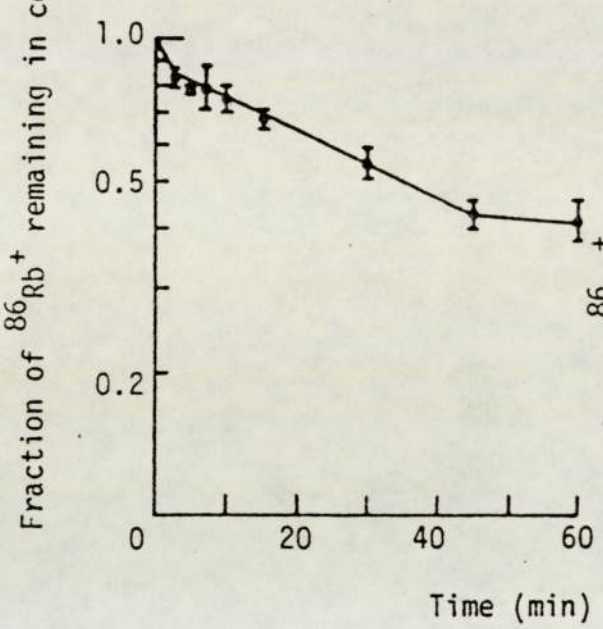


Figure 43

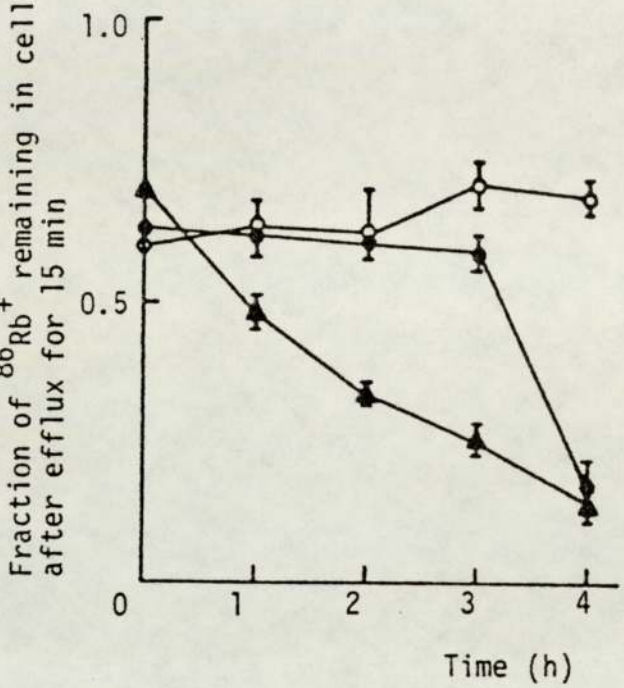


Figure 44

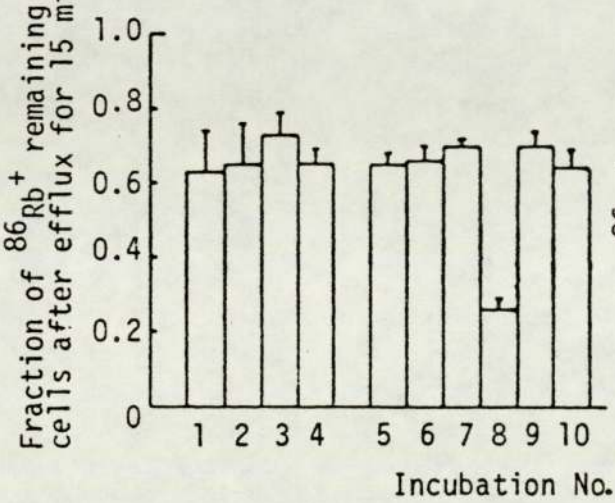
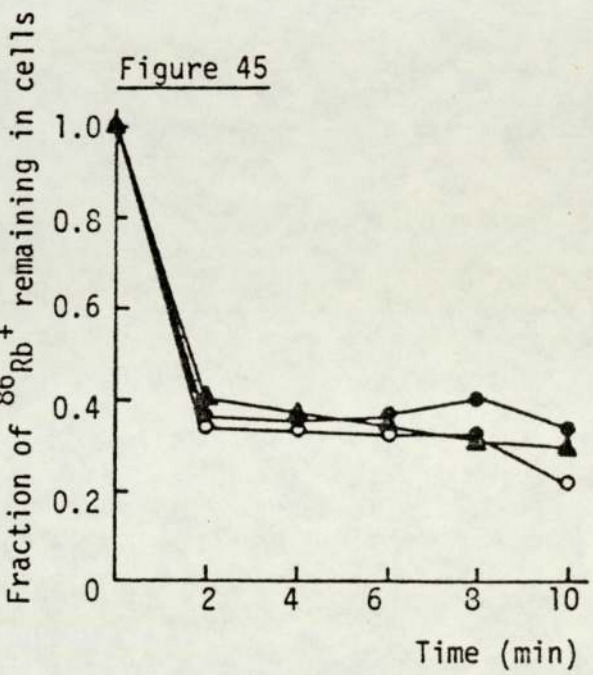


Figure 45



originally loaded $^{86}Rb^+$ being lost to the medium within two minutes; thereafter, further loss was barely measurable. In the absence of Na^+ , K^+ or Cl^- in the external medium, the $^{86}Rb^+$ efflux from L1210 cells was unaffected by preincubation for 3h with 10^{-5}M HN2 or by preincubation for 15 minutes with 10^{-4}M bumetanide (figure 45). This result shows that the bumetanide-sensitive component of Rb^+ & K^+ influx into L1210 cells is not reversible, even when the gradients of all the ions favour efflux.

4.4.2.3 Discussion

The $Na^+K^+Cl^-$ cotransporter of L1210 cells appeared to mediate a net influx of ions under physiological conditions; no evidence was found for diuretic-sensitive $^{86}Rb^+$ efflux or $^{86}Rb^+/K^+$ exchange (results 4.4.1). ^{86}Rubidium-loaded L1210 cells were placed in a K^+-free buffer in an attempt to drive $^{86}Rb^+$ efflux by a favourable K^+ concentration gradient across the cell membrane.

The efflux of $^{86}Rb^+$ from preloaded L1210 cells in K^+-free KR displayed two phases; for the first five minutes $^{86}Rb^+$ was lost with a half-time of approximately 13 minutes, and thereafter a slower component of efflux, with a half-time of approximately 42 minutes became evident. A similar biphasic efflux of $^{86}Rb^+$ was observed by Gargus et al (1978) and Jayme et al (1984) using mouse fibroblasts placed in media of low (0.2mM) K^+ concentrations. The rapid initial phase of $^{86}Rb^+$ efflux may represent loss from rapidly-exchanged pools; for example, $^{86}Rb^+$ associated with negatively-charged groups at the cell surface. The second, slower phase of $^{86}Rb^+$ efflux may represent removal of $^{86}Rb^+$ from compartments which have a partial barrier to such loss; for example, the $^{86}Rb^+$ contained in the cytosol must traverse the cell membrane or be carried out of the cell through the action of specific transport processes. Examination.

of $^{86}Rb^+$ loss at a single time point fifteen minutes after initiation, allowed the comparison of effects of various agents upon the initial rate of the second phase of efflux. Measurement of $^{86}Rb^+$ efflux after longer time intervals would require the consideration of the effects of the same agents on the re-uptake of $^{86}Rb^+$.

An unexpected result was that the efflux of $^{86}Rb^+$ from L1210 cells incubated in K^+ free medium was insensitive to diuretics or nitrogen mustard. In contrast, the slower phase of $^{86}Rb^+$ efflux was largely diuretic-sensitive in 3T3 cells (O'Brien and Krzeminski, 1983), MDCK cells (McRoberts et al, 1983), and mouse L cells (Jayme et al, 1981; Gargus et al, 1978) incubated in media of reduced K^+ concentrations. If the $Na^+K^+Cl^-$ cotranporter is envisaged as a passive exchange pathway under physiological conditions, net efflux would only be possible if the movement of an ion in one direction, whilst coupled to the co-ions, is not obligatorily coupled to the movement of ions in the opposite direction (McManus and Schmidt, 1978). Thus, a diuretic-sensitive efflux of $^{86}Rb^+$ may occur when cells are incubated in the absence of external K^+. This model does not apply to the $Na^+K^+Cl^-$ cotransporter of L1210 cells, which catalyzes the influx of $^{86}Rb^+$ under physiological K^+ concentrations; the system catalyzes no $^{86}Rb^+$ efflux at physiological K^+ concentrations (results 4.4.1), or in the absence of extemal K^+. Indeed, even in the absence of external Na^+, K^+ and Cl^-, the efflux of $^{86}Rb^+$ from L1210 cells was insensitive to bumetanide, indicating that the system did not catalyze net efflux, even when all the transmembrane concentration gradients would favour efflux. This latter conclusion relies upon the assumption that the substituting ions, TMA^+ and $gluconate^-$, which were unable to support diuretic-

sensitive $^{86}Rb^+$ influx (results 4.2.3), were also without "trans" effects, i.e., that the presence of these ions at the exterior face of the cell membrane did not influence the movement of ions at the interior face.

In addition, these conclusions assume that cotransport is inhibited by diuretics under conditions of low, or absent, external K^+. The binding of diuretics to cell membranes is thought to require K^+ (Forbush and Palfrey, 1983). However, nitrogen mustard, which was a potent inhibitor of $^{86}Rb^+$ influx, independently of external K^+ (results 4.3.1), was also without effect upon $^{86}Rb^+$ efflux, suggesting that a $Na^+K^+Cl^-$ cotransporter-mediated component was absent.

Diuretic-sensitive fluxes have been shown to be sensitive to removal of serum (Amsler et al, 1985), and the efflux of $^{86}Rb^+$ from L1210 cells was measured in the absence of serum. Therefore, the efflux of $^{86}Rb^+$ must be examined in the presence of dialysed serum to confirm the results of the present study.

Mutants with altered $Na^+K^+Cl^-$ cotransport activity, deficient in either $^{86}Rb^+$ influx or efflux, have been isolated from MDCK cells (McRoberts et al, 1983) mouse L cells (Gargus and Slayman, 1980, Jayme et al, 1981; 1984; Gargus et al, 1978) and 3T3 cells (Sussman and O'Brien, 1985). An L1210 cell line resistant to HN2 was shown to be deficient in bumetanide-sensitive $^{86}Rb^+$ influx (results 4.16) it will be of interest to examine whether, in common with the sensitive L1210 cell line, it is deficient in $^{86}Rb^+$ efflux. The results of a preliminary study indicate that this is indeed true (results 4.4.3).

The results of the experiments described in this section also demonstrate the stimulation of $^{86}Rb^+$ efflux in ouabain-treated cells (see results 4.4.1). In addition, the lack of dependence of $^{86}Rb^+$

efflux upon external K^+ supports the conclusion that K^+/K^+ exchange is not a major component of $^{86}Rb^+$ efflux from L1210 cells (see results 4.4.1).

4.4.3 THE EFFECT OF CALCIUM ON ^{86}RUBIDIUM EFFLUX FROM L1210 CELLS

4.4.3.1 Introduction

The Ca^{++} activated K^+ channel was first described in human erythrocytes by Gardos (1958). It is insensitive to the cardiac glycoside ouabain, and to the diuretics furosemide and bumetanide, but it is inhibited by quinine (Lew and Ferreira, 1978). The pathway is activated by elevations in intracellular calcium concentrations; the calcium ionophore A23187 is useful for the study of Ca^{++} activated K^+ fluxes. In several cell types the response to hypotonic medium is thought to involve the activation a K^+ channel with identical properties to the Ca^{++}-activated channel (see discussions 4.8.3 and 4.4.3.3).

The effect of the Ca^{++} ionophore A23187 upon $^{86}Rb^+$ efflux from L1210 cells was examined. At the same time, the cell volume was determined, in order to establish whether the Ca^{++}-activated K^+ channel had a role in the maintenance of L1210 cell volume under physiological conditions.

The results described below represent a preliminary study; the experiments were repeated only once or twice (n=2 or 3). Consequently, the experimental results are not statistically significantly different ($p>0.05$) from the controls, except where otherwise stated. Accordingly, the results are presented in a qualitative rather than quantitative manner.

4.4.3.1 Results

Figures 46 and 47 show the effect of various agents on the efflux of $^{86}Rb^+$ from preloaded cells, and the concomitant changes in cell volume, respectively.

Figure 46

The effect of calcium on the efflux of $^{86}Rb^{+}$ from preloaded cells incubated in K^{+}-replete buffer.

(a) L1210 cells from exponentially-growing cultures

(Mean + S.D.; n=3)

b) L1210 cells from stationary cultures

(Mean + S.D.; n=3)

(c) L1210R cells from exponentially-growing cultures

(Mean + S.D.; n=2)

Key:
1. control
2. 10^{-4}M EGTA
3. 10^{-4}M bumetanide
4. 10^{-3}M $MgSO_4$
5. $3x10^{-4}$M quinine
6. 10^{-5}M A23187 + 10^{-4}M EGTA
7. 10^{-5}M A23187 + 10^{-4}M Ca^{++}
8. 10^{-5}M A23187 + 10^{-3}M Ca^{++}
9. 10^{-5}M A23187 + 10^{-3}M Ca^{++} + 10^{-4}M bumetanide
10. 10^{-5}M A23187 + 10^{-3}M Ca^{++} + $3x10^{-4}$M quinine

Figure 46

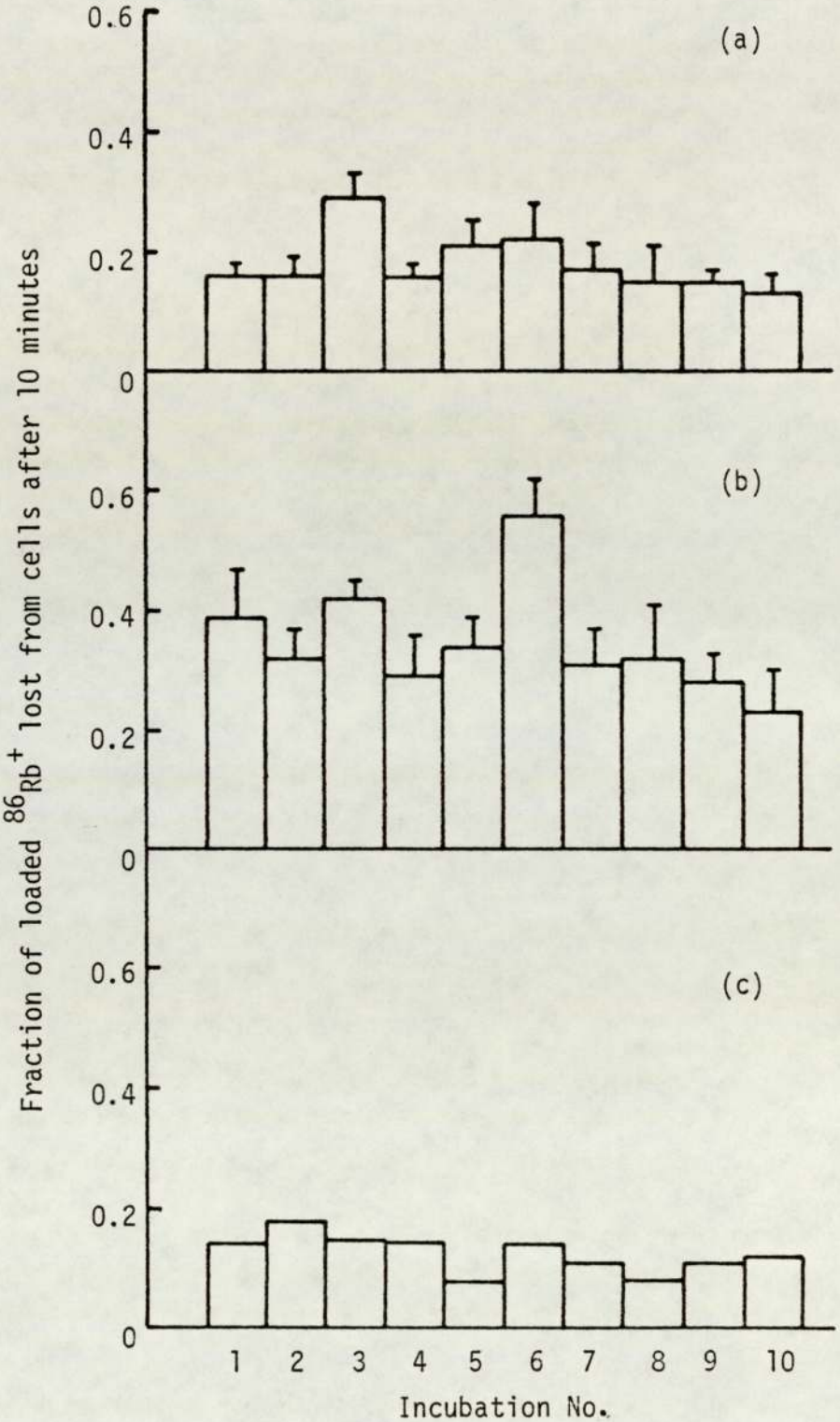

0.6
0.4
0.2
0
(a)
(b)
(c)
Fraction of loaded $^{86}Rb^{+}$ lost from cells after 10 minutes
1 2 3 4 5 6 7 8 9 10
Incubation No.

Figure 47

The effect of calcium on the volume of $^{86}Rb^{+}$-loaded L1210 cells incubated in K^{+}-replete buffer.

(a) L1210 cells from exponentially-growing cultures

(Mean + S.D.; n=3)

(b) L1210 cells from stationary cultures

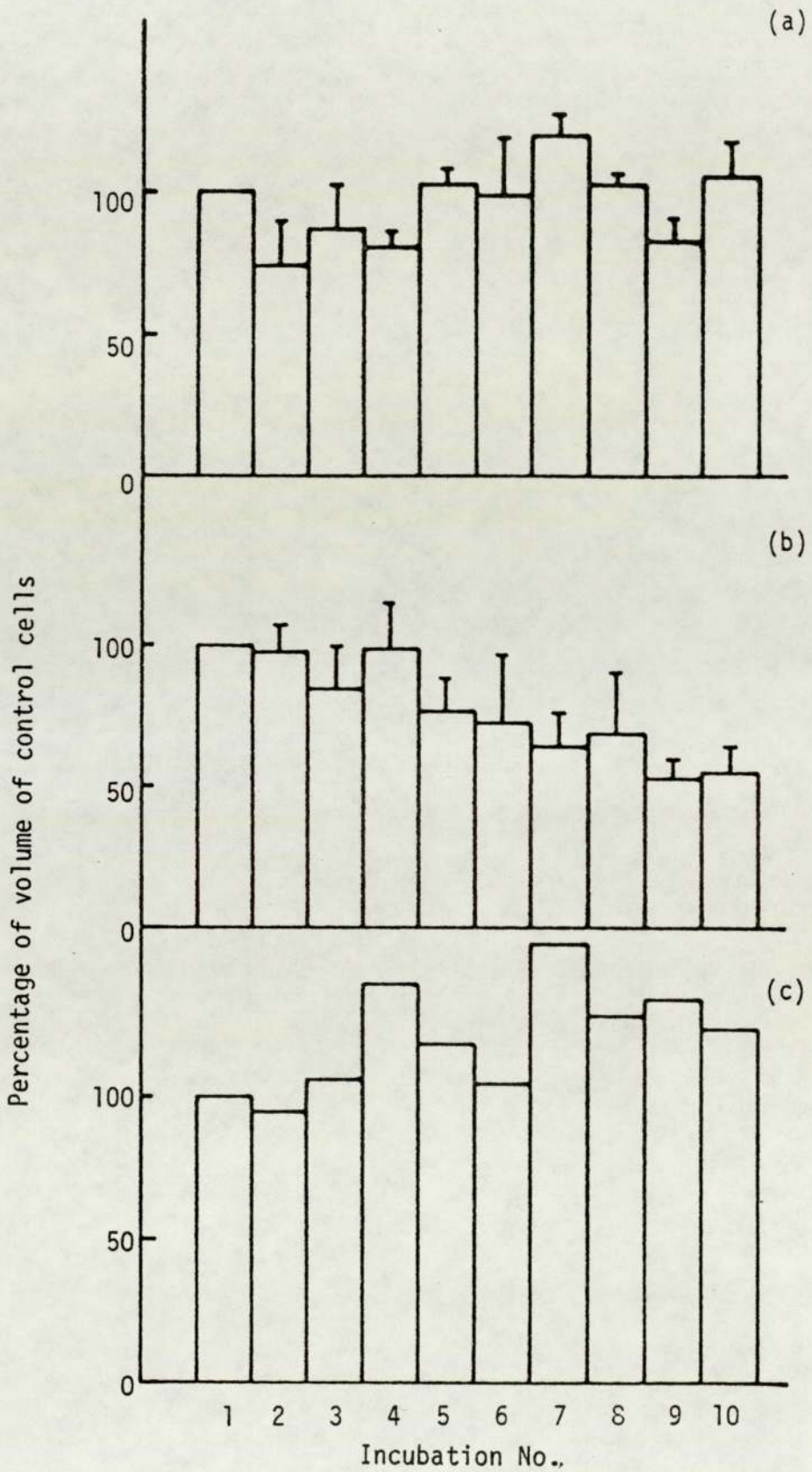
(Mean + S.D.; n=3)

(c) L1210R cells from exponentially-growing cultures

(Mean; n=2)

Key:
1. control
2. 10^{-4}M EGTA
3. 10^{-4}M bumetanide
4. 10^{-3}M $MgSO_4$
5. $3x10^{-4}$M quinine
6. 10^{-5}M A23187 + 10^{-4}M EGTA
7. 10^{-5}M A23187 + 10^{-4}M Ca^{++}
8. 10^{-5}M A23187 + 10^{-3}M Ca^{++}
9. 10^{-5}M A23187 + 10^{-3}M Ca^{++} + 10^{-4}M bumetanide
10. 10^{-5}M A23187 + 10^{-3}M Ca^{++} + $3x10^{-4}$M quinine

Figure 47


(a)
100
50
0
(b)
100
50
0
(c)
100
50
0
Percentage of volume of control cells
1 2 3 4 5 6 7 8 9 10
Incubation No.

Efflux of $^{86}Rb^+$ was allowed to proceed for 10 minutes in the presence of $10^{-3}M$ ouabain. The efflux of $^{86}Rb^+$ was examined in rapidly-dividing L1210 cells, stationary phase L1210 cells, and rapidly-dividing L1210R cells.

Efflux of $^{86}Rb^+$ from rapidly-dividing L1210 and L1210R cells occurred at similar rates; efflux of $^{86}Rb^+$ from stationary phase L1210 cells was more rapid.

In the presence of $10^{-3}M$ ouabain, and in the absence of Ca^{++}, bumetanide ($10^{-4}M$) significantly ($p>0.05$) stimulated $^{86}Rb^+$ efflux from rapidly-dividing L1210 cells, but was without effect upon $^{86}Rb^+$ efflux from stationary phase L1210 cells or growing L1210R cells. The stimulatory effect of bumetanide upon $^{86}Rb^+$ efflux was not observed in other experiments (results 4.4.1 and 4.4.2), it was presumed that the stimulation of efflux arose through prevention of the re-uptake of $^{86}Rb^+$ through the $Na^+K^+Cl^-$ cotransporter, which mediated influx of $^{86}Rb^+$ at physiological K^+ concentrations (results 4.2; 4.4.1 and 4.4.2); the lack of effect of bumetanide on $^{86}Rb^+$ efflux from L1210R cells and stationary phase L1210 cells reflected the reduced or absent activity of the $Na^+K^+Cl^-$ cotransporter in these cells (results 4.2.2.3 and 4.16.3). In common with L1210 cells, there was no diuretic-sensitive $^{86}Rb^+$ efflux from L1210R cells incubated in medium of physiological K^+ concentration.

Bumetanide reduced the volume of L1210 cells to 94±7% of control cells; this was a smaller effect than when volume was measured in medium which did not contain ouabain (results 4.8), and was consistent with antagonistic roles of the $Na^+K^+Cl^-$ cotransporter and Na^+K^+ATPase in the regulation of L1210 cell volume (see results 4.8.3). However, if these results are found to be significant another pathway must also function to regulate volume; if the volume

of bumetanide-treated cells is reduced by the action of Na^+K^+ATPase alone, it would be expected that changes in volume would be absent in cells incubated in the presence of ouabain. Similarly, the volume of stationary phase cells was reduced by bumetanide to 93±6% of controls, again, if the result is significant suggesting that volume was affected by pathways distinct from the $Na^+K^+Cl^-$ cotransporter and Na^+K^+ATPase. The volume of L1210R cells was unaffected by bumetanide.

In order to ensure that extracellular Ca^{++} was absent, 0.1mM EGTA was added to the medium. EGTA was without effect upon $^{86}Rb^+$ efflux from growing L1210 cells, but reduced the volume to 75±15% of controls. In contrast, EGTA reduced $^{86}Rb^+$ efflux from stationary phase cells but had no effect on volume. However, EGTA slightly stimulated $^{86}Rb^+$ efflux from L1210R cells, and slightly reduced volume. These results suggest that low concentrations of external Ca^{++} (or Mg^{++}) may influence K^+ movements and volume regulation; the mechanism by which this occurs awaits more detailed investigation.

Addition of Mg^{++}, in common with EGTA, reduced the volume of growing L1210 cells with no effect upon $^{86}Rb^+$ efflux, and reduced $^{86}Rb^+$ efflux from stationary phase cells with no effect on volume. However, addition of Mg^{++} had no effect on $^{86}Rb^+$ efflux from L1210R cells but produced a large (40%) increase in cell volume.

The addition of calcium ionophore A23187 with EGTA would be expected to deplete intracellular calcium; this treatment stimulated $^{86}Rb^+$ efflux from growing L1210 cells, but had no effect on volume. The $^{86}Rb^+$ efflux from stationary phase L1210 cells was also stimulated by A23187 + EGTA, but, in this case, stimulation was accompanied by a decrease in cell volume. ^{86}Rubidium efflux and volume of L1210R cells were unaffected by A23187 + EGTA. These

results suggest that at physiological external K^+ concentrations, $^{86}Rb^+$ & K^+ efflux from L1210 cells may be affected by changes in intracellular Ca^{++}, but not in a manner consistent with a K^+ channel stimulated by elevations in intracellular Ca^{++}.

Addition of quinine stimulated $^{86}Rb^+$ efflux from growing L1210 cells, but had no effect upon their volume; this effect was similar to the effect of A23187 + EGTA. In stationary phase L1210 cells, quinine inhibited $^{86}Rb^+$ efflux and decreased cell volume; this effect differed from the effect of A23187 + EGTA. Quinine inhibited $^{86}Rb^+$ efflux from L1210R cells, but in this case, increased the cell volume.

In the presence of extracellular Ca^{++}, the addition of calcium ionophore A23187 would be expected to increase intracellular Ca^{++} concentration. Addition of A23187 with 0.1mM Ca^{++} or 1mM Ca^{++} had no marked effect on $^{86}Rb^+$ efflux from growing L1210 cells; the $^{86}Rb^+$ efflux was unaffected by bumetanide or quinine. In the presence of 0.1mM Ca^{++}, but not 1mM Ca^{++}, A23187 caused an increase in cell volume. In the presence of A23187 and 1mM Ca^{++}, quinine was without effect on cell volume, but bumetanide reduced cell volume. In stationary phase L1210 cells, A23187 + Ca^{++} inhibited $^{86}Rb^+$ efflux; quinine and bumetanide both increased the inhibition. A23187 + Ca^{++} reduced stationary phase L1210 cell volume, and both quinine and bumetanide reduced the volume still further. Addition of A23187 + Ca^{++} to L1210R cells also produced inhibition of $^{86}Rb^+$ efflux, but in this case, quinine or bumetanide slightly reduced this effect. In addition, the inhibition of $^{86}Rb^+$ efflux was accompanied by an increase in cell volume which was not markedly inhibited by quinine or bumetanide.

4.4.3.3 Discussion

The calcium-stimulated K^+ channel was described in human erythrocytes by Gardos (1958), and has been demonstrated in a variety of other cell types including Ehrlich ascites tumour cells (Valdeolmillos et al, 1982) and human lymphocytes (Grinstein et al,1982a; 1982b; 1983a; 1983b) in which it has been extensively studied.

The channel may have a role in the regulation of membrane potential of L1210 cells (Hickman et al, 1984) and murine myeloma cells, thereby influencing the electrogenic transport of Na^+-dependent amino acids (Pershadsingh et al, 1985). However, the major role of the channel in a variety of cell types may be in the regulation of cell volume: this phenomenom has been most extensively investigated in cells under osmotic stress rather than under steady-state isosmotic conditions. In response to hypotonic media, cells initially swell, then undergo a decrease in their volume, characterised by the loss of water, K^+ and Cl^-; this process (RVD) has been extensively studied in Ehrlich ascites cells, and human lymphocytes and erythrocytes, and is thought to involve the Ca^{++}-stimulated K^+ channel. The initial swelling of Ehrlich ascites cells induces the calcium-dependent activation of separate conductive K^+ and Cl^- pathways (Hoffmann, 1985a; 1985b), but, in contrast, the loss of Cl^- from lymphocytes is not calcium-dependent (Grinstein et al, 1982a; 1982b; Sarkadi et al, 1984). The calcium-ionophore A23187 has been used to study the effect of activation of the K^+ channel upon the volume of cells incubated in isosmotic media (Hoffmann et al, 1984; Hoffmann, 1985a; 1985b; Grinstein et al, 1983b).

The pathway of K^+ loss from Ehrlich cells during RVD is insensitive to diuretics and is independent of Cl^- (Hoffmann et al,

1984; Hoffmann, 1985a; 1985b). It is also insensitive to the inhibitor of anion-exchange, DIDS (Hoffmann et al, 1984). The calcium-activated K^+ pathway is sensitive to the inhibitor quinine, which is frequently used to define its function (Lew and Ferreira, 1978; Armando-Hardy et al, 1975; introduction 1.2.1).

The calcium ionphore A23187 was used together with quinine to investigate the activity of the K^+ channel of L1210 cells under steady state isosmotic conditions. If the K^+ channel has a role in the maintenance of cell volume under steady-state conditions, it would be expected that inhibition by quinine would increase the cell volume; furthermore, it would be expected that the activity of the channel would respond to changes in intracellular calcium concentrations, and the addition of A23187 + Ca^{++} would be expected to activate the channel and reduce cell volume. Activation and volume changes would be sensitive to quinine.

The results obtained were inconsistent with this model for the K^+ efflux and volume regulation of L1210 or L1210R cells. The movement of $^{86}Rb^+$ out of the cells did not consistently correlate with the concomitant changes in cell volume. The Rb^+ efflux was not dependent upon intracellular Ca^{++}; volume changes and $^{86}Rb^+$ efflux from cells incubated with calcium ionophore A23187 were inconsistent with a Ca^{++}-activated $^{86}Rb^+$ efflux pathway functioning to reduce cell volume. However, the rate of $^{86}Rb^+$ efflux was altered by changes in intracellular and extracellular calcium concentrations; these responses differed between growing and quiescent L1210 cells and between L1210 and L1210R cells, but await further elucidation. Extracellular calcium has been shown to influence intracellular Na^+ and K^+ concentrations (Kino et al, 1985); the mechanism by which it may affect $^{86}Rb^+$ efflux from L1210 cells is unknown.

In conclusion, under steady-state conditions there appears to be no Ca^{++}-senstive $^{86}Rb^{+}$ & K^{+} efflux from L1210 or L1210R cells. In addition, the presence of calcium ionophore A23187 failed to stimulate the activity of a $^{86}Rb^{+}$ channel with characteristics similar to those of the Ca^{++}-activated K^{+} channel of other cell types. However, Hickman et al (1984) found that A23187 induced changes in the membrane potential and volume of L1210 cells which were consistent with activation of a Ca^{++}-stimulated K^{+} efflux pathway. Further studies are required to establish whether the response of the L1210 cells used in the present study to hypotonic medium includes activation of Ca^{++}-stimulated K^{+} channel (see also results 4.8), which is not active under physiological conditions.

4.5 ^{22}SODIUM AND ^{36}CHLORIDE INFLUX INTO L1210 CELLS

4.5.1 Introduction

Rapidly-dividing L1210 cells were found to possess a diuretic-sensitive transport system for $^{86}Rb^+$ & K^+ influx which was wholly dependent upon extracellular Na^+ and Cl^- ions (results 4.2), indicating that these ions were required for K^+ transport. These properties are characteristic of $Na^+K^+Cl^-$ cotransport systems described in a number of different cells (introduction 1.3). However, the experiments described in sections 4.2 and 4.4 did not confirm whether Na^+ and Cl^- ions were cotransported with $^{86}Rb^+$ & K^+ into L1210 cells. The experiments described here represent attempts to examine Na^+ and Cl^- fluxes in L1210 cells using tracer amounts of the radioisotopes $^{22}Na^+$ and $^{36}Cl^-$ respectively.

4.5.2 Results

Figures 48 and 49 show the initial uptake of $^{22}Na^+$ into exponentially-dividing L1210 cells incubated in Krebs-Ringer bicarbonate buffer at 37°, in the absence and presence of 10^{-3}M ouabain, respectively. The accummulation of $^{22}Na^+$ was measured by use of the oil barrier method developed for measurements of $^{86}Rb^+$ transport.

The influx of $^{22}Na^+$ into L1210 cells was not linear with time, and no initial rate of $^{22}Na^+$ + Na^+ transport could be determined. Ouabain was without effect. The distribution of $^{22}Na^+$ appeared to approach isotopic equilibrium within 15 seconds of addition to the cell suspension; this time point represented the shortest interval after addition of isotope over which it was possible to determine in triplicate the distribution of $^{22}Na^+$ in cells and supernatent fractions. The results shown in figures 48 and 49 are representative of several attempts in each case; however, no consistent effect of HN2 upon $^{22}Na^+$ uptake was observed.

Figure 48

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the uptake of $^{22}Na^{+}$ into L1210 cells.

Key: ○ control

● 10^{-5}M HN2

(Mean of triplicate determinations of a single experiment)

Figure 49

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the uptake of $^{22}Na^{+}$ into L1210 cells incubated with 10^{-3}M ouabain.

Key: ○ control

● 10^{-5}M HN2

(Mean of triplicate determinations of a single experiment)

Figure 48

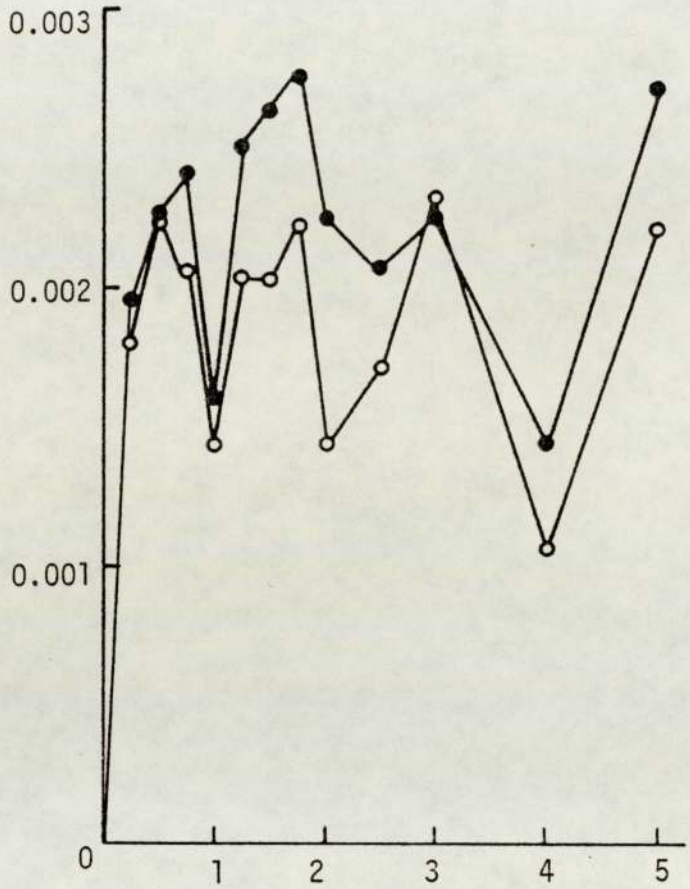


Figure 49

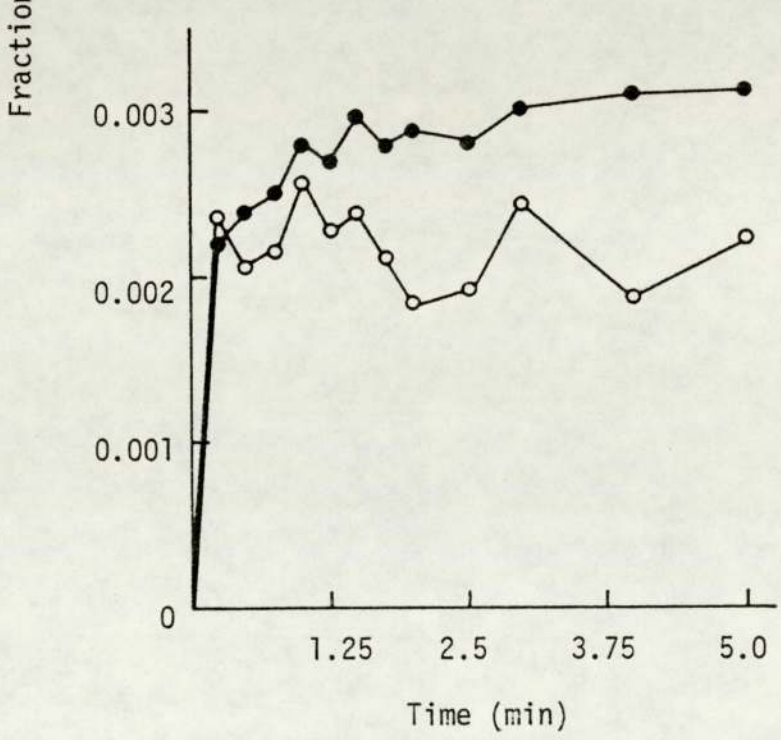


Figure 50

The uptake of $^{22}Na^{+}$ into L1210 cells incubated in RPMI at 20^{o}.

(Mean of triplicate determinations of a single experiment)

Figure 51

The effect of preincubation for 3h with 10^{-5}M HN2 on the uptake of $^{22}Na^{+}$ into L1210 cells, measured by ultra-filtration.

Key: ● control

○ 10^{-5}M HN2

(Mean of duplicate determinations of a single experiment)

Figure 50

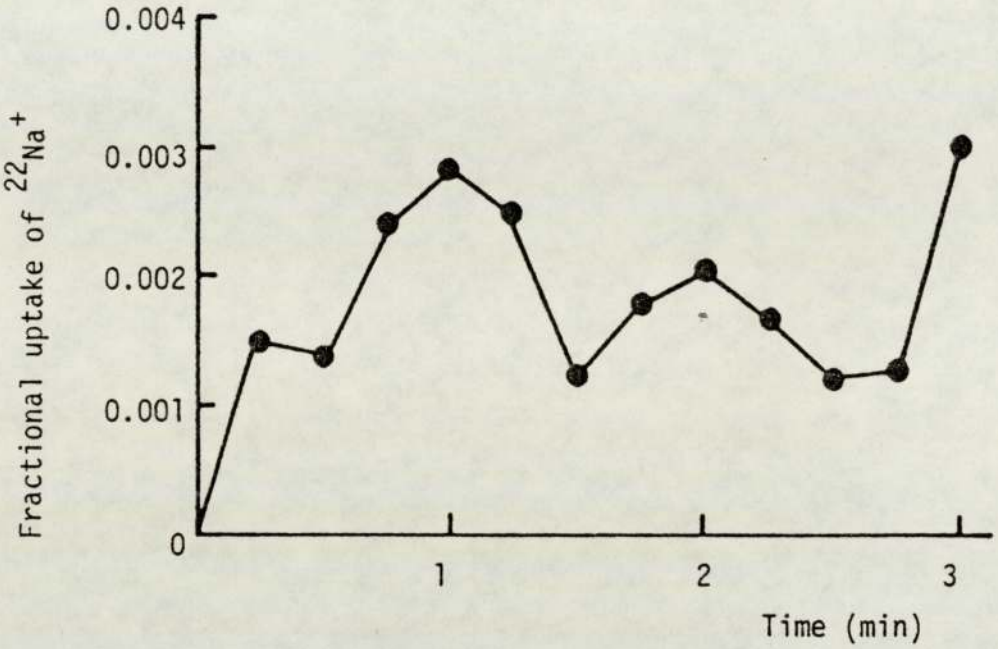


Figure 51

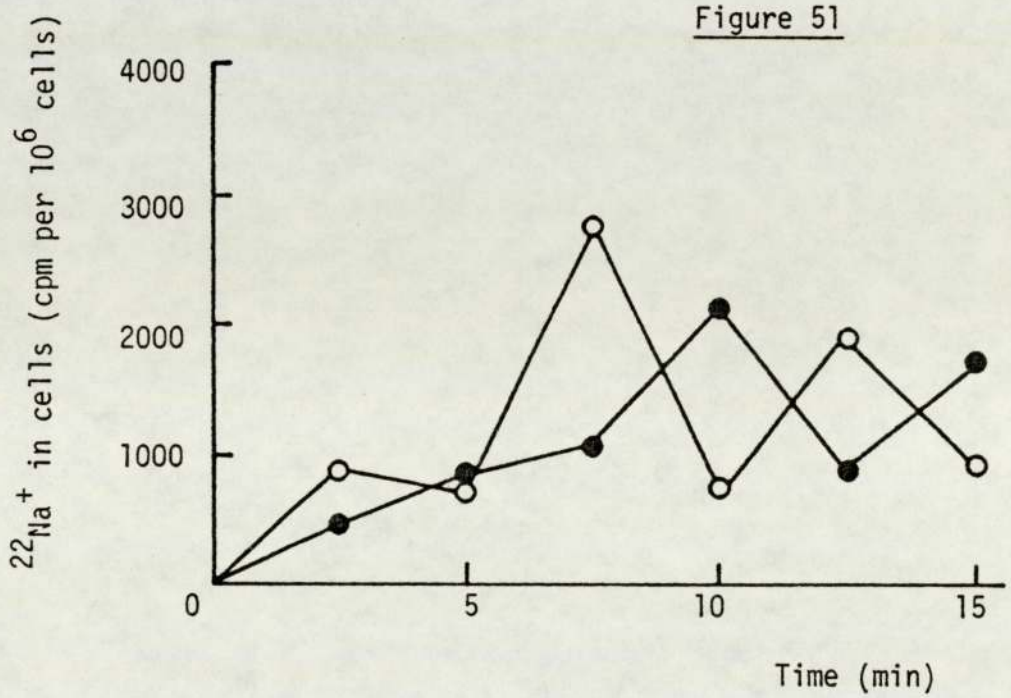


Figure 52

The effect of bumetanide on the uptake of $^{22}Na^{+}$ into L1210 cells incubated in Krebs-Ringer buffer in the presence of 10^{-3}M ouabain.

Key: ● control

▲ 10^{-4}M bumetanide

(Mean of triplicate determinations of a single experiment)

Figure 53

The effect of bumetanide on the uptake of $^{22}Na^{+}$ into L1210 cells incubated in HEPES-buffered medium in the presence of 10^{-3}M ouabain.

Key: ● control

▲ 10^{-4}M bumetanide

(Mean of triplicate determinations of a single experiment)

Figure 54

The effect of bumetanide on the change in equilibrium distribution of $^{22}Na^{+}$ in L1210 cells upon addition of 10^{-3}M ouabain.

Key: ● control

▲ 10^{-4}M bumetanide

↓ denotes point of addition of 10^{-3}M ouabain or 10^{-3}M ouabain + 10^{-4}M bumetanide

(Mean of triplicate determinations of single experiment)

Figure 52

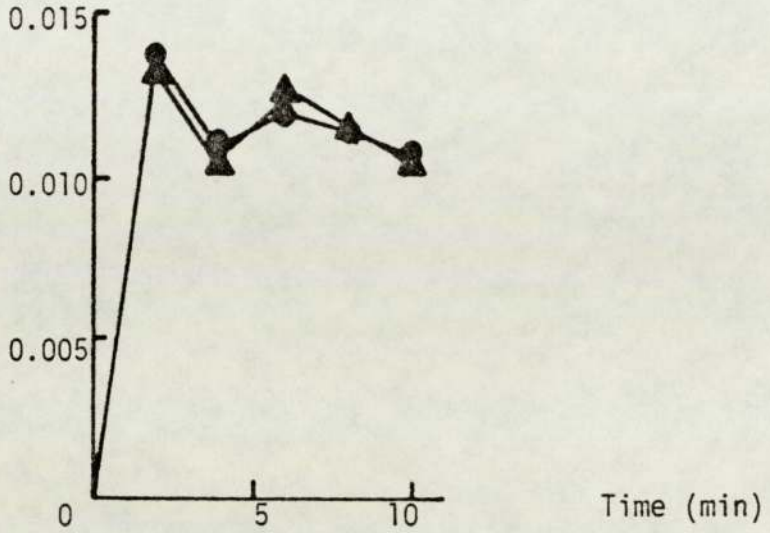


Figure 53

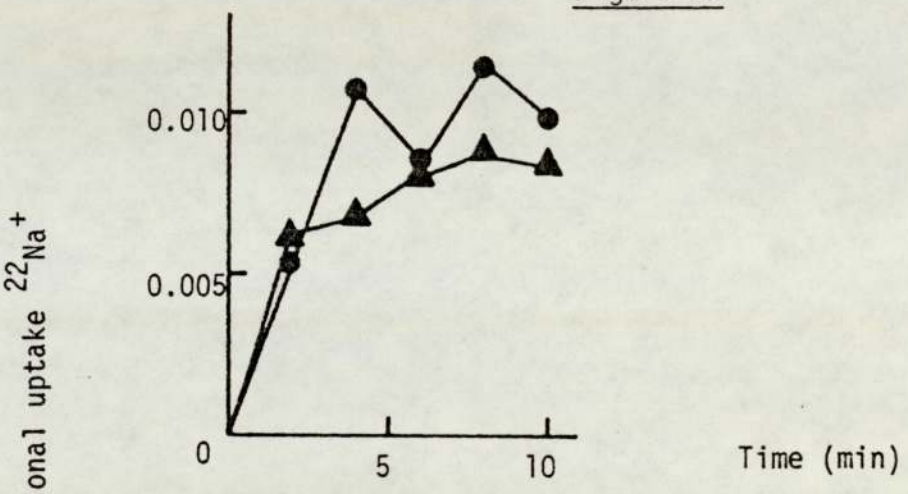


Figure 54

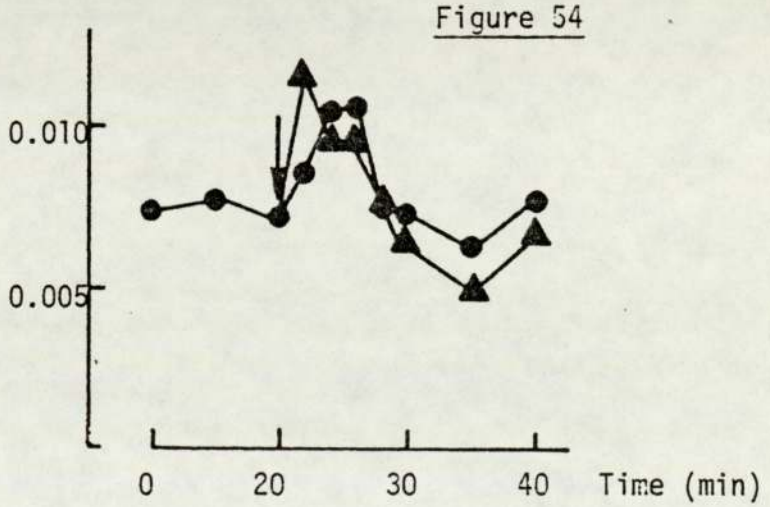


Figure 55

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the initial uptake of $^{22}Na^{+}$ into L1210 cells incubated in the presence of various agents.

Key: 1 control

2 10^{-3}M ouabain

3 10^{-3}M furosemide

4 10^{-4}M bumetanide

5 10^{-4}M amiloride

\+ incubated with 10^{-5}M HN2

\- incubated in absence of HN2

(Mean of triplicate determinations of a single experiment)

Figure 56

The effect of various agents on the uptake of $^{36}Cl^{-}$ into L1210 cells.

Key: ○ control

● 3h 10^{-5}M HN2

▲ 15 min, 10^{-3}M furosemide

△ 15 min, 10^{-4}M DIDS

(Mean of triplicate determinations of a single experiment)

Figure 55

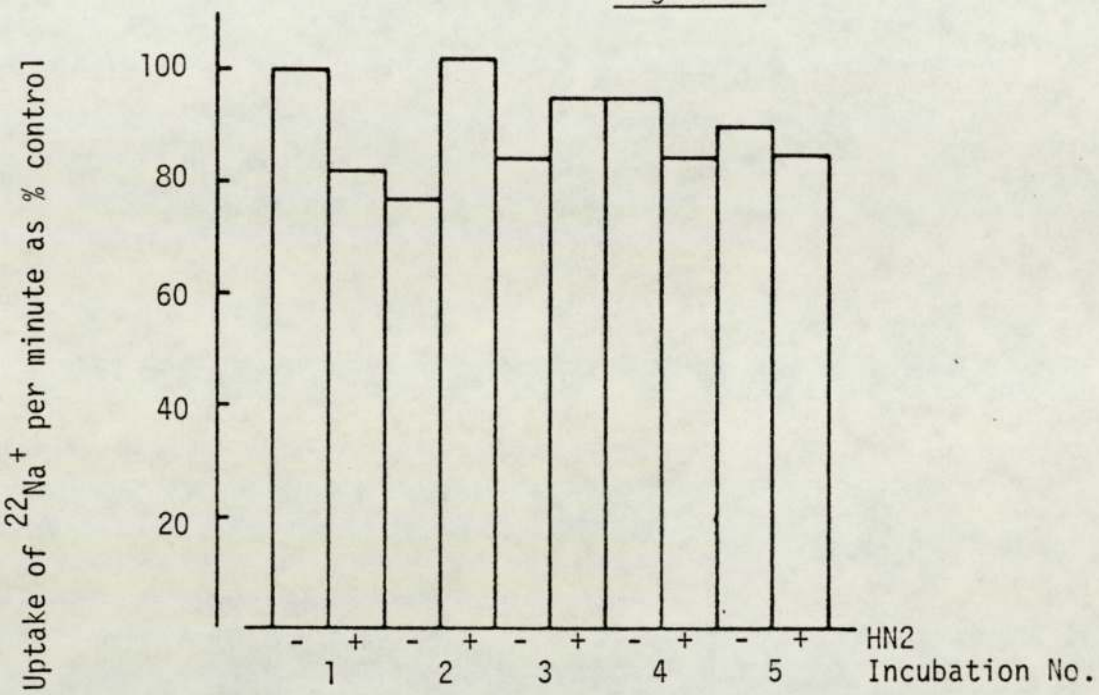
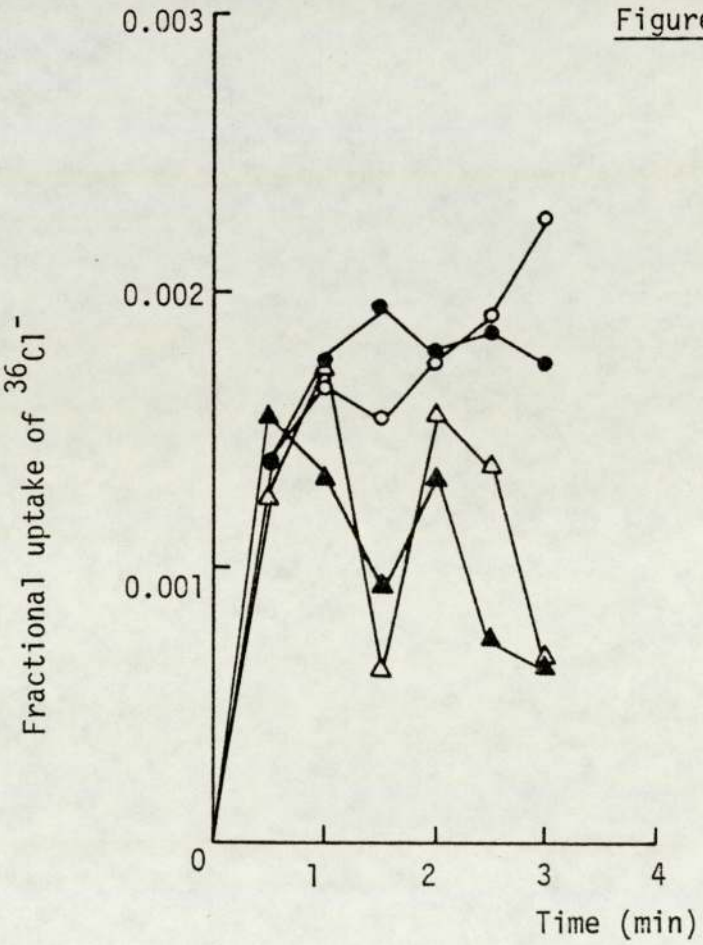


Figure 56



The rationale for the use of a medium free of amino acids, KR buffer, was that Na^+ influx which may occur concomitantly with amino acid uptake (see introduction 1.2.1) would be eliminated. However, no differences were observed in the movement of $^{22}Na^+$ into L1210 cells incubated in KR and RPMI (data not shown); although it was not attempted for the present study, the measurement of $^{22}Na^+$ uptake into L1210 cells incubated for short periods in glucose-free medium may be of use in future experiments, by similarly preventing cotransport of Na^+ and glucose.

In an attempt to slow the attainment of isotopic equilibrium, L1210 cells were incubated for short periods at 20^o. Figure 50 shows the uptake of $^{22}Na^+$ into exponentially-dividing L1210 cells incubated in RPMI at 20^o. The distribution of $^{22}Na^+$ in the cells rapidly reached isotopic equilibrium, and no initial rate of $^{22}Na^+$ accummulation could be determined. A similar result was obtained when the cells were incubated in KR buffer (data not shown) and the presence of $10^{-3}M$ ouabain was without effect upon $^{22}Na^+$ distribution in cells incubated in either medium (data not shown).

Whilst the method for separation of cells from supernatent fractions by centrifugation through an oil barrier offered rapid sampling of isotope distribution, it was not possible to wash the cells free of isotope which was thought to be associated with the glycocalyx, but not transported into the cytoplasm. For this reason, the distribution of $^{22}Na^+$ in L1210 cells was examined by use of ultrafiltration under negative pressure. One disadvantage of this method is that duplicate samples cannot readily be taken at intervals of less than one minute. Several attempts, of which the data in figure 51 are representative, were made, but no constant rate of $^{22}Na^+$ accumulation was detected. Nitrogen mustard was not shown

to have a consistent effect upon $^{22}Na^+$ uptake (figure 51) and 10^{-3}M ouabain was without effect (data not shown).

In order to increase the specific activity of $^{22}Na^+$ & Na^+ inside the cells, and so increase sensitivity, the uptake of $^{22}Na^+$ into L1210 cells incubated in Na^+-free KR was examined. Choline chloride and potassium bicarbonate were substitued for the sodium salts. Uptake was measured in the presence of 10^{-3}M ouabain by the oil-barrier method. Again, a linear rate of $^{22}Na^+$ accumulation was not demonstrated (figure 52).

The experiment was repeated in a modified KR buffer with HEPES substituted for $NaHCO_3$, so that the pH was not dependent upon a gaseous phase. However, no linear rate of $^{22}Na^+$ uptake was obtained; this result eliminated the possibility that the variation in uptake into cells incubated in KR was due to fluctuations in activity of Na^+/H^+ exchange in response to fluctuations of extracellular pH (figure 53).

Figure 55 shows the effect of addition of 10^{-3}M ouabain, furosemide, bumetanide or amiloride on the accumulation of $^{22}Na^+$ by L1210 cells incubated for one minute in Na^+-free KR in the presence of isotope. The cells were preincubated for 3h in RPMI with and without HN2. The majority of the accumulation of $^{22}Na^+$ by control and treated cells was insensitive to these agents; other experiments confirmed that $^{22}Na^+$ influx was largely unaffected by HN2 (figures 48, 49 and 51) or bumetanide (figure 48). These observations suggested that the majority of $^{22}Na^+$ influx occurred by passive movement down the transmembrane Na^+ concentration gradient, independently of specific carrier mechanisms. In an attempt to eliminate the influence of these fluxes upon the results, L1210 cells were incubated with $^{22}Na^+$ for sufficient time (15 minutes) to allow

attainment of isotopic equilibrium. Ouabain (10^{-3}M) was then added, thus preventing the efflux of $^{22}Na^+$ through the action of Na^+K^+ATPase; it would be expected that the cells would begin to accumulate $^{22}Na^+$. If accumulation of $^{22}Na^+$ occurs through the action of the $Na^+K^+Cl^-$ cotransporter, addition of bumetanide should inhibit the process. This approach produces linear rates of $^{22}Na^+$ uptake into Friend erythroleukaemia cells (personal communication, Dr L Cantley); however, a linear rate of $^{22}Na^+$ uptake into L1210 cells was not detected (figure 54).

The accummulation of $^{36}Cl^-$ by L1210 cells was examined by use of the oil barrier method; the influx of $^{36}Cl^-$ was reduced by the diuretic furosemide and by DIDS, the inhibitor of anion exchange. However, it was impossible to quantify the inhibition since initial uptake of $^{36}Cl^-$ in the presence of these agents did not occur at a constant rate. Preincubation for 3h with 10^{-5}M was without effect upon the accummulation of $^{36}Cl^-$. A typical result is shown in figure 56.

It was demonstrated by Geck et al (1980) that Na^+, K^+, and Cl^- ions were transported in a stoichiometric ratio of 1:1:2 into K^+-depleted, Na^+-loaded Ehrlich ascites tumour cells. In an attempt to reproduce this observation, L1210 cells were first preincubated for various times in a modified KR buffer with Na^+ salts replacing K^+ salts. Under these conditions, the cells rapidly became permeable to trypan blue, and influx of $^{86}Rb^+$ & K^+ was barely measurable (data not shown), suggesting that Na^+ loaded L1210 cells had limited viability.

4.5.3 Discussion

The influx of $^{22}Na^+$ into L1210 cells occurred rapidly, indicating that the plasma membrane is readily permeable to Na^+ ions. The accumulation of Na^+ was largely unaffected by inhibitors

of Na^+/H^+ and $Na^+K^+Cl^-$ transporters, suggesting that under steady-state, isosmotic conditions, these processes contribute little to the total movement of Na^+ into L1210 cells. This property would tend to make the processes of carrier-mediated Na^+ transport less amenable to dissection by use of inhibitors, in the manner analogous to that described for pathways of K^+ transport (results 4.2.2). Indeed, many technical problems were encountered in attempts to quantify the rate of $^{22}Na^+$ accumulation, even in the presence of ouabain to inhibit loss of $^{22}Na^+$, and when cells were subjected to manipulation of external ion concentrations.

Other workers have experienced difficulty in their attempts to measure $^{22}Na^+$ fluxes in cells incubated under physiological conditions (Paris and Pouyssegur, 1986; Palfrey and Rao, 1983; Dissing et al, 1985). Measurements of $^{22}Na^+$ fluxes have been made upon cells incubated under non-physiological conditions, such as altered concentrations of internal and external ions (Geck et al, 1980; Haggerty et al, 1985; Vigne et al, 1984; Paris and Pouyssegur, 1984; 1986), or in the presence of digitoxin (Owen and Villereal, 1983) or ouabain (Cassel et al, 1983; Burns and Rozengurt, 1984; Grinstein et al, 1983b; Owen, 1985; Paris and Pouyssegur, 1986), which may perturb intracellular ion concentrations (results 4.7). Attempts to reduce the rate of $^{22}Na^+$ influx by reduction of temperature (see results 4.5.2 above) may also cause changes in intracellular ion concentrations (Boonstra et al, 1984).

The rapid equilibration of the distribution of $^{22}Na^+$ with intracellular and extracellular Na^+ limits the use of this isotope to measure Na^+ movements. Owen and Villereal (1983b) found that in cultured human fibroblasts, the rates of net Na^+ influx and $^{22}Na^+$ uptake differred greatly, suggesting that a large proportion

of $^{22}Na^+$ uptake was due to $^{22}Na^+/Na^+$ exchange. This system is sensitive to phloretin, and does not result in net cation movements in erythrocytes (Haas et al, 1975; Sarkadi et al, 1978); thus phloretin may prove to be of use in eliminating the contribution of $^{22}Na^+/Na^+$ exchange to measurements of $^{22}Na^+$ transport.

The lack of a successful method for the measurement of $^{22}Na^+$ and $^{36}Cl^-$ fluxes into L1210 cells prevented the confirmation that the Na^+ and Cl^- ions required for bumetanide-sensitive $^{86}Rb^+$ influx (results 4.2.3) were cotransported into the cell, and also prevented the determination of the stoichiometry of cotransport.

There is a body of conflicting evidence concerning the stoichiometry of $Na^+K^+Cl^-$ cotransport in different cell types. McManus and Schmidt (1978) suggested that the diuretic-sensitive Na^+K^+ system transported ions in the ratio 1:1 in the avian erythrocyte. Geck et al (1980) found evidence for a fixed stoichiometric ratio of 1:1:2 for $Na^+K^+Cl^-$ cotransport into Ehrlich ascites tumour cells. However, these cells were depleted of K^+ and loaded with Na^+, so both intracellular and extracellular ion concentrations could not be regarded as physiological. In flounder intestinal epithelial cells, the stoichiometry of K^+Cl^- influx was calculated to be 1:2 (Musch et al, 1983); later measurements suggested that Na^+ and K^+ were transported in a ratio of 2:1, and Na^+ and Cl^- were cotransported in the ratio of 1:3:1, suggesting a net transport of $2Na^+ : 1K^+ : 2Cl^-$, although the dependence of $^{86}Rb^+$ influx upon external Na^+ suggested a 1:1:2 ratio (Palfrey and Rao, 1983). Atlan et al (1984) found a variable stoichiometric relationship between Na^+ and $^{86}Rb^+$ ions transported into NIH 3T3 cells, which depended upon the external ion concentration.

It appears that the stoichiometry of the ions for transport differs for unidirectional efflux and influx, and net fluxes. For example, McManus and Schmidt (1978) suggested $1Na^+ : 1K^+$ cotransport into avian erythrocytes, based on measurements of net fluxes over a wide range of external Na^+ and K^+ concentrations, which were not all physiological. In contrast, Kregenow (1977) measured unidirectional fluxes, and found that the two ions became uncoupled as the external ion concentrations were varied, and that the Na:K ratio could vary up to 1:3; thus, these results were not consistent with a fixed stoichiometric relationship between the transported ions. In human erythrocytes, the unidirectional inward furosemide-sensitive Na^+ K^+ cotransport has been shown to proceed with stoichiometry ranging from 0.2:1 (Dunham et al, 1980) to 1:1 (Wiley and Cooper, 1974). Atlan et al (1984) found the unidirectional Na^+K^+ transport into NIH 3T3 cells to proceed with a stoichiometry of 1:1, but the net fluxes did not comply with this model, since no evidence was found for a net Na^+ efflux coupled to net K^+ fluxes at physiological ion concentrations. Both K^+ influx and K^+ efflux were dependent upon Na^+, leading these authors to speculate that inward K^+ movement was accompanied by inward Na^+ movement, but that the outward movement of K^+ required the binding of Na^+ at a cooperative site without transport of the Na^+ ion. External Cl^- was also required, and the complex probably bound two Cl^- ions to maintain electroneutrality, although movement of Cl^- was not investigated (Atlan et al, 1984). Gargus and Slayman (1980) suggested that the variations in the stoichiometry of Na^+K^+ cotransport with varying ion concentration might be explained by the existence of a K^+/K^+ exchange mechanism, sensitive to furosemide and dependent upon external Na^+. However, in the present study, no evidence was found for the presence of

furosemide-sensitive K^+/K^+ exchange, or furosemide-sensitive K^+ efflux in L1210 cells (results 4.4.1, 4.4.2), although the dependence of $^{86}Rb^+$ efflux on extracellular Na^+ was not investigated. Attempts were made to drive the cotransport to mediate net efflux of ions (results 4.4.2), and the rates of efflux of Na^+ and K^+ from L1210 cells were determined (data not shown). The ratio of Na^+ :K^+ appearing in the extracellular medium was found to vary with time, but was not found to be sensitive to bumetanide (data not shown)

In conclusion, the results obtained in the present study do not provide information of the stoichiometry of $Na^+K^+Cl^-$ cotransport into L1210 cells, and do not show whether diuretic-sensitive $^{86}Rb^+$ & K^+ influx is coupled to the movement of Na^+ & K^+ ions.

4.6 THE CYTOTOXICITY OF FUROSEMIDE AND BUMETANIDE TO L1210 CELLS

4.6.1 Introduction

Nitrogen mustard inhibited the diuretic-sensitive uptake of $^{86}Rb^+$ & K^+ into rapidly-dividing L1210 cells; the inhibition of $^{86}Rb^+$ & K^+ transport was concentration-dependent and only cytotoxic concentrations of HN2 were effective (results 4.3.1), suggesting a pharmacological role for inhibition of $Na^+K^+Cl^-$ cotransport in the cytotoxic mechanism of HN2. If inhibition of the $Na^+K^+Cl^-$ cotransporter is a lethal event for the dividing cell, it would be expected that the diuretics furosemide and bumetanide would be inhibitory to cell growth.

The effect of the diuretics upon the growth of L1210 cells in suspension culture and the growth of single cells in soft agar were examined.

4.6.2 Results

Figure 57 shows the effect of continuous exposure to various concentrations of furosemide and bumetanide upon the proliferation of L1210 cells during 72h in suspension culture; for each inhibitor the IC_{50} was approximately $10^{-3}M$.

Figure 58 shows the effect of preincubation for 1h with various concentrations of furosemide and bumetanide upon the formation of colonies from single L1210 cells in soft agar; for each inhibitor the concentration causing 50% reduction of colony formation was approximately $2x10^{-3}M$.

4.6.3 Discussion

The 'loop' diuretics, including bumetanide, furosemide and their analogues, are so named because their primary action is exerted upon the thick ascending loop of Henle in the kidney. The potency with which these compounds induce diuresis in the dog has been shown to

Figure 57

The effect of continuous incubation with furosemide or bumetanide on the proliferation of L1210 cells during 72h *in vitro*.

Key: ● bumetanide (n=5)

□ furosemide (n=4) (Mean ± S.D.)

Figure 58

The effect of preincubation for 1h with furosemide or bumetanide on the formation of L1210 or L1210R cell colonies in soft agar.

Key: L1210 cells

● furosemide (Mean; n=5)

○ bumetanide (Mean; n=4)

L1210R cells

■ furosemide (Mean; n=3)

Figure 57

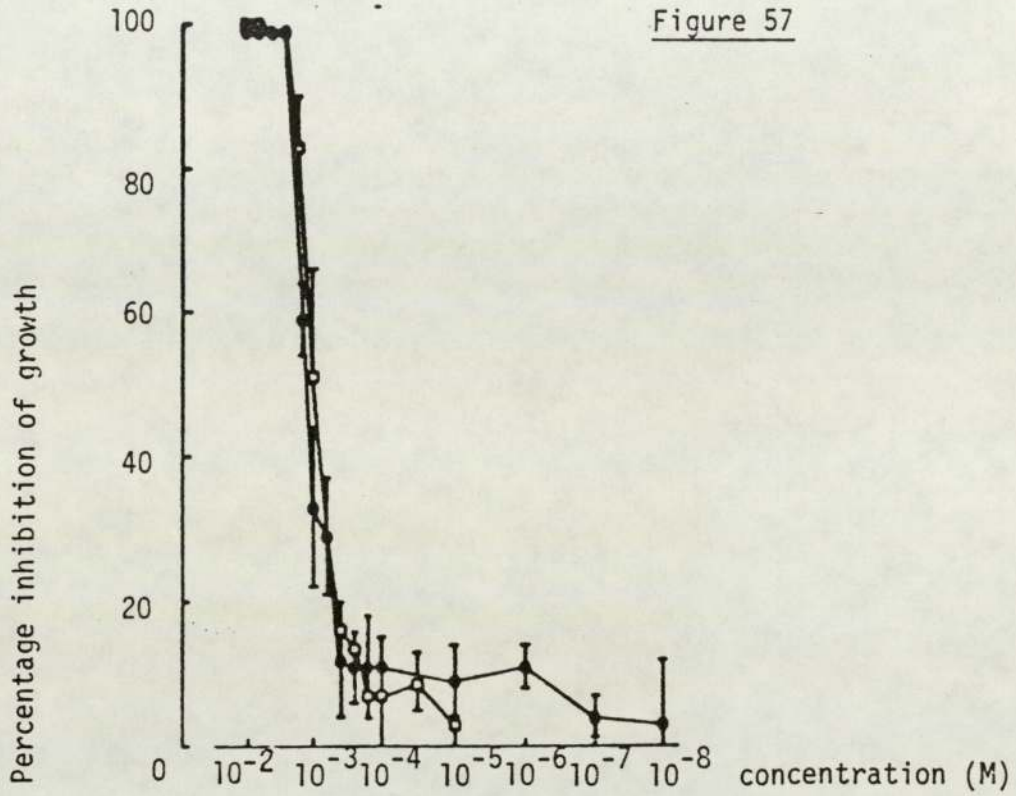
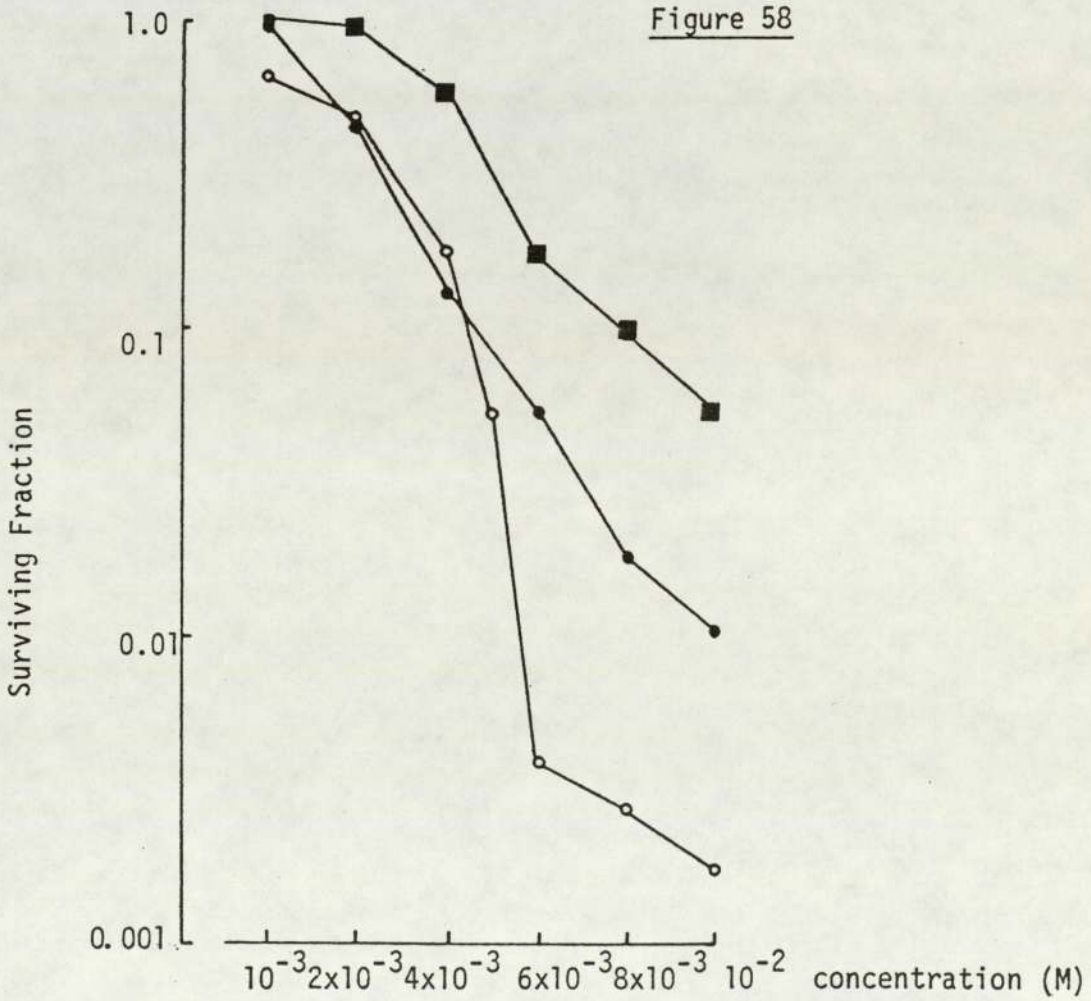


Figure 58



correlate well with their ability to inhibit the cAMP-stimulated Na^+K^+ cotransport system of avian erythrocytes (Palfrey et al, 1980). For example, furosemide at concentrations of 10^{-3}M - 10^{-5}M completely inhibited the stimulation of this system (Palfrey et al, 1980; McManus and Schmidt, 1978), but bumetanide was at least fifty times more effective in producing this inhibition, in producing diuresis in dogs, and in clinical trials (Ostergard et al, 1972). The difference in potency between furosemide and bumetanide appeared to result from differences in their affinity for a specific site (Palfrey et al, 1980).

The diuretics inhibit the function of $Na^+K^+Cl^-$ cotransport by direct interaction with the cotransporter itself. Palfrey et al (1980) suggested that the diuretics did not compete for Na^+ or K^+ binding sites, and the presence of extracellular Na^+ and K^+ were found to promote the inhibition of cotransport by these compounds (Palfrey et al, 1980; Haas and McManus, 1982). Forbush and Palfrey (1983) and Jorgensen et al (1985) confirmed that Na^+ and K^+, and low concentrations of Cl^-, were able to promote the binding of bumetanide to kidney membranes. High concentrations of Cl^- were found to inhibit binding. These observations have lead to the suggestion that the diuretics bind to a complex consisting of the transport protein + $1Na^+$ + $1K^+$ + $1Cl^-$, and compete for binding at the second anion site (Forbush and Palfrey, 1983; Haas and McManus, 1982; 1983). Jorgensen et al (1985) suggested that bumetanide was bound to a protein of 34000 Daltons molecular weight which was associated with the cytoskeleton, which is itself vital in the control of cell proliferation (Folkman and Moscona, 1978). Despite such evidence for the direct associations of the diuretics with the cotransporter itself, it has been reported that the $^{86}Rb^+$ influx into a mouse

macrophage cell line deficient in adenylate cyclase was ten times less sensitive to inhibition by bumetanide, than that of the parent cell, or that of a mutant deficient in cAMP-dependent protein kinase (Bourrit et al, 1985). Further, furosemide has been shown to inhibit a cAMP-dependent protein kinase (Ferguson and Twite, 1974), although the diuretics were found to block the stimulation of $Na^+K^+Cl^-$ cotransport in avian erythrocytes whatever stimulus was applied (Palfrey et al, 1980).

If the activity of the $Na^+K^+Cl^-$cotransporter has an important role in the proliferation of L1210 cells in vitro, it would be expected that the cytotoxicity of the diuretics would reflect their different ability to inhibit the system, their different potency as diuretics and their different affinity for binding to kidney membranes; this difference is not evident in the results of the present study. There was no difference in the potency with which furosemide and bumetanide inhibited the proliferation of L1210 cells in suspension culture. There was no correlation with the inhibition of proliferation and inhibition of $^{86}Rb^+$ influx; 10^{-3}M furosemide was found to produce maximum inhibition of ouabain-resistant $^{86}Rb^+$ & K^+ transport into L1210 cells (data not shown), but inhibited cell proliferation by only 50%. Similarly, 10^{-4}M bumetanide, also found to maximally inhibit ouabain-resistant $^{86}Rb^+$ & K^+ influx (data not shown), inhibited proliferation by only 10%.

Furosemide and bumetanide at concentrations of $1x10^{-3}$ - $4x10^{-3}$M were similarly effective inhibitors of L1210 colony formation (figure 58). At higher concentrations (up to 10^{-2}M), bumetanide was more effective than furosemide in inhibiting colony formation.

Bumetanide appears to be a more specific inhibitor of $Na^+K^+Cl^-$ cotransport than furosemide, which has been shown to affect a number

of other processes, including DIDS-sensitive Cl^- exchange (Brazy and Gunn, 1976; Palfrey et al, 1980), glucose transport (Jung and Mookerjee, 1976), Na^+-dependent glycine uptake (Palfrey et al, 1980), Na^+K^+ATPase (Wiley and Cooper, 1974; Palfrey et al, 1980), and membrane permeability (Walter, 1981). Palfrey et al (1980) found that 10^{-5}M bumetanide completely inhibited Na^+ K^+Cl^- cotransport, but was without effect upon Na^+K^+ATPase, glycine transport, or anion exchange in avian erythrocytes. The different slopes of the plot of survival in soft agar versus concentration (figure 58) would suggest that cytotoxicity of furosemide and bumetanide arise by different mechanisms.

A mutant L1210 cell line, L1210R, was selected for resistance to nitrogen mustard (see results 4.16), and was found to be altered in $Na^+K^+Cl^-$ cotransport function (results 4.16.3). It was also more resistant to the cytotoxicity of furosemide (figure 58). However, the similar slopes of the plots of survival of L1210 and L1210R cells after incubation with furosemide may indicate that toxicity arose by similar mechanisms. This result suggests either that in the mutant L1210R cell line, the function of $Na^+K^+Cl^-$ was absent, and so furosemide was toxic to both L1210 and L1210R cells by an independent mechanism; or, that the cotransporter of L1210R cells was altered in some way which altered the affinity of furosemide for its binding site. In the latter case, this alteration would also have to account for the increased resistance to the cytotoxicity of nitrogen mustard observed in L1210R cells; since HN2 and furosemide appear to inhibit cotransport by different mechanisms, this possibility seems unlikely.

It would be of interest to obtain a more extensive range of analogues of furosemide, and to study their binding affinities to cell membranes, their ability to inhibit ouabain-resistant $^{86}Rb^+$ & K^+

transport, and their cytotoxicity to L1210 and L1210R cells. Such a study would provide insight into the importance of the function of $Na^+K^+Cl^-$ cotransport in cell proliferation, and the nature of the mutation in L1210R cells, which would in turn provide further information regarding the mechanism of action of nitrogen mustard.

The different potency of the diuretics and HN2 in the inhibition of L1210 cell growth is discussed further in section 5.

4.7 THE EFFECT OF NITROGEN MUSTARD ON INTRACELLULAR SODIUM AND POTASSIUM CONCENTRATIONS OF L1210 CELLS

4.7.1 Introduction

Nitrogen mustard inhibited the influx of $^{86}Rb^+$ & K^+ into L1210 cells by means of a selective interference with the activity of a $Na^+K^+Cl^-$ cotransporter (results 4.3.1). It was considered that this interference may cause a reduction of the intracellular content of Na^+ and K^+. Perturbation of the homeostasis of these ions may then have lethal effects upon the cell (Hickman, 1982) and may account for the cytotoxicity of HN2. In order to investigate this possibility, the intracellular contents of Na^+ and K^+ of L1210 cells were measured after incubation with or without 10^{-5}M HN2. It was of interest to perform these measurements on L1210 cells taken from cultures in both the exponential and stationary phases of growth, because of their different $Na^+K^+Cl^-$ cotransport activities (results 4.2.2.3) and the different sensitivities of $^{86}Rb^+$ & K^+ influx to inhibition by HN2 (results 4.3.1; 4.3.2). The effects of the K^+ transport inhibitors, ouabain and furosemide, upon intracellular Na^+ and K^+ concentrations were also examined. The Na^+ and K^+ contents of L1210 cells were each estimated by two methods; firstly, they were measured directly by use of atomic absorption spectrophotometry; secondly, they were inferred from the equilibrium distributions of tracer amounts of radioactive isotopes:- $^{86}Rb^+$ for K^+, and $^{22}Na^+$ for Na^+.

4.7.2 Results

Figure 59 shows the effect of 10^{-5}M HN2 and 10^{-3}M ouabain on the equilibrium distribution of $^{86}Rb^+$ in L1210 cells from exponentially-growing cultures, incubated in KR buffer. The $^{86}Rb^+$ present in the cells is expressed as a fraction of total $^{86}Rb^+$ in cells and supernatent fractions. Both HN2 and ouabain reduced $^{86}Rb^+$, and

therefore K^+ (results 4.2.2), content of L1210 cells. In neither case was this reduction accompanied by a significant increase in the number of cells permeable to trypan blue (tables 5 and 8). The potassium content of L1210 cells was estimated from the equilibrium distribution of $^{86}Rb^+$. It was assumed that the radioactivity of $^{86}Rb^+$ was proportional to the known K^+ content of the external medium, and the unknown K^+ content of the cells was then calculated from their $^{86}Rb^+$ activity (results 4.2.1). The intracellular K^+ concentration, $[K]_i$, was calculated by reference to the estimate of total cell water. The effect of 10^{-5}M HN2 on $[K^+]_i$ of L1210 cells from exponentially-growing cultures incubated in KR buffer is shown in table 9. During incubation, $[K^+]_i$ decreased significantly with time in the presence ($p<0.01$) and absence ($p<0.01$) of HN2, but after 3h the $[K^+]_i$ of treated and untreated L1210 cells were not significantly different ($P>0.5$) from each other. No significant depletion of $[K^+]_i$ of L1210 cells was observed during incubation in RPMI in the absence ($p>0.05$) or presence of HN2 ($p>0.05$; table 10). After incubation for 3h, the $[K^+]_i$ of L1210 cells incubated with or without 10^{-5}M HN2 was not significantly different ($p>0.5$). Further experiments to measure $[K^+]_i$ and $[Na^+]_i$ were carried out using RPMI as the incubation medium.

Although incubation of L1210 cells with 10^{-5}M HN2 reduced the K^+ content (figure 63) in comparison with initial control values, it did not significantly ($p>0.05$) reduce $[K]_i$ in comparision with controls after 3h (table 10); $[K^+]_i$ was maintained by a reduction of cell water content (tables 17 and 19; results 4.8). Measurement of $[K^+]_i$ by atomic absorption spectrophotometry confirmed that incubation with 10^{-5}M HN2 did not reduce $[K^+]_i$ (table 11); in fact, according to these measurements, the $[K^+]_i$ of L1210 cells was increased by

comparison with initial values during incubation for 3h in the absence ($p<0.01$) or presence ($p\pm0.05$) of HN2 (table 10). At the 3h time point, the $[K^+]_i$ of cells incubated with and without HN2 was not significantly different ($p>0.1$). The $[K^+]_i$ of L1210 cells from exponentially-growing cultures was reduced in a time-dependent manner during incubation with 10^{-3}M ouabain, but not with 10^{-3}M furosemide (table 10); the $[K^+]_i$ of L1210 cells incubated for 3h with 10^{-3}M furosemide was not significantly different from the $[K^+]_i$ of cells at the start of incubation ($p>0.1$) or that of untreated cells after the same period of incubation ($p>0.5$).

Nitrogen mustard had no significant effect on $[K^+]_i$ of L1210 cells from stationary cultures (tables 12 and 13). Incubation with 10^{-3}M furosemide also had no effect of $[K^+]_i$ of these cells (table 12). Incubation with 10^{-3}M ouabain reduced $[K^+]_i$ in comparision with that of controls, although to a lesser extent than in L1210 cells from exponentially-growing cultures (compare tables 12 and 10).

The intracellular Na^+ concentration, $[Na^+]_i$, of L1210 cells from exponentially-growing cultures was not significantly different ($p>0.1$, table 14 and $p>0.05$ table 15) after incubation for 3h with or without 10^{-5}M HN2. When $[Na^+]_i$ was measured by distribution of $^{22}Na^+$, the results suggested that incubation for 3h with 10^{-5}M HN2 elevated $[Na^+]_i$ in comparison to the initial $[Na^+]_i$ ($p<0.05$; table 14). However, there was no difference between $[Na^+]_i$ of control and treated cells at the 3h time point (see above, table 14). In addition, the $[Na^+]_i$ measured by atomic absorption spectrophotometry, was not significantly different for L1210 cells at the start of incubation and HN2-treated L1210 cells after incubation for 3h (table 15). It was concluded that the apparently significant elevation of $[Na^+]_i$ during incubation for 3h with HN2 in comparison with initial

$[Na^+]_i$ (table 14) was probably due to the large standard deviations of the results. Incubation for 3h with 10^{-5}M HN2 had no significant effect on the $[Na^+]_i$ of L1210 cells from stationary cultures, in comparison to controls either at the same time point ($p>0.5$) or at the start of incubation ($p>0.1$; table 16).

Table 8

The effect of 10^{-3}M ouabain on the exclusion of trypan blue by L1210 cells incubated in KR

Time	Percentage of cells impermeable to trypan blue	
h	Control	10^{-3}M ouabain
0.5	93 ± 2	97 ± 0
1.0	93 ± 4	93 ± 1
2.0	89 ± 4	91 ± 2
2.5	90 ± 4	90 ± 5
3.0	86 ± 1	85 ± 5
3.5	83 ± 5	77 ± 4
4.0	77 ± 4	68 ± 5[a]

Mean ± S.D., n=3

[a] p value for comparison with control at 4h : $p>0.05$.

Table 9

The effect of 10^{-5}M HN2 on $[K^+]_i$ of exponentially-growing L1210 cells incubated in KR

Time	Potassium concentration, mM	
h	control	10^{-5}M HN2
0	135 ± 17	146 ± 17
1	83 ± 15	95 ± 16
2	86 ± 9	69 ± 5
3	65 ± 20[a]	71 ± 10 [b,c]

Mean ± S.D.; n-3

[a] p value for comparison with control at t=0 : $p<0.01$

[b] p value for comparison with control at t=0 : $p<0.01$

[c] p value for comparison with control at t=3 : $p>0.5$

Table 10

The effect of 10^{-5}M HN2 on $[K^+]_i$ of exponentially-growing L1210 cells incubated in RPMI, measured by equilibrium distribution of $^{86}Rb^+$

Time	Potassium Concentration, MM			
h	**control**	**10^{-5}m HN2**	**10^{-3} M FUR**	**10^{-3}M OUA**
0		144 ± 23		
1	150 ± 13	150 ± 12	128 ± 14	58 ± 4
2	120 ± 3	143 ± 14	128 ± 4	45 ± 13
3	118 ± 19[1,a]	113 ± 12[1,b]	127 ± 13[c,d]	22 ± 2

Mean ± S.D.; n=3, except [1]n=9.

FUR = furosemide

OUA = ouabain

[a]p value for comparison with control at t=0 : p>0.05

[b]p value for comparison with control at t=0 : p>0.05

[c]p value for comparison with control at t=3 : p>0.1

[d]p value for comparison with control at t=3 : p>0.5

Table 11

The effect of 10^{-5}M HN2 on $[K^+]_i$ of exponentially-growing L1210 cells incubated in RPMI (measured by atomic absorption spectrophotometry)

Time	Potassium concentration, mM	
h	control	10^{-5}M HN2
0	138 ± 22[1]	
1	134 ± 16	155 ± 20
2	159 ± 17	123 ± 20
3	174 ± 15[2,a]	163 ± 24[2,b,c]

Mean ± S.D.; n=3 except [1]n=12, [2]n=6

[a]p value for comparison with control at t=0 : $p<0.01$

[b]p value for comparison with control at t=0 : $p=0.05$

[c]p value for comparison with control at t=3 : $p>0.1$

Table 12

The effect of 10^{-5}M HN2 on $[K^+]_i$ of L1210 cells from stationary cultures, incubated in RPMI (measured by equilibrium distribution of $^{86}Rb^+$)

Time	Potassium concentration, mM			
h	control	10^{-5}M HN2	10^{-3}M FUR	10^{-3}M OUA
0	137 ± 14[1]			
1	134 ± 19	139 ± 25	165 ± 16	98 ± 16
2	211 ± 10	218 ± 18	202 ± 28	80 ± 14
3	120 ± 10	138 ± 10	128 ± 15	80 ± 15

Mean ± S.D.; n=6 except [1]n=8

Table 13

The effect of 10^{-5}M HN2 on $[K^+]_i$ of L1210 cells from stationary cultures, incubated in RPMI (measured by atomic absorption spectrophotometry).

Time	Potassium concentration, mM	
h	control	10^{-5}M HN2
0	183 ± 19[1]	
1	192 ± 18	144 ± 10
2	135 ± 15	121 ± 14
3	171 ± 16	169 ± 4

Mean ± S.D.; n=3, except [1]n=6.

Table 14

The effect of 10^{-5}M HN2 on $[Na^+]_i$ of exponentially-growing L1210 cells incubated in RPMI (measured by equilibrium distribution of $^{22}Na^+$)

Time	Sodium concentration, mM	
h	control	10^{-5}M HN2
0	30 ± 9[1]	
1	25 ± 8	37 ± 20
3	38 ± 18	56 ± 22[a,b]

Mean ± S.D.; n=6, except [1]n=17.

[a]p value for comparison with control at t=0 : $p<0.05$

[b]p value for comparison with control at t=3 : $p>0.1$

Table 15

The effect of 10^{-5}M HN2 on $[Na^+]_i$ of exponentially-growing L1210 cells incubated in RPMI (measured by atomic absorption sepctrophotometry)

Time	Sodium concentration, mM	
h	control	10^{-5}M HN2
0	27 ± 10[1]	
1	34 ± 17	34 ± 15
3	34 ± 18	41 ± 17 [a,b]

Mean ± S.D.; n=6 except [1]n=12

[a]p value for comparison with control at t=0 : p>0.05

[b]p value for comparison with control at t=3 : p>0.5

Table 16

The effect of 10^{-5}M HN2 on $[Na^+]_i$ of L1210 cells from stationary cultures, incubated in RPMI (measured by atomic absorption spectrophotometry)

Time	Sodium concentration, mM	
h	control	10^{-5}M HN2
0	59 ± 20	
1	50 ± 15	57 ± 13
3	44 ± 23	45 ± 24

Mean ± S.D.; n=6

Figure 59

The effect of preincubation with 10^{-5}M nitrogen mustard or 10^{-3}M ouabain on the fraction of total $^{86}Rb^{+}$ loaded into L1210 cells.

(Mean + S.D.; n=3)

Figure 60

The size distribution of 10^{4} L1210 cells incubated for 3h with or without 10^{-5}M nitrogen mustard.

A typical result.

Figure 61

The size distribution of L1210 cells after various periods in vitro.

A typical result.

Figure 59

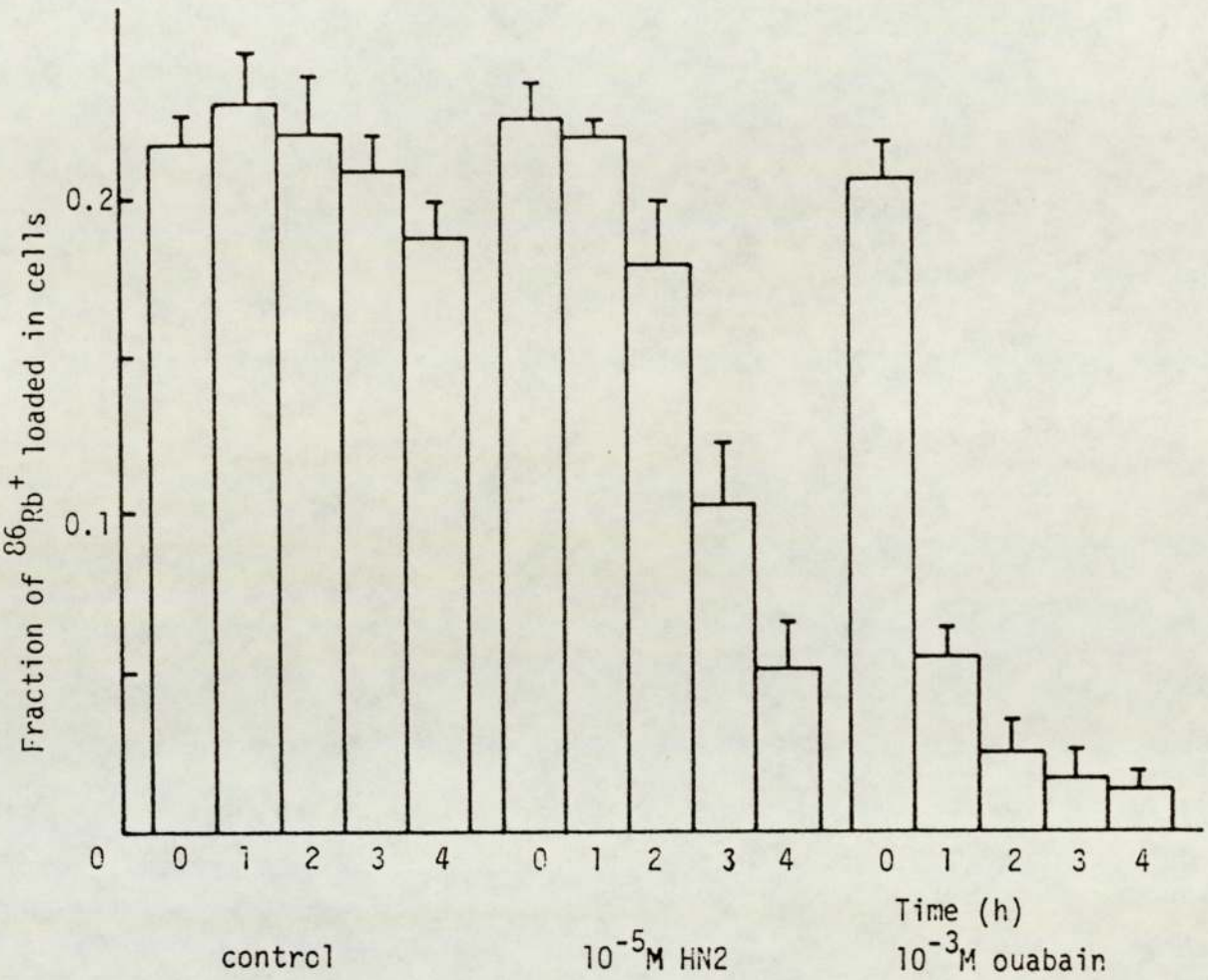


Figure 60

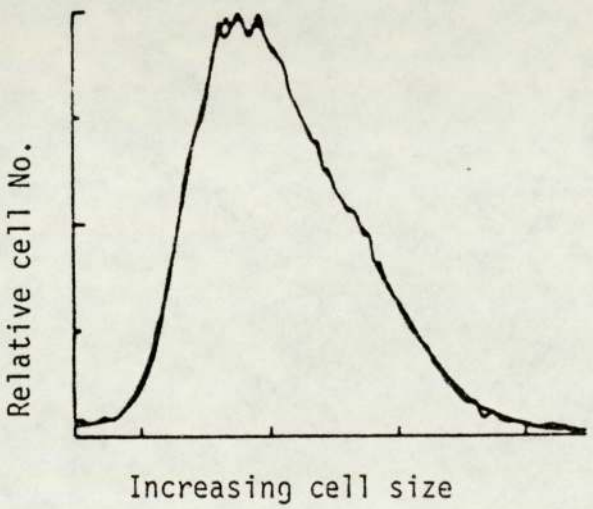
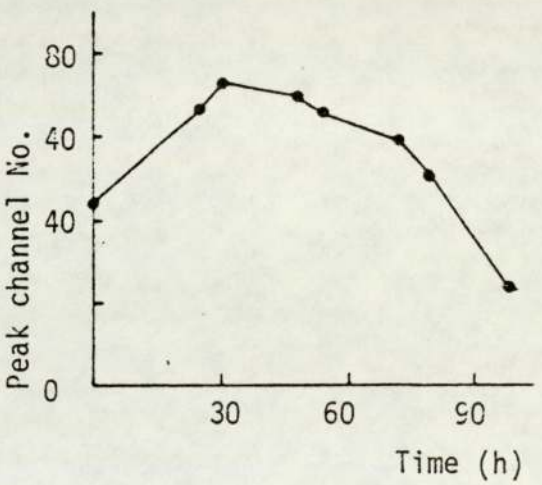


Figure 61



4.7.3 Discussion

Both the isotopic equilibriation (methods 3.8.1 and 3.8.2) and spectrophotometric (methods 3.8.3) techniques for the measurement of $[Na^+]_i$ and $[K^+]_i$ produced results with high standard deviations. Large variations in results obtained by spectrophotometry have been reported before (Smith and Robinson, 1981), and a large variation in measurement of Na^+ concentration was to be expected, given the variation in the results of measurement of $^{22}Na^+$ influx (results 4.6).

The reduction in cellular K^+ concentration in control L1210 cells incubated in KR buffer may explain the more limited survival of cells in this buffer in comparison with RPMI (results 4.1.3.3; table 5). Further experiments to measure $[Na^+]_i$ and $[K^+]_i$ were therefore performed with L1210 cells incubated in RPMI.

L1210 cells from exponentialy-growing cultures had $[K^+]_i$ values of 144 and 138 mM, measured by isotopic and spectrophotometric methods (3.8.1 and 3.8.3) respectively. These values are larger than the value of 117mM for the $[K^+]_i$ of L1210 cells (Hickman et al, 1984), which was obtained by a similar spectrophotometric technique (Smith and Robinson, 1981). A survey of intracellular K^+ concentrations (determined by atomic absorption spectrophotometry) of normal, tumour and transformed cells revealed a range of $[K^+]_i$ values of 95 - 169mM (Moyer et al, 1982). The intracellular K^+ concentrations of exponentially-growing and stationary phase L1210 cells were similar ; this finding conflicts with evidence reviewed by Boonstra et al (1982) that $[K^+]_i$ decreases as confluence is attained. It is also at variance with the proposal of Rozengurt (1980, 1981) that low cellular K^+ concentrations are limiting for the progression of cells through the cell cycle. Thus, the mean $[K^+]_i$ of

the population L1210 cells may be maintained at a constant value during growth in culture, despite variations in the rates of $^{86}Rb^+$ & K^+ transport. Total $^{86}Rb^+$ & K^+ flux into 10^6 exponentially-growing cells was 1.99 ± 0.22 nmol min $^{-1}$, in comparison to 0.88±0.13 nmol min^{-1} in stationary phase cells; exponentially-growing cells exhibited greater activity of Na^+K^+ATPase and $Na^+K^+Cl^-$ cotransport and less passive K^+ diffusion than stationary cells (results 4.2.2.3). These observations are consistent with other reports (reviewed by Boonstra et al, 1982; see also introduction 1.2.2) of changes in K^+ fluxes during the cell cycle of several cell types. Other experiments revealed differences in the rates of $^{86}Rb^+$ efflux from exponentially-growing and stationary cells (results 4.3.3). Clearly, the mechanisms by which the cell maintains K^+ homeostasis may vary throughout the cell cycle.

There is some evidence to suggest that a reduction of $[K^+]_i$ leads to inhibition of cell proliferation and may inhibit DNA synthesis (Boonstra et al, 1982; introduction 1.2.2). Nitrogen mustard inhibited the $^{86}Rb^+$ & K^+ influx into L1210 (results 4.3), PC6A (results 4.1.1) K562 (results 4.15), and Ehrlich ascites cells (Grunicke et al, 1985), and inhibits DNA synthesis in a variety of cell types (introduction 1.1.4.2). It was therefore important to determine the effect of HN2 on $[K^+]_i$.

The $^{86}Rb^+$ influx into exponentially-growing L1210 cells is more sensitive to inhibition by HN2 or the diuretics (results 4.3.1 and 4.2.2.3), than the $^{86}Rb^+$ influx into stationary cells; however, neither HN2 nor furosemide reduced the $[K^+]_i$ of either exponentially-growing or stationary cells.

The inhibition of $^{86}Rb^+$ & K^+ influx by HN2 or furosemide was accompanied by a reduction of cell volume (results 4.8) so the

intracellular K^+ concentration was maintained. Aiton et al (1981) showed that intracellular cation concentrations of HeLa cells and MDCK cells were unchanged after incubation for 12h with 10^{-4}M furosemide, although furosemide caused substantial inhibition of $^{86}Rb^+$ influx. However, Duhm and Gobel (1984) noticed that the effects of furosemide on ion contents and volume of erythrocytes tended to disappear at extracellular K^+ concentrations of above 5mM; for the present study, experiments to examine ion content and cell volume were not performed using extracellular K^+ concentrations of less than 6mM.

Grunicke et al (1985) and Doppler et al (1985) demonstrated that HN2 inhibited $^{86}Rb^+$ influx into Ehrlich ascites cells, but they reported no change in cell volume. They did not speculate on the consequences of this inhibition on K^+ homeostasis or on the maintenance of cell volume (see discussion, section 5).

Ouabain, in contrast to furosemide, caused marked reduction of $[K^+]_i$ of L1210 cells; this observation is in agreement with the results of other workers (Lubin, 1967; 1980; Lamb and McCall, 1972; Ledbetter and Lubin, 1977; Kaplan, 1978). The $[K^+]_i$ of exponentially-growing L1210 cells was reduced to a greater extent by ouabain, consistent with the increased activity of the Na^+K^+ATPase in comparison with stationary cells (results 4.2.2.3). Incubation of L1210 cells with ouabain caused an increase in cell volume (results 4.8) which thereby exacerbated the reduction in K^+ content brought about by the inhibition of $^{86}Rb^+$ & K^+ influx.

The different responses of L1210 cell volume and $[K^+]_i$ to ouabain and furosemide are consistent with the operation of two distinct Na^+-K^+ transporters, which were previously identified by the pharmacological separation of components of $^{86}Rb^+$ & K^+ flux (results

4.2.2.3). The effects of HN2 on $[K^+]_i$ and cell volume provide further evidence that the $Na^+K^+Cl^-$ cotransporter, and not the Na^+K^+ATPase, is the locus for the inhibition of $^{86}Rb^+$ influx in L1210 cells.

From the predictions of Cone (1971) it might be expected that $[Na^+]_i$ of exponentially-growing cells would be higher than that of stationary cells; the results obtained here suggest that the converse is true.

The maintenance of a Na^+ gradient (outside > inside) across the cell membrane provides the driving potential energy for several membrane transport processes (introduction 1.2.1). Nitrogen mustard inhibited the Na^+-dependent transport of $^{86}Rb^+$ into L1210 cells (results 4.3.1) which was shown to be sensitive to the diuretics (results 4.2.3). It may be assumed, then, that it also inhibited Na^+ transport. However, experimental confirmation of this hypothesis proved to be difficult (results 4.5). Other results showed that HN2 interfered with amino acid transport into PC6A (results 4.11) and L1210 cells (results 4.14) in a manner which suggested that the Na^+ gradient was reduced. It was therefore of great interest to measure directly the intracellular Na^+ concentration of L1210 cells incubated with HN2.

Nitrogen mustard had no effect upon the intracellular Na^+ concentration of L1210 cells; any loss of cellular Na^+ due to the inhibition of the $Na^+K^+Cl^-$ cotransporter was offset by a reduction of cell volume (results 4.8). Alternatively, any tendency of the decrease in volume to elevate $[Na^+]_i$ may have been accompanied by Na^+ efflux through some other mechanism. The latter possibility seems unlikely for the following reasons: firstly, there was no stimulation of ouabain-sensitive $^{86}Rb^+$ influx after incubation with HN2 (results

4.3.1.2), and therefore no increased efflux of Na^+ via the activity of the Na^+K^+ATPase; secondly, the Na^+/Ca^{++} exchanger, which is probably electrogenic (introduction 1.2.1), did not appear to be activated to extrude Na^+ since HN2 had no effect on L1210 cell membrane potential (results 4.10) or total cellular calcium concentration (results 4.9). Lastly, Na^+/H^+ exchange did not appear to be activated, since there was no change in intracellular pH_i of L1210 cells after incubation with HN2, and the possibility that such action subsequently activated the anion exchanger (Cl^-/HCO_3^-) to maintain pH was considered unlikely (results 4.11).

In conclusion, no evidence was found to suggest that HN2 dissipated the Na^+ gradient across the plasma membrane of L1210 cells. The inhibition of Na^+-dependent amino acid transport into L1210 cells after incubation with HN2 (results 4.14) may arise from a direct interaction of the drug with the transport proteins; this possibility is discussed in detail in later sections (results 4.14.1.2.3; discussion, 5). From the evidence presented here it seems unlikely that toxicity of HN2 to L1210 cells arises from perturbation of intracellular Na^+ or K^+ concentrations.

4.8 THE EFFECT OF NITROGEN MUSTARD ON L1210 CELL VOLUME

4.8.1 Introduction

Anion-coupled cation transport systems have been implicated in the mechanisms by which cell volume is maintained and regulated (Geck and Pfeiffer, 1985; MacKnight and Leaf, 1985). Nitrogen mustard interfered with the function of a $Na^+K^+Cl^-$ cotransport system in L1210 cells *in vitro* (results 4.3). It was important to investigate the effect of this action of HN2 upon the steady-state volume of L1210 cells. Cell volume was estimated by the separation of cells from supernatant by use of the oil barrier technique (methods 3.4.1); $[^3H]$-H_2O and $[^{14}C]$-inulin carboxylic acid were used as markers of the total and extracellular water respectively (methods 3.11). Total cell water was calculated by subtraction and assumed to represent cell volume.

L1210 cell volume was estimated after incubation with HN2, HN2-1 or potassium transport inhibitors. Experiments were performed with L1210 cells taken from cultures in both exponential and stationary phases of growth, since these cells differred in their $Na^+K^+Cl^-$ cotransport activity (results 4.2.2.3) and in the sensitivity of their $^{86}Rb^+$ influxes to interference by HN2 (results 4.3.1; 4.3.2).

4.8.2 Results

Table 17 shows the effect of 10^{-5}M HN2 on the volume of 10^6 exponentially-growing L1210 cells incubated in Krebs-Ringer buffer. Cell volume is expressed as microlitres of water per 10^6 cells; the volume of HN2-treated L1210 cells is also expressed as the percentage of the volume of control cells at the same time point for each experiment. The mean volume of L1210 cells after incubation for 3h with 10^{-5}M HN2 was 73 ± 13% of control volume. A similar reduction in cell volume was observed when L1210 cells were incubated in RPMI with 10^{-5}M HN2 (table 19).

Both furosemide and bumetanide at concentrations which inhibited $^{86}Rb^{+}$ and K^{+} transport (results 4.2.2) reduced the volume of exponential phase L1210 cells (table 17); cells incubated with these agents or with 10^{-5}M HN2 all underwent an initial reduction in cell volume followed by a partial recovery of volume and finally by a second reduction.

L1210 cells from stationary cultures were smaller than those from exponentially-growing cultures (table 19; compare tables 17 and 18). Stationary phase cell volume was reduced to a lesser extent by HN2 or furosemide (table 18) than exponentially-growing cell volume (compare tables 17 and 18). Ouabain caused a more pronounced swelling of stationary phase cells (table 18) in comparison to exponentially-growing cells (compare tables 17 and 18).

The change in L1210 cell volume after incubation with 10^{-5}M HN2 was not detected by use of the Coulter Channelyzer (figure 60), although changes in the size distributions of cells during growth in culture were apparent from this method; figure 61 shows the number of the channel of the Channelyzer which contained the peak number of cells when aliquots of cells were counted after various periods of culture.

The distributions of the intensity of light scattered by individual cells, as detected by flow cytofluorimetric techniques, were shown to differ between L1210 cells taken from exponential and stationary cultures, and between L1210 cells incubated with and without 10^{-5}M HN2 (results 4.1.3.4; figure 11). Stationary phase L1210 cells scattered less light than exponentially-growing L1210 cells, and HN2-treated cells scattered less light than controls.

Table 17

The volume of 10^6 exponential-phase L1210 cells during incubation in KR buffer with HN2, HN2-1, or inhibitors of K^+ transport (measured by distribution of [^{3}H]-H_2O and [^{14}C]-inulin carboxylic acid)

Incubation	Time, h	Volume of 10^6 cells, μl	% control
Control	0	0.905 ± 0.108	
	0.5	1.007 ± 0.165	
	1.0	0.810 ± 0.144	100
	2.0	0.757 ± 0.102	
	3.0	0.883 ± 0.131	
HN2, 10^{-5}M	0.5	0.670 ± 0.149	66 ± 15
	1.0	0.652 ± 0.073	81 ± 9
	2.0	0.501 ± 0.136	64 ± 18
	3.0	0.630 ± 0.174	73 ± 13
HN2-1, 10^{-4}M	0.5	0.930 ± 0.168	93 ± 17
	1.0	0.945 ± 0.109	117 ± 7
	2.0	0.767 ± 0.105	108 ± 19
	3.0	0.855 ± 0.197	100 ± 13
Furosemide, 10^{-3}M	0.5	0.811 ± 0.198	77 ± 10
	1.0	0.711 ± 0.112	81 ± 12
	2.0	0.704 ± 0.048	91 ± 6
	3.0	0.605 ± 0.135	67 ± 23
Bumetanide, 10^{-4}M	0.5	0.583 ± 0.175	59 ± 18
	1.0	0.718 ± 0.162	88 ± 3
	2.0	0.735 ± 0.197	96 ± 8
	3.0	0.635 ± 0.196	71 ± 9

Table 17 (continued)

Incubation	Time, h	Volume of 10^6 cells, μl	% control
Ouabain, $10^{-3}M$	0.5	0.961 ± 0.095	98 ± 12
	1.0	0.838 ± 0.170	120 ± 13
	2.0	0.913 ± 0.162	122 ± 18
	3.0	0.952 ± 0.174	125 ± 17

Mean ± S.D.; n = 6

Table 18

The volume of 10^6 L1210 cells from stationary cultures during incubation in KR buffer with HN2 or inhibitors of K^+ transport (measured by distribution of $[^3H]$-H_2O and $[^{14}C]$-inulin carboxylic acid).

Incubation	Time, h	Volume of 10^6 cells, µl	% control
Control	0	0.707 ± 0.177	
	1.0	0.700 ± 0.183	
	2.0	0.646 ± 0.146	100
	3.0	0.697 ± 0.118	
HN2, 10^{-5}M	1.0	0.710 ± 0.130	104 ± 18
	2.0	0.679 ± 0.140	96 ± 10
	3.0	0.627 ± 0.150	93 ± 15
Furosemide, 10^{-3}M	1.0	0.649 ± 0.125	94 ± 2
	2.0	0.602 ± 0.094	96 ± 12
	3.0	0.683 ± 0.076	87 ± 8
Ouabain, 10^{-3}M	1.0	0.896 ± 0.016	99 ± 15
	2.0	0.961 ± 0.136	152 ± 16
	3.0	1.160 ± 0.118	162 ± 13

Mean ± S.D; n = 5

Table 19

The volume of 10^6 L1210 cells incubated in RPMI with 10^{-5}M HN2 (measured by distribution of $[^3H]$-H_2O and $[^{14}C]$-inulin carboxylic acid)

Incubation	Time, h	Volume of 10^6 cells, µl	% control
Exponential phase			
L1210			
Control	0	0.812 ± 0.197	
Control	3	0.988 ± 0.152	
10^{-5}M HN2	3	0.752 ± 0.139	75 ± 12 (n = 32)
Stationary phase			
L1210			
Control	0	0.622 ± 0.155	
Control	3	0.625 ± 0.153	
10^{-5}M HN2	3	0.632 ± 0.129	104 ± 25 (n = 5)

Mean ± S.D.

4.8.3 Discussion

The changes in L1210 cell volume during incubation with ouabain or the diuretics are consistent with the pharmacologically distinct actions of these agents on two separate Na^+ & K^+ transport systems. Geck et al (1980) proposed that the antagonistic functions of the membrane Na^+K^+ATPase and the $Na^+K^+Cl^-$ cotransporter may be involved in the regulation of the volume of Ehrlich ascites cells; this concept may explain the drug-induced changes in the volume of L1210 cells, on condition that the reversible $Na^+K^+Cl^-$ cotransporter (introduction 1.2.1) mediates a net influx of ions. The results of experiments to examine $^{86}Rb^+$ transport across the membrane of L1210 cells incubated in KR buffer (external K^+ concentration 6.17 mM) suggested that there was no $^{86}Rb^+$ or K^+ efflux through the cotransporter under these conditions (results 4.3).

If the cotransporter operates exclusively in the influx direction, the next effect of each "cycle" of action will be the influx of 4 ions into the cell (Geck et al 1980; introduction 1.2.1) Water passes into the cell to maintain osmolarity. In contrast, the action of the Na^+K^+ATPase results in the net extrusion of 1 ion per "cycle" (introduction 1.2.1) so water passes out of the cell. Therefore the steady-state volume. (i.e. in isotonic medium.), of Ehrlich ascites cells and possibly that of L1210 cells may depend upon the antagonistic nature of these processes. The results of the present study support this possibility. Inhibition of the Na^+K^+ATPase by ouabain caused an increase in cell volume because of the continued action of the $Na^+K^+Cl^-$ cotransporter. Conversely, inhibition of the $Na^+K^+Cl^-$ cotransporter by furosemide or bumetanide caused a decrease in cell volume, because of the continued action of the Na^+K^+ATPase. The observation that HN2 produced a decrease in L1210 cell volume

provided further evidence that the $Na^+K^+Cl^-$ cotransporter, rather than the Na^+K^+ATPase, was the locus for the inhibition of $^{86}Rb^+$ uptake by HN2 (results, 4.3). However, Geck et al (1980) examined cotransport into Ehrlich ascites cells which had been preincubated in K^+-depleted medium. Under physiological conditions, in K^+ replete medium, Tupper (1975) and Bakker-Grunwald (1978) found no net flux through the cotransporter of Ehrlich ascites cells. Therefore the model of Geck et al (1980) may be over-simplified.

The progressive inhibition of $^{86}Rb^+$ influx into L1210 cells during incubation with HN2 (results 4.3.1.1) did not proceed at the same rate as the changes in cell volume; rather, the cell volume initially decreased, then partially recovered, suggesting that other volume-regulatory fluxes had been activated. Aiton and Simmons (1983) reported that inhibition of Na^+K^+ATPase may increase cotransporter activity; it may be that these two processes act in a compensatory manner in L1210 cells. The possible nature of this and other compensatory fluxes is further discussed below.

Furosemide reduced the volume of stationary phase L1210 cells to a lesser extent than that of exponential L1210 cells; this observation is consistent with the reduced activities of the $Na^+K^+Cl^-$ cotransporter and Na^+K^+ATPase in stationary phase L1210 cells in comparison with exponentially-growing cells (results 4.2.2.3; introduction 1.2.2). Nitrogen mustard also reduced the volume of stationary phase L1210 cells to a lesser extent than that of exponential phase cells, consistent with the interaction of HN2 with the $Na^+K^+Cl^-$ cotransporter.

The distribution of $[^3H]$-H_2O and $[^{14}C]$-inulin carboxylic acid as markers of the total water and extracellular space (methods 3.11) produced mean values for the volume of 10^6 cells.The distribution of cell size, detected by the Coulter Counter with Channelyzer, shows

that the population of cells in a culture are not of uniform size (figures 60 & 61); this is also reflected in differences in the amount of light scattered by individual cells, which was detected by flow cytofluorimetry (results 4.1.3.4) In addition, recent reports have suggested that L1210 cells exhibit heterogenous changes in cell volume in response to various agents which perturb ion fluxes (Hickman et al, 1984). Therefore, the values of cell volume obtained by the distribution of markers, must be accepted only as an average value for the population.

Changes in volume of L1210 cells incubated with HN2 were detected by distribution of markers and implied from the forward-scattered light in the flow cytofluorimeter (results 4.1.3.4); it is puzzling that no changes were detected by use of the Channelyzer. Grunicke and his coworkers (Grunicke et al, 1985; Doppler et al, 1985) reported that HN2 produced no change in the steady-state volume of Ehrlich ascites cells, measured by use of a Coulter Counter and a Channelyzer (see discussion, section 5).

The mechanisms for the maintenance of cell volume under steady state conditions have received less attention than those mechanisms which operate to regulate cell volume after osmotic shock. In addition to a role in the maintenance of steady-state volume of L1210 cells, the stimulation of $Na^+K^+Cl^-$ cotransport may have a role in the regulation of cell volume after osmotic shock. It would therefore be of interest to examine the effects of HN2 on regulatory volume changes in L1210 cells.

If cells are subjected to a hypertonic extracellular medium, water leaves under osmotic pressure. When normal tonicity is restored, the recovery of cell volume is accompanied by net gain of water, K^+ and Cl^- (Hoffmann, 1985a; 1985b), a phenomenon referred to as the regulatory volume increase (RVI). Activation of the $Na^+K^+Cl^-$

cotransporter has been demonstrated during RVI in avian erythrocytes (McManus and Schmidt, 1978). However, RVI in Ehrlich ascites cells seems to include the activation of an otherwise quiescent, bumetanide-sensitive and K^+-independent, Na^+Cl^- cotransport system (Hoffman et al, 1983; Hoffmann, 1985a; 1985b); the concomitant increase in cellular K^+ content arises from the activation of the Na^+K^+ATPase by the increase in cellular Na^+. These proposals are based on the observation that K^+ influx during RVI was almost completely abolished in the presence of ouabain.

In L1210 cells under steady-state conditions in isosmotic medium, all of the HN2-sensitive $^{86}Rb^+$ uptake is Na^+-dependent (results 4.3.1.3); it would be of interest to examine the ion transport mechanisms involved in RVI of L1210 cells, and the effects of HN2 upon them. If $Na^+K^+Cl^-$ cotransport is activated during RVI, it would be expected that RVI would be blocked in HN2-treated cells; if Na^+Cl^- transport and then Na^+K^+ATPase are activated during RVI, HN2 may be without effect on the process.

When cells are subjected to osmotic shock in hypotonic medium, there is an initial increase in cell volume but subsequent recovery, a phenomenon referred to as the regulatory volume decrease (RVD). Shank and Smith (1976) proposed that this was accounted for by the activation of Na^+K^+ATPase. Other reports have suggested that RVD was accompanied by a net loss of water, K^+ and Cl^-. In some cell types the loss of K^+ was Cl^--dependent and furosemide sensitive; in other types, K^+ and Cl^- are lost through the action of independent pathways (Hoffmann,1985).

Hoffmann (1985b) reviewed evidence (Hoffmann et al, 1984a; 1984b) that, during RVD of Ehrlich ascites cells, K^+ is lost through the action of the Ca^{++}-activated K^+ channel, which may be involved in the regulation of volume of other cell types. Hoffmann (1985)

suggested that the loss of K^+ from Ehrlich ascites cells during RVD was activated by the calmodulin-mediated release of Ca^{++} from intracellular stores.

Whilst the Ca^{++}-activated K^+ channel may be involved in RVD of L1210 cells, it appeared to have no role in the maintenance of the cell volume of exponentially-growing L1210 cells under steady-state conditions. Firstly, the addition of quinine, an inhibitor of the channel (introduction 1.2.1) had no effect on the volume of L1210 cells incubated in isotonic medium. Secondly, the addition of the calcium ionophore A23187 and 1mM Ca^{++} did not reduce the volume of exponential phase L1210 cells (results 4.4.3). These results were obtained from measurement of cell volume by distribution of $[^3H]$-H_2O and $[^{14}C]$-inulin carboxylic acid and therefore represent average values for a sample of 10^6 cells (methods 3.11). However, when volume and membrane potential changes of individual cells were examined by use of a flow cytofluorimetic technique, Hickman et al (1984) found that A23187 and Ca^{++} produced changes in the steady-state volume of exponential phase L1210 cells, in contrast to the results presented here. Clearly their technique will be of much use to further elucidate the pathways involved in maintenance of cell volume.

It was of interest to investigate a possible role for the Ca^{++}-activated K^+ channel in the maintenance of steady-state volume of stationary phase L1210 cells, since activities of the pathways which regulate K^+ homeostasis and cell volume may alter during growth in culture (see above). The addition of A23187 and Ca^{++} reduced volume of stationary phase L1210 cells, although this was not blocked by 0.3mM quinine. The addition of quinine also reduced volume of stationary phase cells in the absence of ionophore (results 4.4.3), which may suggest that it is not a specific inhibitor of the

Ca^{++} -activated K^+ channel. Additionally, the volume of exponential phase L1210 cells was reduced when external Ca^{++} was replaced by Mg^{++} or removed by EGTA, but the volume of stationary phase L1210 cells was unaffected by these changes. The mechanisms which may explain these observations are unknown, and the preliminary experiments (results 4.4.3) must be elaborated to determine the role, if any, of the Ca^{++}-activated K^+ channel in the maintenance of the steady-state volume of exponential or stationary phase L1210 cells.

Spurgin (1981) reported that 10^{-4}M HN2 inhibited RVD of PC6A cells after hypotonic shock. It would be of interest to define the mechanisms for RVD of L1210 cells and examine the effect of HN2 upon them. However the present results provide no evidence to suggest that the HN2-induced decrease in L1210 cell volume is due to activation of the Ca^{++}-stimulated K^+ channel. Nitrogen mustard did not stimulate K^+ or $^{86}Rb^+$ efflux from L1210 cells incubated in isotonic medium (results 4.4.1) nor did it cause an increase in the total cellular Ca^{++} content (results 4.9). The possibility that Ca^{++} was mobilised from intracellular stores was not investigated, because of difficulty in measuring cytoplasmic free Ca^{++} concentrations (results 4.9). However, if, as Hoffmann (1985a;1985b) suggests, the K^+ efflux channel was activated by liberation of free Ca^{++}, the K^+ efflux itself would have been detectable; also, there was no evidence for activation of the Na^+/Ca^{++} exchange pathway (results 4.7; 4.9; 4.10). The Na^+K+Cl^- cotransporter of some cells may be sensitive to regulation by changes in cytoplasmic free Ca^{++} (Garay, 1982). The results discussed above provide evidence that the HN2-induced decrease of cell volume was not due to changes in Ca^{++} concentration, which subsequently inhibited the $Na^+K^+Cl^-$ cotransporter.

A role has been proposed for the amiloride-sensitive Na^+/H^+ exchanger in the regulation of cell volume (Roos and Boron, 1981).

No evidence was found for an effect of HN2 on the Na^+/H^+ exchanger of L1210 cells; intracellular Na^+ concentration (results 4.7) and intracellular pH (results 4.11) did not differ from controls after incubation for 3h with 10^{-5}M HN2.

Grunicke and his coworkers (Grunicke et al, 1985; Doppler et al, 1985) reported that incubation for 4h with 2×10^{-6}M HN2 inhibited by 50% the furosemide-sensitive $^{86}Rb^+$ influx into Ehrlich ascites cells, but did not affect the steady state cell volume. They did not speculate on the consequence of inhibition of $^{86}Rb^+$ (and K^+) influx to the intracellular K^+ concentration in the absence of a decrease in cell volume. Their findings and conclusions will be discussed in detail, and compared with the results presented in this thesis, in section 5. It may be possible that the cell volume of HN2-treated Ehrlich cells was maintained by activation of some unknown, furosemide-sensitive K^+ transport mechanism. The authors did not consider this possibility, even though the processes which regulate volume have been very well defined for the Ehrlich ascites cell (reviewed by Hoffmann, 1985b).

The $Na^+K^+Cl^-$ cotransporter is involved in ion movements during cell proliferation (introduction 1.2.2). However, it seems unlikely that the inhibition of cotransport activity per se is causal to the cytotoxicity of HN2 to L1210 cells; HN2 did not affect Na^+ or K^+ homeostasis (results 4.7), and concentrations of bumetanide and furosemide which completely inhibited cotransport (results 4.2.2) caused only 10-50% inhibition of cell growth (results 4.6).

The different survival of cells after similar inhibition of cotransport by HN2 and the diuretics may be associated with the different types of interaction of the compounds with cellular constituents. Nitrogen mustard forms irreversible covalent bonds with a variety of cellular compounds (introduction 1.1.1.4) and is

not removed from L1210 cells by repeated washing (results 4.1.3.1); bumetanide and furosemide bind reversibly to cell membranes (Palfrey et al, 1980; introduction 1.2.1). If HN2 is cytotoxic because it reduces volume and imposes a physical restriction of cell growth, the survival of cells incubated with diuretics may depend upon a recovery of cell volume. Jayme et al (1981) reported that the bumetanide-sensitive K^+ fluxes of mouse fibroblasts were increased after incubation for 24h with bumetanide (concentration not given); this result suggests an increase in the number of active $Na^+K^+Cl^-$ cotransporter units on the cell membrane, in response to the stress of inhibition of the cotransporter. From the results presented in this thesis, it cannot be determined whether a similar up-regulation of cotransport activity occurs in L1210 cells incubated with HN2 or bumetanide. Grunicke et al (1985) and Doppler et al (1985) reported that incubation of Ehrlich ascites cells with HN2 initially inhibited $Na^+K^+Cl^-$ cotransport activity, but that after incubation for 4-12h there was recovery of this activity (discussion, 5). However, the concentration of HN2 (2×10^{-6}M) used in these experiments inhibited growth of Ehrlich cells in vitro by only 50%, in contrast to the more toxic concentration of HN2 (10^{-5}M) used in the present study. A crucial future experiment will be to examine cotransport activity of L1210 cells after prolonged periods of incubation with concentrations of HN2 and diuretics which produce less than 100% inhibition of growth.

The different toxicities of HN2 and the diuretics and the involvement of changes in cell volume in the toxic mechanism of HN2 are discussed further in section 5.

4.9 THE EFFECT OF NITROGEN MUSTARD ON INTRACELLULAR CALCIUM CONCENTRATIONS OF L1210 CELLS

4.9.1 Introduction

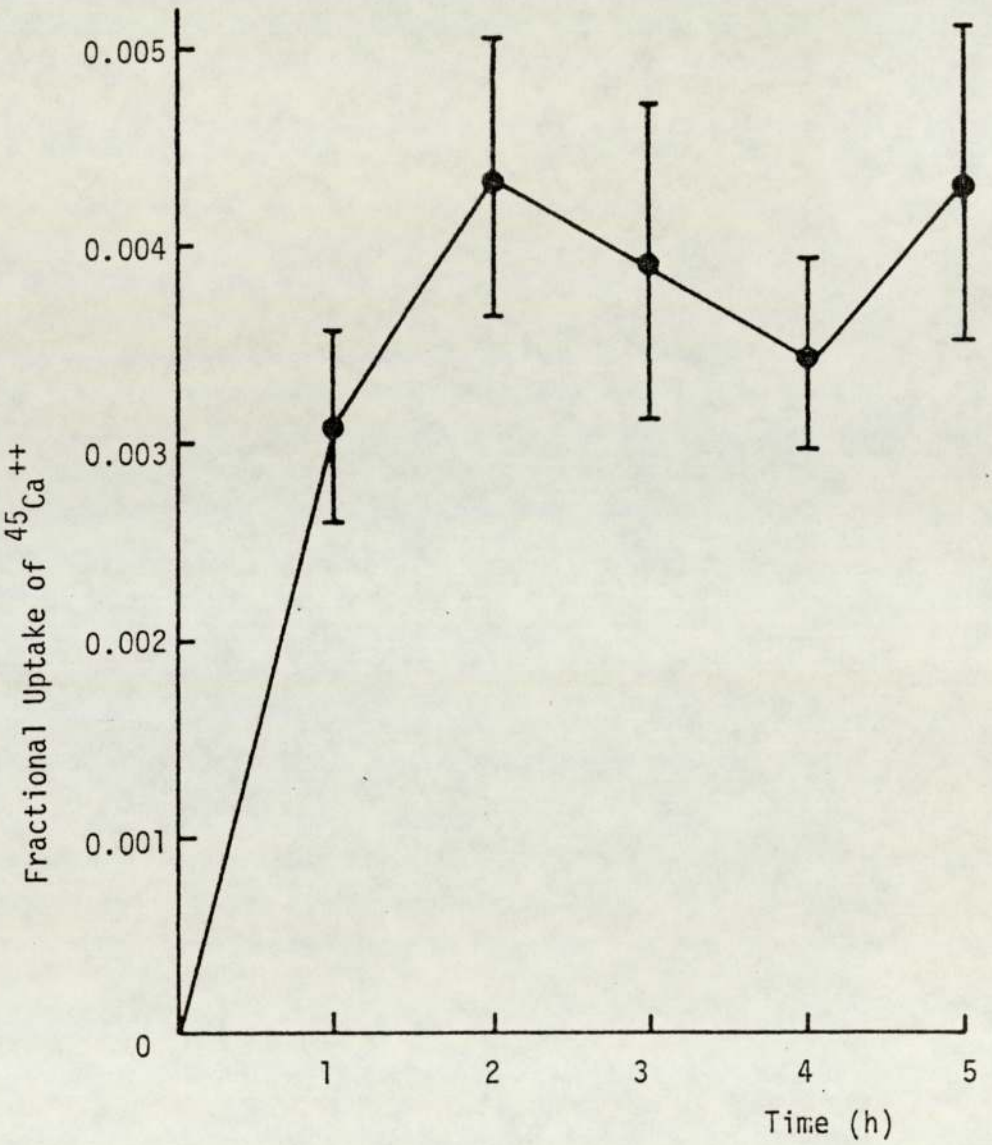
Intracellular free calcium ions perform many functions as secondary messengers in regulatory biochemical processes; therefore the concentration of Ca^{++} is also tightly regulated. Extracellular Ca^{++} is maintained at approximately 10^{-3}M; in contrast, intracellular free Ca^{++} concentrations are of the order 10^{-5}-10^{-7}M (Blaustein and Nelson, 1982). Therefore, the low intracellular Ca^{++} concentrations are maintained against a large transmembrane concentration gradient of Ca^{++} by active transport of Ca^{++} out of the cell, and by sequestration of Ca^{++} in intracellular organelles, such as mitochondria and endoplasmic reticulum. Inhibition of these transport processes, or the energy-generating processes of the cell, leads to the loss of the transmembrane Ca^{++} gradient, causing an increase in intracellular Ca^{++} concentrations, which has been proposed as a common factor in different mechanisms of cell death (Schanne et al, 1979). In addition, changes in Ca^{++} concentrations may affect the processes of K^+ transport (Gardos, 1958; Garay, 1982; Kino et al, 1985; Epstein and Whittam, 1966; Schwartz et al, 1975; Lindenmayer and Schwartz, 1975; Hoffman, 1962; Yingst and Hoffman, 1981). It was therefore important to determine whether the effects of HN2 upon $^{86}Rb^+$&K^+ transport and proliferation of L1210 cells were mediated by changes in intracellular calcium concentrations.

The total intracellular calcium concentrations of L1210 cells were estimated from the distribution, at isotopic equilibrium, of the radioisotope $^{45}Ca^{++}$. It was also intended that free cytoplasmic calcium concentration would be measured by the use of Quin-2, a fluorescent probe for free intracellular Ca^{++} ions (see discussion 4.9.3 below). The retention of Quin-2 inside cells requires the

Figure 62

The uptake of $^{45}Ca^{++}$ into L1210 cells.

(Mean ± S.D.; n=4).



action of cellular esterase enzymes (see discussion 4.9.3 below); as a preliminary experiment, L1210 cells were stained histochemically for the presence of non-specific esterases.

4.9.2 Results

The initial influx of $^{45}Ca^{++}$ into L1210 cells did not occur at a constant or reproducible rate; in addition to high inter-assay variation, there was large variation between triplicate measurements within each assay, using the oil barrier method (data not shown). Examination of $^{45}Ca^{++}$ content of cells by use of an ultrafiltration method also failed to detect linear rates of influx (data not shown). Reproducibility was not improved by the use of cells from different, synchronously established cultures (data not shown). However, when $^{45}Ca^{++}$ content of washed cells was measured at hourly intervals, isotopic equilibrium appeared to be reached within 5h of addition of $^{45}Ca^{++}$ (figure 62). Subsequently, L1210 cells were incubated for 5h in RPMI with $^{45}Ca^{++}$ with and without 10^{-5}M HN2 for the final 3h; the distribution of $^{45}Ca^{++}$, in the cellular and supernatant fractions was then measured. It was assumed that at isotopic equilibrium, the activity of the sample was proportional to the total calcium content; thus the radioactivity measured in the supernatant fraction was assumed to be proportional to the Ca^{++} content of the medium, and a value was calculated for the radioactivity per mole Ca^{++} + $^{45}Ca^{++}$. In this manner the Ca^{++} content of the cells was determined, and with reference to the concomitant measurement of cell volume, the intracellular total calcium concentration was calculated.

The intracellular total Ca^{++} concentration of L1210 cells was 0.306±0.127mM; after incubation with 10^{-5}M HN2, the Ca^{++} concentration was 0.476±0.206mM (means ± S.D.; n = 6), which was not statistically significantly different from controls ($p > 0.05$). This

result suggested that HN2 did not significantly elevate intracellular total Ca^{++} concentrations of L1210 cells.

When L1210 cells were stained histochemically for the presence of non-specific esterases, the cells were green in colour, which indicated that esterase activity was absent (see discussion 4.9.3 below); the measurement of intracellular free Ca^{++} by fluorescence of Quin-2 was not attempted.

4.9.3 Discussion

Free cytosolic calcium ions influence a wide variety of cellular processes (Scarpa and Carafoli, 1978; Carafoli et al, 1975) including stimulation of excitable cells (Kostyuk, 1982; Cavero and Spedding, 1983), cellular organisation (Cavero and Spedding, 1983; Weber and Murray, 1973; Buridge and Feramisco, 1981), and the coupling of cellular responses to a variety of stimuli (Cavero and Spedding 1983), including mitogens (Moolenaar et al, 1984; see introduction 1.2.2). Elevation of intracellular free Ca^{++} concentration, $[Ca^{++}]_i$, causes changes in cell volume (Passow, 1963), membrane potential (Glynn and Warner, 1972; Hoffman and Knauf, 1973), carbohydrate metabolism, phosphatidylinositol turnover, microtubule organisation, mitochondrial metabolism, cellular cAMP levels, protease activity and cyclic nucleotide phosphodiesterase activity (Ralph, 1983), as well as changes in the activity of potassium transport systems located in the plasma membrane. Increases in $[Ca^{++}]_i$ increase the K^+ permeability (Gardos, 1958; Blum and Hoffman, 1981), inhibit Na^+K^+ATPase (Hoffman, 1962; Yingst and Hoffman 1981), and interfere with the function of the $Na^+K^+Cl^-$ cotransporter (Garay, 1982). A common denominator in the action of many toxins may be an elevation of $[Ca^{++}]_i$ (Schanne et al,1979); in addition, the protein calmodulin, which binds calcium ions and regulates the activity of Ca^{++}-ATPase, thereby regulating $[Ca^{++}]_i$ (Schatzmann, 1982), has functionally

important methionine groups (Walsh and Stevens, 1977; 1978a; 1978b; Richman and Klee, 1978a; 1978b) which may be susceptible to alkylation by HN2 (see introduction 1.1.4.1). In addition, HN2 may affect cellular thiol levels (see results 4.1.3.3), which appear to be important to maintain thiol groups of proteins in the normal state, via the cytosolic thiol transferase (Mannervik et al, 1981). This mechanism may be important in the control of sequestration of calcium in the endoplasmic reticulum (Jewell et al 1982). Thus it was important to determine whether the effects of HN2 upon K^+ transport (results 4.3) were mediated by changes in $[Ca^{++}]_i$.

Nitrogen mustard reduced the volume of proliferating L1210 cells (results 4.8) but Na^+ homeostasis was maintained (results 4.7). Since the Na^+/Ca^{++} exchanger may contribute to the regulation of cellular Na^+ (Villereal, 1981) and Ca^{++} (Blaustein and Nelson, 1982), it was desirable to measure Ca^{++} influx and so determine whether this transport system was activated in HN2-treated cells. In addition, the reduction in cell volume may be expected to cause elevation of cell Ca^{++} concentrations.

Attempts to measure Ca^{++} influx by use of tracer amounts of $^{45}Ca^{++}$ were not satisfactory; reproducible results were not obtained. In a single experiment to investigate loss of $^{45}Ca^{++}$ from preloaded L1210 cells, similar problems were encountered (data not shown). One explanation for these difficulties may be the rapid exchange of $^{45}Ca^{++}$ with Ca^{++} bound at the glycocalyx; it is possible that this pool of isotope was partially disrupted through damage to the glycocalyx during the rapid centrifugation of samples. However, a method which involved no centrifugation, and allowed washing of loaded cells, ultrafiltration, was similarly unsuccessful (data not shown). A large fraction of total cellular calcium is associated with the cell membrane and glycocalyx (Harrison and Long, 1968;

Tupper et al, 1978); this pool is exchanged rapidly (Uchikawa and Borle,1978) and presents problems with techniques using radioisotopic tracers, (Yingst and Hoffman,1984a). The metallochromic indicator, Arsenazo III, has been used to monitor Ca^{++} influx into human red blood cell ghosts (Yingst and Hoffman, 1984a; 1984b), but this approach is obviously unsuitable for use with intact cells at homeostasis.

Attention was then turned to estimation of total cellular Ca^{++} from the distribution of $^{45}Ca^{++}$ at isotopic equilibrium. Such a calculation requires that the labelling period is sufficient to allow attainment of equilibrium between all the cellular Ca^{++} pools; from the data in figure 62, it appears that this requirement was fulfilled after a labelling period of five hours. However, the work of Uchikawa and Borle (1978) suggests that this may not be so. These authors proposed that the kinetics of $^{45}Ca^{++}$ efflux from isolated kidney cells were consistent with three cellular compartments: a rapidly-exchanged pool of Ca^{++} associated with the cell surface or glycolalyx; a cytoplasmic pool, consisting of free Ca^{++} ions and Ca^{++} bound to soluble ligands and subcellular structures; and a third pool of insoluble Ca^{++}, sequestered in organelles such as ER and mitochondria. A similar conclusion was drawn by Claret-Berthon et al (1977), who suggested that Ca^{++} existed in a dynamic cytoplasmic pool, a slowly-exchanged mitochondrial pool and a non-exchanged mitochondrial pool. Uchikawa and Borle (1978) calculated that the loss of $^{45}Ca^{++}$ from the most slowly exchanged pool had a half-life of several hundred minutes, and thus that the labelling period required to achieve equilibrium would be many hours long, and therefore impractical. On the basis of these results, they devised a technique whereby the cells were labelled with isotope for two hours only; then the efflux, measured over several hours, was assumed to be

proportional to the initial concentration of isotope (Studer and Borle, 1982; Uchikawa and Borle, 1978; Borle et al, 1981). However, this method relies on the assumption that the experimental treatment is without effect upon the Ca^{++} transport systems. Since nitrogen mustard has been shown to affect transmembrane transport processes (introduction 1.3), this assumption may not necessarily be valid for the present study, although Spurgin (1981) found that preincubation for 1h with 10^{-4}M HN2 was without effect upon Ca^{++}-ATPase of PC6A murine plasmacytoma cells.

The problem of association of isotope with the cell surface has been overcome by use of a technique whereby the calcium ionophore A23187 is used to release cytoplasmic Ca^{++}, then the uncoupler FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) is added to release mitochondrial calcium; the released calcium is then detected by Arsenazo III (Murphy et al 1980; Bellomo et al, 1982; Jewell et al, 1982; Keyes et al, in preparation). Using this method, Keyes et al found the total cytoplasmic Ca^{++} of proliferating L1210 cells to be approximately 2.5×10^{-7}M; extramitochondrial Ca^{++} was approximately 1.0×10^{-7}M. The estimate of total cellular Ca^{++} of 3×10^{-4}M obtained in the present study must indicate that a large portion of this Ca^{++} is associated with the cell membrane.

The subcellular distribution of Ca^{++} cannot be detected by use of atomic absorption spectroscopy, because the Ca^{++} concentrations involved are so small, but it has been studied by use of Ca^{++} microelectrodes (Simon et al, 1978) by measurement of Ca^{++} in different fractions obtained after cell disruption (Tischler et al, 1977), and by use of the photoprotein aequorin (Blinks, 1978), but the most frequently used probe for free Ca^{++} ions is Quin-2 (Tsien, 1980; 1983; Tsien et al, 1982; 1984). This compound, 2-[2-[bis(carboxymethyl)amino]-5-methyl-phenoxy]-methyl]-6-methoxy-8-

[bis(carboxymethyl)amino] quinoline, displays a shift in the spectrum and an enhancement of the intensity of the fluorescence upon binding calcium, but is not without its limitations (Grynkiewicz et al, 1985; Tsien et al, 1982; 1984). Cells are incubated with a membrane-permanent ester derivative, and cytoplasmic non-specific esterases cleave off the ester groups to leave the impermeant Quin-2 in the cytoplasm. In preparation for measurement of L1210 cell Ca^{++} by Quin-2, the cells were stained histochemically for non-specific esterases (Yan et al, 1971).

The action of the enzymes cleave α-naphtholacetate to release α-naphthol, which reacts with the hexazotized pararosanilin to produce an insoluble, red-brown azo dye, and so localize the enzymes (Davis and Ornstein, 1959; Nachlas and Seligman, 1949). The cells are counterstained with methyl green; thus a green stain represents a negative result. Unfortunately, the presence of esterases in L1210 cells was not detected in this manner, and the Quin-2 assay was not carried out. However, since completion of the experimental work for the present study, Quin-2 has been used to measure cellular Ca^{++} in HL60 human leukaemia cells, which also failed to stain positively for the presence of nonspecific esterases (Dr. M. Thompson, personal communication). Therefore, a future attempt to assay Ca^{++} in L1210 cells using Quin-2 may prove successful. Such a measurement is of particular importance because, although HN2 had no significant effect upon total cell Ca^{++}, the result provides no information on the movement of Ca^{++} between the subcellular compartments. The increases in cytoplasmic free Ca++ in response to various stimuli are thought to be primarily due to mobilisation of intracellular Ca^{++} stores (Prentki et al, 1984; Moolenaar et al, 1984; Lopez-Rivas and Rozengurt, 1983; Owen and Villereal, 1983a; Sawyer and Cohen, 1981; Tupper et al, 1978; Blaustein and Nelson, 1982).

4.10 THE EFFECT OF NITROGEN MUSTARD ON L1210 CELL MEMBRANE POTENTIAL

4.10.1 Introduction

The plasma membrane potential depends upon the electrogenic movement of ions across the membrane, which is brought about by the action of several transporters and the passive diffusion of ions down their concentration gradients; it is exquisitely sensitive to changes in ion fluxes of femtomolar amounts (introduction 1.2.1). Therefore, it may be expected that agents which perturb the action of electrogenic ion transporters may cause readily detectable changes in the membrane potential. Incubation for 3h with 10^{-5}M HN2 was sufficient to completely inhibit the furosemide-sensitive $^{86}Rb^{+}$ influx into L1210 cells (results 4.3). It has been reported previously (Okada et al, 1980) that the bioactivated alkylating agent, mitomycin C (Carter and Crooke, 1979), reduced the hepatocyte membrane potential in rats injected with therapeutic amounts. It was of interest to examine whether the marked effect of HN2 on $^{86}Rb^{+}$ & K^{+} fluxes had effects upon cell membrane potential. For the present study, L1210 cells were incubated for 3h with and without 10^{-5}M HN2, and the membrane potential was estimated from the distribution of the lipophilic, radiolabelled cation, $[^{3}H]$-$TPMP^{+}$, into cells incubated in buffers of different K^{+} concentration (methods 3.12).

4.10.2 Results

The use of $[^{3}H]$-$TPMP^{+}$ as a probe for the membrane potential of L1210 cells has been described previously (Chahwala and Hickman, 1985). The accumulation of $[^{3}H]$-$TPMP^{+}$ was initially rapid, but reached equilibrium and steady state by 60 minutes. This was true for L1210 cells incubated in high K^{+} or low K^{+} Krebs-Ringer buffers (methods 3.12). The $[^{3}H]$-$TPMP^{+}$ content of the cells was proportional

to the $TPMP^+$ concentration. $TPMP^+$ had no effect on L1210 cell viability on the basis of impermeability to trypan blue (Chahwala and Hickman, 1985).

The membrane potential of L1210 cells was measured at a single 60 minute time point (methods 3.12). After incubation for 3h with 10^{-5}M HN2 the membrane potential of L1210 cells was -67±10mV; the membrane potential of cells incubated in the absence of HN2 was -58±11 mV. (mean ± S.D.; n=11; $p>0.05$). Nitrogen mustard produced no significant change in the membrane potential of exponentially-growing L1210 cells.

4.10.3 Discussion

Perturbation of electrogenic transmembrane ion transport processes may be reflected in changes in the plasma membrane potential. Maintenance of plasma membrane potential is vital for the processes of nutrient transport and secretion, and may play a role in regulation of cell growth (introduction 1.2.2). It was therefore of interest to examine the effect of nitrogen mustard on the plasma membrane potential of L1210 cells, to determine whether the cytotoxic mechanism of the drug depends upon extensive metabolic inhibition mediated by changes in membrane potential. The plasma membrane potential of L1210 cells was estimated after incubation for 3h with 10^{-5}M HN2, under which conditions the $Na^+K^+Cl^-$ cotransporter was completely inhibited (results 4.3).

Calculation of membrane potential from the distribution of the lipophilic cation $[^3H]$-$TPMP^+$ requires the assumption that there is passive equilibration of the cation with the membrane potential (Skulachev, 1971; Haydon and Hladky, 1972; Lichtshtein et al, 1979); this assumption was supported by the dependence of $TPMP^+$ accumulation on $TPMP^+$ concentration, which implied a non-saturable process of transport (Chahwala and Hickman, 1985; Lichtshtein et al, 1979).

Accumulation of [^{3}H]-TPMP$^+$ was reduced by 50% when L1210 cells were incubated in high K^+ medium, which is consistent with depolarisation to the extent of the potassium membrane potential (Chahwala and Hickman, 1985), and suggests that, in part, the resting membrane potential of L1210 cells arises from the transmembrane K^+ diffusion gradient (Lichtshtein et al, 1979). However, the residual accumulation of [^{3}H]-TPMP$^+$ into cells incubated in high K^+ medium, observed by Chahwala and Hickman (1985), indicates that L1210 cells do not regulate resting membrane potential as simple K^+ electrodes.

The electrogenic activity of the Na^+K^+ATPase (introduction 1.2.1) may regulate plasma membrane potential in certain cell types. The plasma membrane potentials of Lettre cells (Bashford and Pasternak, 1984) and human neutrophils (Bashford and Pasternak, 1985) were unaltered by changes in external K^+, but ouabain produced depolarisation. The action of Na^+K^+ATPase appears to be important for the maintenance of membrane potential in Ehrlich ascites cells (Geck et al, 1980; Heinz et al, 1981). In contrast, ouabain had no effect on the resting membrane potential of L1210 cells (Chahwala and Hickman, 1985), suggesting that the Na^+K^+ATPase contributes nothing to the resting membrane potential. This result is in agreement with those obtained with neuroblastoma-glioma hybrid cells (Lichtshtein et al, 1979). The addition of amiloride, which has been shown to inhibit Na^+/H^+ exchange (Roos and Boron, 1981; Vigne et al, 1984; and others), Na^+/Ca^{++} exchange (Smith et al, 1982; Swanson et al, 1983, Dethmers et al, 1983), and Na^+K^+ATPase (Soltoff and Mandel, 1983) was without effect upon the resting membrane potential of L1210 cells (Hickman et al, 1984), which may suggest that none of these pumps contributed to the resting membrane potential.

Different methods for measurement of membrane potential may yield different results (Rink et al, 1980), and each technique is not without criticism (Johnstone et al 1982; Smith, 1982; Brauner et al, 1984; Lichtshtein et al,1979). Distribution of $TPMP^+$ may be affected by non-specific binding (Chahwala and Hickman, 1985), and mitrochondrial membrane potential (Grinius et al, 1970). It is probably advisable to regard the result of the present study as a probe for qualitative changes in membrane potential, and not as a quantitiatively absolute measurement.

The value of -58mV found here for the membrane potential of L1210 cells is less negative that the value of -71±7mV reported by Chahwala and Hickman (1985), who used the same method; it is in good agreement with the value of -52.3 mv obtained by Hickman et al (1984), by use of the "null point" assay of Deutsch and Price (1982). The value found here for the membrane potential of L1210 cells is less negative than the value of -65mv, obtained by distribution of a lipophilic cation, for the membrane potential of normal mouse lymphocytes (Kiefer et al, 1980). When L1210 cell membrane potential was measured by the distribution of chloride ions, a value of -5.62mv was obtained (Goldman et al, 1968; Fyfe and Goldman, 1973).

The results obtained in the present study suggest that nitrogen mustard is without effect on the resting membrane potential of L1210 cells; this result is consistent with an interference of the function of an electrically silent $1Na^+:1K^+:2Cl^-$ cotransport system (results 4.3). Geck et al (1980) reported that the membrane potential of Ehrlich ascites cells remained unchanged over a wide range of furosemide-sensitive fluxes of Na^+, K^+ and Cl^- and, conversely, that changes in membrane potential were without effect on those fluxes.

It has been suggested that the cotransporter of some cells types may mediate electroneutral exchanges of Na^+ & Na^+, K^+ & K^+ and Na^+ & K^+ (introduction 1.2.1). However, studies of $^{86}Rb^+$ efflux from preloaded L1210 cells suggest that there is no bumetanide-sensitive K^+ exchange (results 4.4). Exchange of Na^+ was not examined due to practical difficulties (see results 4.5).

The use of the distribution of [^{3}H]-TPMP$^+$ to estimate membrane potential is limited by two factors. Firstly, the relatively slow attainment of equilibrium distribution may preclude its use to detect rapid changes in membrane potential. Secondly, it provides only an average value of the membrane potential of a population of cells. It is unlikely that all the cells in the sample will have identical membrane potential. In addition, there is evidence that a population of L1210 cells may exhibit heterogeneity of response to agents which perturb ion fluxes (Hickman et al, 1984). L1210 cells were labelled for flow cytofluorimetry with a fluorescent dye, DiOC6(3). Addition of the calcium ionophore A23187 induced simultaneous hyperpolarisation (by activation of Ca^{++}-stimulated K^+ efflux) and depolarisation (by activation of the Na^+/Ca^{++} exchanger) in the same population of L1210 cells. Under the same conditions, A23187 caused no change in L210 membrane potential as detected by the distribution of [^{3}H]-TPMP (Chahwala and Hickman, 1985). The addition of A23187 to human lymphocytes and rat thymocytes induced simultaneous hyper- and de-polarisation, as detected by the use of the fluorescent dye diS-C_3-(5) (Gukovskaya and Zinchencko, 1985). It may be of interest to examine the possibility of a heterogeneous response of the membrane potential of L1210 cells after incubation with HN2, by use of the flow cytofluorimetric method described by Hickman et al (1984). A cellular basis for any heterogeneous response may be examined by the use of cells synchronised in different stages of the cell cycle.

4.11 THE EFFECT OF NITROGEN MUSTARD ON THE INTRACELLULAR pH OF L1210 CELLS

4.11.1 Introduction

Changes in intracellular pH (pH_i) are among the first events observed upon the stimulation of quiescent cells by growth factors and upon fertilization of oocytes (Roos and Boron, 1981). Small changes of pH_i can alter membrane function, and may serve as regulatory stimuli for enzymes at control points in metabolic pathways (introduction 1.2.2). The effect of HN2 upon intracellular pH was investigated, to determine whether the cytotoxic or cytostatic action of HN2 may be mediated by changes in pH_i. Changes of pH_i may also be accompanied by changes in the activity of the Na^+/H^+ antiport and the HCO_3^-/Cl^- anion exchanger which function to regulate pH_i in many cell types (Roos and Boron, 1981). The radiolabelled weak acids [^{14}C]-benzoic acid (BA) and [^{14}C]-dimethyloxazolidine (DMO) were used as probes of intracellular pH of L1210 cells after incubation with or without HN2.

4.11.2 Results

During the time course of the experiments to measure pH_i, the permeability to trypan blue of HN2-treated L1210 cells did not differ significantly from that of controls (table 20). Figures 63 and 64 show the accumulation of [^{14}C]-DMO and [^{14}C]-BA, respectively, by L1210 cells incubated in bicarbonate-free saline (methods, 3.13). The distribution of both probes rapidly reached equilibrium. Intracellular pH was calculated from the distribution of the probes at the single 15 minute time point (methods, 3.13). Tables 21 and 22 show the effect of nitrogen mustard on intracellular pH estimated by the distribution of [^{14}C]-DMO and [^{14}C]-BA respectively (methods 3.13). The estimate of pH_i was consistently lower when DMO was used as the probe, but nitrogen mustard produced no significant ($p>0.05$) change in pH_i estimated by either probe.

Figure 63

The uptake of [^{14}C]-dimethyloxazolidine-2,4,-dione into L1210 cells incubated in bicarbonate-free saline.

(Mean ± S.D.; n=3)

Figure 64

The uptake of [^{14}C]-benzoic acid into L1210 cells incubated in bicarbonate-free saline.

(Mean ± S.D.; n=3)

Figure 63

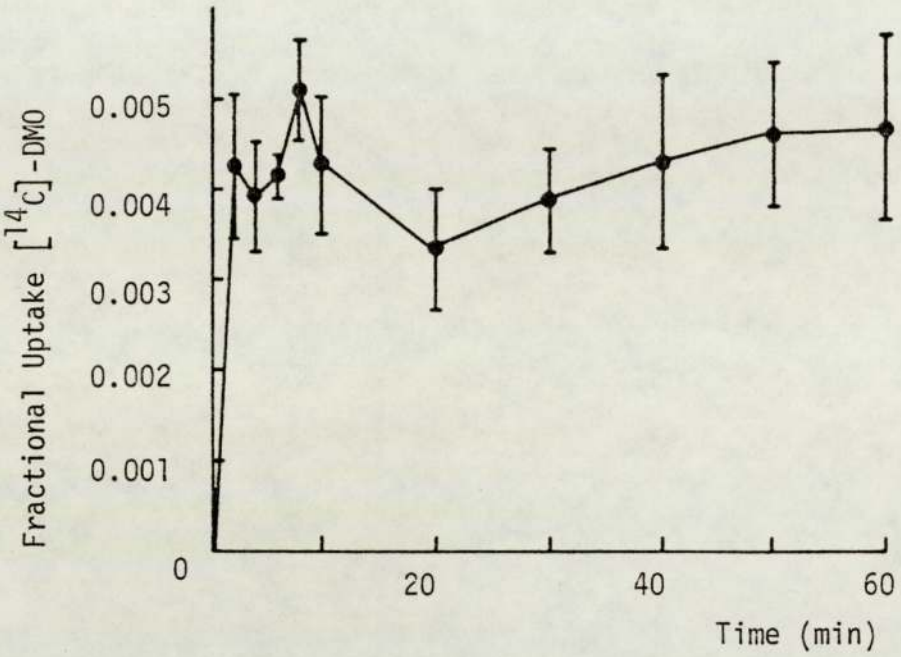


Figure 64

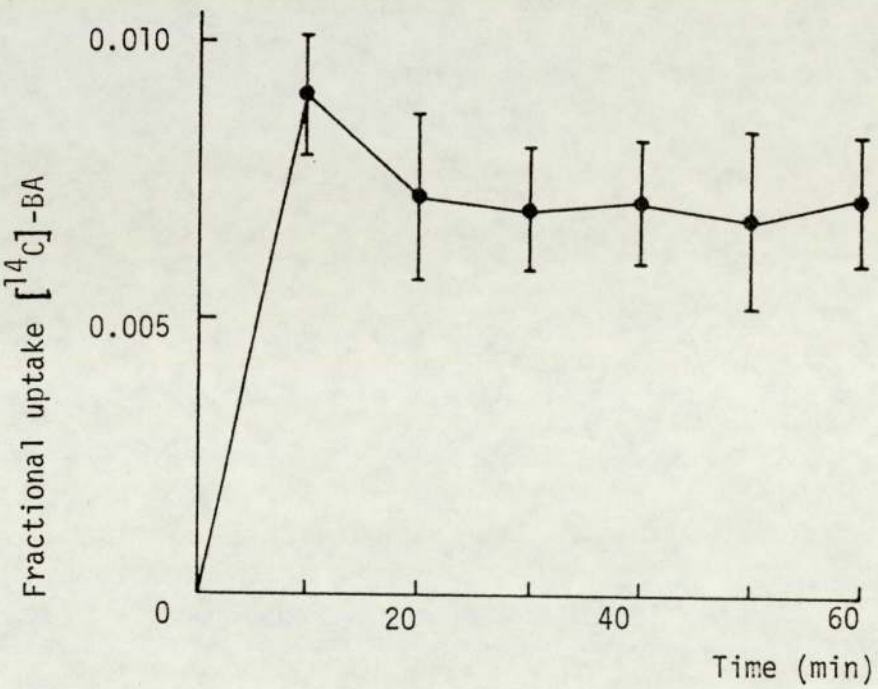


Table 20

The effect of nitrogen mustard on the exclusion of trypan blue by L1210 cells incubated in HCO_3^--free saline.

Time	Percentage of cells excluding trypan blue	
h	control	10^{-5}M HN2
0	96 ± 5	96 ± 5
0.5	96 ± 2	94 ± 7
1.0	94 ± 5	90 ± 6
1.5	93 ± 3	92 ± 5
2.0	88 ± 9	91 ± 8
3.0	82 ± 9	86 ± 7

Mean ± S.D.; n=3

Table 21

The effect of nitrogen mustard on intracellular pH of L1210 cells (measured by the equilibrium distribution of [^{14}C]-DMO)

Time	pH_i (units)		
h	control	10^{-5}M HN2	ΔpH_i
0	7.51 ± 0.11	7.42 ± 0.29	-0.09 ± 0.18
0.5	7.43 ± 0.24	7.45 ± 0.14	+0.02 ± 0.18
1.0	7.42 ± 0.29	7.32 ± 0.12	-0.11 ± 0.16
2.0	7.49 ± 0.16	7.25 ± 0.11	-0.24 ± 0.05
3.0	7.45 ± 0.31	7.30 ± 0.06[1]	-0.14 ± 0.16

External pH (pH_0) = 7.38 ± 0

[1] p value for comparision with control : p>0.1

Mean ± S.D.; n=3

Table 22

The effect of nitrogen mustard on intracellular pH of L1210 cells (measured by the distribution of [^{14}C]-BA.).

Time	pH_i (Units)		
h	control	10^{-5}M HN2	$\triangle pH_i$
0	7.73 ± 0.17	7.73 ± 0.39	+0.02 ±0.08
1	7.64 ± 0.18	7.66 ± 0.14	+0.04 ± 0.05
2	7.62 ± 0.22	7.66 ± 0.20	+0.05 ± 0.13
3	7.73 ± 0.15	7.81 ± 0.14[1]	+0.06 ± 0.09

External pH (pH_0) = 7.38 ± 0

[1]p value for comparision with control: p>0.1

Mean ± S.D.; n=3

4.11.3 Discussion

The distribution of weak acids has been used as a probe for intracellular pH in a variety of cells and tissues (Roos and Boron, 1981). The calculation of pH_i (methods 3.13) relies on assumptions that: only the uncharged form of the acid enters the cells; the concentration of the uncharged form is the same in intracellular and extracellular water; the dissociation constant of the acid in intracellular water is the same as in extracellular water; the acid itself does not affect pH_i.

The cells and supernatent fractions were separated by centrifugation through an oil barrier (methods 3.4.1). The use of this method obviated the need to wash unabsorbed tracer from the cells and the consequent correction for tracer efflux (L'Allemain et al, 1984b). The method was also employed for the simultaneous measurement of cell volume and extracellular water in the cell pellet. The distribution of the probe was corrected for the fraction of the probe trapped in extracellular water.

The distribution of [^{14}C]-DMO or [^{14}C]-BA was measured in L1210 cells incubated in HCO_3^- -free buffer, for the following reasons: firstly, extracellular pH did not depend on continuous gassing with CO_2, so minor pH_o fluctuations were minimised; secondly, a Na^+-dependent anion exchanger for HCO_3^-/Cl^- may be involved in the regulation of pH_i (Pouyssegur et al, 1984), in a similar manner to that described in invertebrate systems (Roos and Boron, 1981: Boron, 1983). Changes in the activity of the Na^+/H^+ exhanger, after incubation with HN2, may then be revealed by changes in pH_i, and not obscured by activation of the anion exchanger, which may occur in the presence of extracellular HCO_3^- in vivo. Lastly, measurements of the distributions of DMO and NH_3 yielded different values of pH_i of nucleated avian erythrocytes in a bicarbonate buffer, but the estimates of pH_i were in good agreement when cells were incubated in a phosphate buffer (Roos and Boron, 1981).

The distribution of [^{14}C]-DMO and [^{14}C]-BA in L1210 cells rapidly reached steady values at equilibrium. The distribution of [^{14}C]-BA in Chinese hamster lung fibroblasts also reached a steady value at equilibrium, which was interpreted to indicate that there was no significant accumulation of [^{14}C]-BA in intracellular organelles (L'Allemain et al, 1984b). L'Allemain et al (1984b) studied the distribution of benzoic acid as a marker of pH_i of Chinese hamster lung fibroblasts. They found that the addition of digitonin, which selectively permeabilised the plasma membrane, caused a rapid equilibration of intracellular BA with extracellular pH, and a similar equilibration occurred when the cells were placed in Na^+- and Li^+-free medium, which reversed the Na^+/H^+antiport. The rapid equilibration in both cases suggested that there was no accumulation of the probe in intracellular organelles, and therefore

no contribution to the measurement of pH_i. Intracellular pH of 3T3 cells, measured by distribution of [^{14}C]-DMO or [^{14}C]-BA, was unchanged after addition of mitrochondrial inhibitors (Schuldiner and Rozengurt, 1982). It should be noted that the distribution of [^{14}C]-DMO or [^{14}C]-BA yields only an average value of pH_i for a population of cells, but there is evidence that agents which perturb ion fluxes may elicit hetergeneous responses of the cellular mechanisms for ion homeostasis (Hickman et al, 1984) within the same population of cells. If these responses include activation of those pathways involved in the regulation of pH_i, heterogeneity would not be detected by the method used for the present study.

The estimate of pH_i obtained by distribution of [^{14}C]-DMO was consistently lower than that obtained by [^{14}C]-BA. The reasons for this are unknown. The short period of incubation of cells with the probes probably minimised their metabolism, and the steady values at equilibrium provided evidence for this assumption. The calculation of pH_i required the assumption that the pK_a of the probe in the intracellular fluid was the same as that in the extracellular medium (methods 3.13), but differences in pK_a may arise due to differences in ionic composition inside and outside the cell (Roos and Boron, 1981). The calculation of pH_i also relied upon the assumption that the plasma membrane of the cell was impermeable to the charged form of the probe (Roos and Boron, 1981). Weak acids may themselves affect pH_i, and thus influence the measurement (Roos and Boron, 1981). Therefore, the different values for pH_i, obtained by the distribution of DMO and BA may arise from different permeabilities of their charged forms, or from their different effects on pH_i. These possibilities are all disadvantages of the use of the distribution of weak acids to estimate pH_i. However, the techniques are simple,

rapid, allow changes in pH_i to be monitored, and are suitable for use with isolated cells and organelles which are too small to allow direct electrophysiological methods (Roos and Boron, 1981). Where comparative measurements have been made, the estimate of pH_i agrees well with that obtained by direct methods.

The intracellular pH of rapidly dividing cells is usually greater than extracellular pH (Roos and Boron, 1981) because of the increased efflux of H^+ through the activity of the Na^+/H^+ antiport (introduction 1.2.2). This observation was confirmed by the present results.

Nitrogen mustard had no significant effect on pH_i in the absence of extracellular HCO_3^-. It was concluded that the toxicity of HN2 to dividing cells did not arise from an effect on pH-sensitive metabolic processes, or from interference with the Na^+/H^+ antiport. The results may also provide indirect evidence that HN2 had no effect on the enzymes of metabolic pathways which themselves affect cellular pH (Roos and Boron, 1981). The lack of effect of HN2 on pH_i indirectly provided evidence against an increase in free cytoplasmic Ca^{++} concentration during incubation of L1210 cells with HN2 (see also results 4.9.3); an increase in free Ca^+ concentration has been shown to cause a rapid fall in pH_i, as it is sequestered into mitochondria in exhange for H^+ (Roos and Boron, 1981).

4.12 THE EFFECT OF NITROGEN MUSTARD ON INTRACELLULAR ATP CONCENTRATION OF L1210 CELLS

4.12.1 Introduction

The energy for many reactions of cellular metabolism is provided by the cleavage of ATP; it would be expected that any agent which interfered with the mechanisms which maintain normal cellular concentrations of ATP might have widespread and damaging consequences for cell function.

The plasma membrane Na^+K^+ATPase uses energy from cleavage of ATP to maintain the cellular concentrations of Na^+ and K^+ against their transmembrane concentration gradients (introduction 1.2.1). In contrast, the action of the $Na^+K^+Cl^-$ cotransporter does not require metabolic energy, since the movement of the ions is driven by the sum of their concentration gradients across the cell membrane (Geck et al, 1980; introduction 1.2.1). However, the transporter does require ATP for its activity; it has been postulated that ATP may regulate cotransport function. Changes in intracellular ATP concentrations may therefore be reflected in changes in the diuretic-sensitive Rb^+ and K^+ fluxes.

In addition, the ATP-generating processs of glycolysis may be sensitive to changes in transmembrane ion fluxes (Solomon, 1977). Nitrogen mustard has been shown to interfere with the ATP-generating processes of glycolysis and respiration (Ochoa and Hirschberg, 1967; introduction 1.1.4.3; 1.1.4.4). It was therefore important to ascertain whether the inhibition of $^{86}Rb^+$ influx into HN2-treated L1210 cells was the indirect result of depletion of cellular ATP concentrations. Cellular ATP contents of L1210 cell extracts were determined by measurement of the luminescence provided by the firefly luciferase-luciferin reaction.

4.12.2 Results

Under the conditions of the assay, the intensity of luminescence of the reaction was found to be constant over a 15-30 second time interval after initiation of the reaction by addition of firefly luciferin-luciferase reagent; the luminescence of further samples was measured over this time interval.

Figure 65 shows the relationship between the intensity of luminescence, as a percentage of that of the internal standard of the luminometer, and the concentration of ATP; this was proportional to ATP concentation over the range of 0-0.5 $\mu g ml^{-1}$ ATP, and samples were diluted with buffer to produce a luminescence of 10-50% of the internal standard.

The ATP concentration of L1210 cells from exponentially growing cultures was 9.8±5.6 mM (mean ± S.D.; n=13). After incubation for 3h in RPMI the ATP concentration was 3.6±2.6mm (mean ± S.D; n=20). The ATP concentration of L1210 cells incubated for 3h with 10^{-5}M HN2 was 5.5±2.8 mM (mean ± S.D.; n=20), or 153 ± 9 % (mean ± S.D.; n=20) of the ATP concentration of control L1210 cells at the same time point in each experiment. Total cellular ATP was unchanged by incubation with HN2; the apparent increase in intracellular ATP concentration was due to a reduction of cell volume (results 4.8).

4.12.3 Discussion

The basis of the assay for ATP measurement is shown in figure 66. Firefly luciferase catalyzes activation of D-luciferin by ATP and its oxidation. Light is released upon transition of oxyluciferin to its ground state, and the assay conditions have been manipulated such that light emission occurs slowly, and at a constant intensity which is proportional to ATP concentration (Lundin et al, 1976). A photomultiplier tube in the luminometer converts the

Figure 65

The relationship between intensity of luminescence of the firefly luciferase-luciferin reaction and ATP content of standard solutions.

A typical result; each point is the mean of triplicate readings.

Figure 66

Schematic diagram of the mechanism of the firefly luciferase-luciferin reaction.

Firefly luciferase, E, catalyzes activation of the D-luciferin (LH_2) by ATP and subsequent oxidation to the excited state (L^*). Light is released upon the transition of luciferin to the ground state (L).

Figure 67

The relationship between rate of change of absorbance and glutathione content of standard solutions.

A typical result; each point is the mean of duplicate readings.

Figure 65

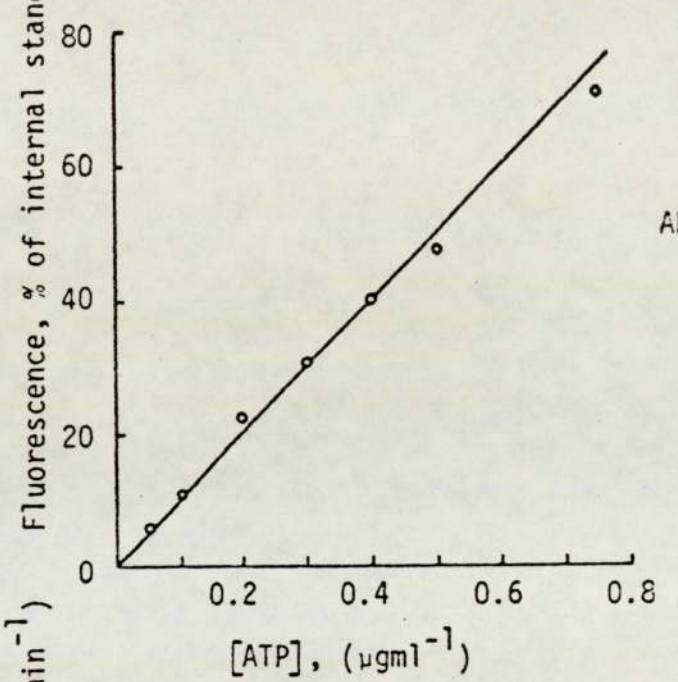


Figure 66

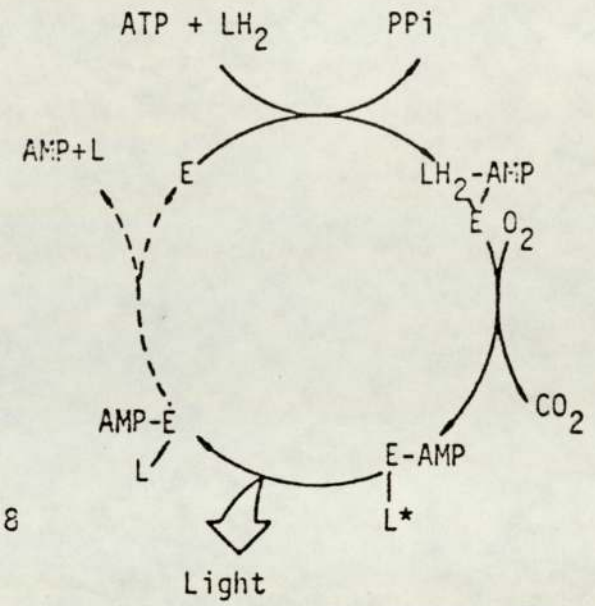
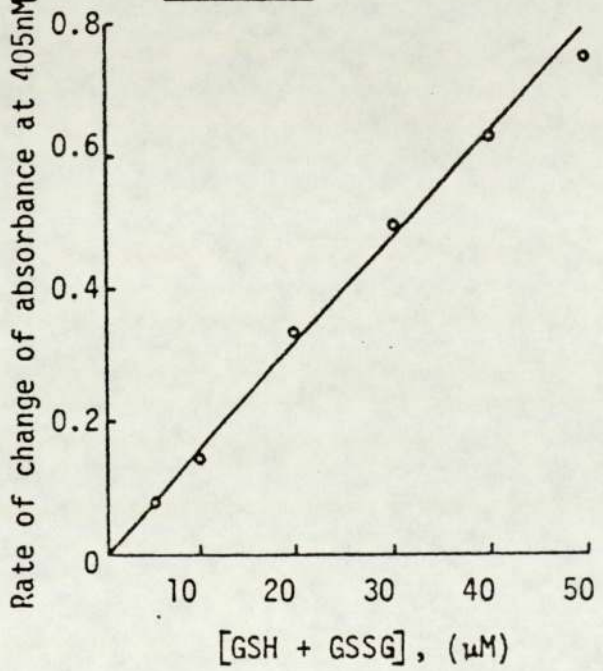


Figure 67



emitted light to a potential difference, which is detected by a chart recorder calibrated against an internal standard of the luminometer.

The large standard deviation of the results of 20 determinations reveal the high inter-assay variation of intracellular ATP concentrations. Intra-assay variation was much lower; each sample was assayed two or three times and individual results were within 5% of the mean. The small intra-assay variation is also well illustrated by the small standard deviation from the mean of the ATP concentration of HN2-treated cells expressed as a percentage of the control at the same time point of each experiment (153 ± 9%).

The lack of effect of HN2 on the total ATP content of L1210 cells suggested that HN2 did not interfere with any cellular process which generated or consumed ATP; or that, if it did, cellular mechanisms were able to maintain normal ATP concentration.

It has been reported that ATP supply is not rate-limiting to the activity of the Na^+K^+ATPase of HeLa cells (Pollack et al, 1981), but there is more recent evidence that ouabain-sensitive $^{86}Rb^+$ influx is proportional to total ATP content of HeLa cells (Ikehara et al, 1984). The results of the present study suggest that, within the range of change of ATP concentrations observed here, the ATP concentration was not rate-limiting to the activity of the Na^+K^+ATPase of L1210 cells. Firstly, the activity of the Na^+K^+ATPase, estimated by the initial rate of ouabain-sensitive $^{86}Rb^+$ influx, was not elevated in HN2-treated cells (results 4.3.1.2) which are now shown to have elevated ATP concentrations. Secondly, the initial rate of total $^{86}Rb^+$ influx into L1210 cells incubated in the absence of HN2 did not change significantly after 3h (figure 30), but the present results show that intracellular ATP concentration was depleted during this incubation.

A study of $^{86}Rb^+$ fluxes in HeLa cells (Ikehara et al, 1984) also revealed a tendency for $^{86}Rb^+$ influx in the presence of ouabain to increase with an increase in ATP content, although the authors did not call attention to it. Reduction in ATP content has been shown to reduce $Na^+K^+Cl^-$ cotransport in several systems (introduction 1.2.2). The $Na^+K^+Cl^-$ cotransport activity of squid axon was not supported by the replacement of ATP by hydrolysable or non-hydrolysable analogues (Russell, 1980). Furosemide had no effect on ATP consumption by Ehrlich cells (Geck et al, 1980). These results imply a regulatory function for ATP in the activity of $Na^+K^+Cl^-$ cotransport. In some systems, ATP may be involved in a phosphorylation event in the regulation of $Na^+K^+Cl^-$ cotransport (introduction 1.2.2), but there appears to be heterogeneity of the regulatory mechanisms for cotransport activity of different cells (introduction 1.2.1). Therefore caution must be excercised when the results from any study are extrapolated to other cell types. Consequently, the results presented for this study show that the inhibition of bumetanide-sensitive $^{86}Rb^+$ influx into HN2-treated L1210 cells was not caused by a depletion of cellular ATP; however, the same results do not eliminate the possiblity that the cotransporter was inhibited by the elevated cellular ATP concentrations. However, the possibility may be ruled out because there is no correlation between intracellular ATP concentration and inhibition of $^{86}Rb^+$ influx; after incubation for 3h with 10^{-5}M HN2, the total $^{86}Rb^+$ influx was inhibited not only in comparison to controls at the same time point, which had comparatively lower ATP levels, but also in comparison to controls at the initial time point, which had higher ATP levels.

These results suggest that inhibition of the cotransporter of L1210 cells after incubation with 10^{-5}M HN2 was unrelated to changes in intracellular ATP concentration, and that the activity of the cotransporter of L1210 cells is not subject to close regulation by cellular ATP . The latter of these conclusions may be confirmed by measurements of diuretic-sensitive $^{86}Rb^{+}$ influx into L1210 cells in response to the manipulation of intracellular ATP concentrations, by the methods of Ikehara et al (1984).

Changes in cellular ATP concentrations may also produce changes in cAMP concentrations (Tisdale, 1974; Kaplan, 1978). The $Na^{+}K^{+}Cl^{-}$ cotransporters of some cell types appear to be sensitive to regulation by changes in cAMP concentrations (introduction 1.2.1) The results described here, showing no correlation between $^{86}Rb^{+}$ influx and intracellular ATP concentrations may provide further indirect evidence to support the view that the $Na^{+}K^{+}Cl^{-}$ cotransporter of L1210 cells is not regulated by changes in cAMP concentrations (results 4.2.5). The mechanisms of regulation of cotransport in L1210 cells and the possible effects of nitrogen mustard on these mechanisms will be discussed in further detail in section 5.

4.13 THE EFFECT OF NITROGEN MUSTARD ON INTRACELLULAR GLUTATHIONE CONCENTRATION IN L1210 CELLS

4.13.1 Introduction

The tripeptide glutathione, γglutamylcysteinylglycine, is important for such processes as the detoxification of xenobiotics, removal of free radicals, the maintenance of sulphydryl groups in proteins, and the regulation of enzyme activity through disulphide interchange (Akerboom and Sies, 1981). Glutathione may be involved in the detoxification of alkylating agents (introduction 1.1.3), and several studies have suggested that HN2-resistant cells may have elevated non-protein sulphydryl levels (Brockman, 1974). It is possible that incubation with HN2 may deplete cellular glutathione concentrations, which may in turn cause changes in the sulphydryl status of proteins and enzymes. Some of the proteins affected may be those responsible for membrane transport processes, several of which have been shown to be sensitive to agents which bind to sulphydryl groups. The total glutathione (reduced and oxidised forms) content of L1210 cells was assayed after incubation for 3h in RPMI or KR, with and without 10^{-5}M HN2. RPMI contains $1mgl^{-1}$ glutathione, and was able to sustain cell survival for longer periods than the simple buffer KR (results 4.1.3.3). A concomitant measurement of cell volume allowed calculation of the intracellular glutathione concentration.

4.13.2 Results

Figure 67 shows the relationship between the rate of change of absorbance (units per minute) and the glutathione content of standard solutions. Samples were assayed at appropriate dilutions to produce reaction rates of between 0.1 and 0.6 absorbance units min^{-1}, over which interval the rate of reaction was directly proportional to the

glutathione content. The concentration of "glutathione equivalents" (see discussion 4.13.3) in L1210 cells was 5.2±0.4mM and 5.6±1.9mM after 3h incubation in KR in the absence and presence respectively, of 10^{-5}M HN2, and 3.9±1.4mM and 6.9±0.7mM after incubation in RPMI in the absence and presence, of HN2. (All values are mean ± S.D., of three determinations).

4.13.3 Discussion

The assay for the measurement of glutathione exploits the following reactions (Akerboom and Sies, 1981):-

$$2GSH + DTNB \xrightarrow{\text{non-enzymic}} GSSG + 2TNB$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2GSH + NADP^+$$

the sum of which is:

$$NADPH + H^+ \; DTNB \xrightarrow[\text{GSSH reductase}]{\text{GSSG/GSH}} 2TNB + NADP^+$$

Catalytic amounts of reduced (GSH) and oxidised (GSSG) glutathione caused the reduction of DTNB by NADPH (Deakin, 1963), and the formation of TNB (5-thio-2-nitrobenzoate) was followed spectrophotometrically.

The results are expressed as "glutathione equivalents" i.e., GSH + 2GSSG. It was assumed that no other thiols interfered with the assay and that glutathione reductase was specific for its substrate. Excess cysteine has been shown to be without effect on the results of the assay (Akerboom and Sies, 1981).

The values obtained for intracellular glutathione content were in good agreement with the results obtained previously for L1210 cells (Suzukake et al, 1982; 1983) and CHO cells (Begleiter et al, 1983). In both cell lines, resistance to the alkylating agent, melphalan (L-phenylalanine mustard), corresponded to an increased intracellular glutathione concentration. The ability of resistant

L1210 cells to dechlorinate the drug corresponded to the glutathione content, and was inhibited by N-ethylmaleimide (Suzukake *et al*, 1983). Endresen *et al* (1983) showed that increased resistance of human epithelial and mouse fibroblastic cells to chlorambucil was associated with increased cellular concentrations of metallothioneins, a cysteine-rich group of low molecular weight proteins (Kagi *et al*, 1980). The presence of thiol-containing compounds in incubation media has been shown to protect several different cell types against the toxic effects of HN2 *in vitro* (introduction 1.1.2). Elevated cellular sulphydryl concentrations have been implicated in a mechanism of resistance to HN2 (Brockman, 1974) and other alkylating agents (introduction 1.1.2).

Nitrogen mustard had no effect on the total intracellular glutathione concentration of L1210 cells; thus the cells were able to maintain glutathione levels, even if some glutathione had reacted with HN2. From this result, it seems unlikely that the toxicity of HN2 to L1210 cells is mediated by a glutathione-dependent pathway. Nitrogen mustard reacts covalently with sulphydryl groups (introduction 1.1.4.1); it seems unlikely that the redox status of glulathione would be altered by HN2. Such changes may have consequences for the re-dox status of protein sulphydryl groups, including those important to the operation of membrane transport systems. From the results described here, which show no significant fall in concentrations of cellular non-protein sulphydryl compounds after incubation with nitrogen mustard, it was concluded that the effects of HN2 on membrane transport processes were not mediated *via* a change in the cellular non-protein sulphydryl content.

4.14 THE EFFECT OF NITROGEN MUSTARD ON THE TRANSPORT OF AMINO ACIDS INTO L1210 CELLS.

4.14.1 THE EFFECT OF NITROGEN MUSTARD ON THE TRANSPORT OF [^{14}C]-α-AMINOISOBUTYRIC ACID

4.14.1.1 The influx of [^{14}C]-α-AIB into L1210 cells incubated in RPMI or Krebs-Ringer buffer.

4.14.1.1.1 Introduction

Previous results had shown that the transport of [^{14}C]-α-AIB into PC6A cells was inhibited after incubation with 10^{-5}M HN2 in RPMI for 4h (results 4.1.1.2) The experiment was repeated with L1210 cells. However, reaction of HN2 with certain components of RPMI medium may have effectively reduced the concentration of HN2 to which the cells were exposed (results 4.1.3.3.3). In addition the amino acids in RPMI would compete for transport with radiolabelled [^{14}C]-α-AIB. It was desirable to measure the effect of HN2 on [^{14}C]-α-AIB transport into L1210 cells incubated in KR buffer.

Aminoisobutyric acid has been used as a marker for Na^+-dependent amino acid transport, but it may not be absolutely specific (discussion 4.14.1.1.3 below), and so non-radiolabelled cycloleucine was added to the assay in an attempt to reduce the transport of [^{14}C]-α-AIB by Na^+-independent mechanisms.

4.14.1.1.2 Results

Figure 68 shows the transport of [^{14}C]-α-AIB into L1210 cells incubated in RPMI or KR. The initial rate of [^{14}C]-α-AIB uptake was 276% greater into L1210 cells incubated in KR rather than RPMI, consistent with the absence of competing amino acids. The ability of L1210 cells to exclude trypan blue during incubation in KR is shown in Table 5 (results 4.1.3.3); incubation time was limited to a maximum of 3h, because of the limited survival of L1210 cells in KR

Figure 68

The uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in RPMI or Krebs-Ringer buffer.

Key: ○ RPMI

● Krebs-Ringer buffer (Mean ± S.D; n=3)

Figure 69

The uptake of [^{14}C]-α-aminoisobutryic acid into L1210 cells incubated in Krebs-Ringer buffer in the presence or absence of cycloleucine.

Key: ○ control

● 15 minute preincubation, 0.1 mgml^{-1} cycloleucine

▲ simultaneous addition, 0.1 mgml^{-1} cycloleucine

(Mean ± S.D.; n=3)

Figure 70

The effect of preincubation for 4h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in RPMI.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=4)

Figure 68

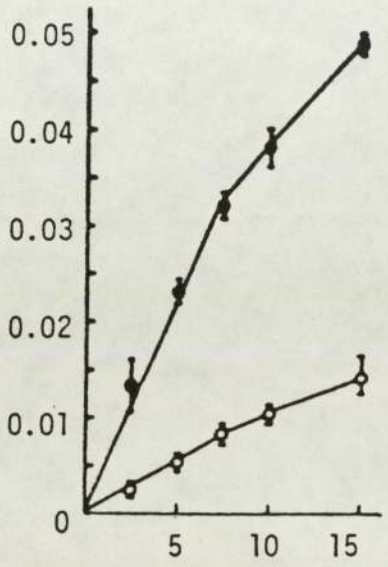


Figure 69

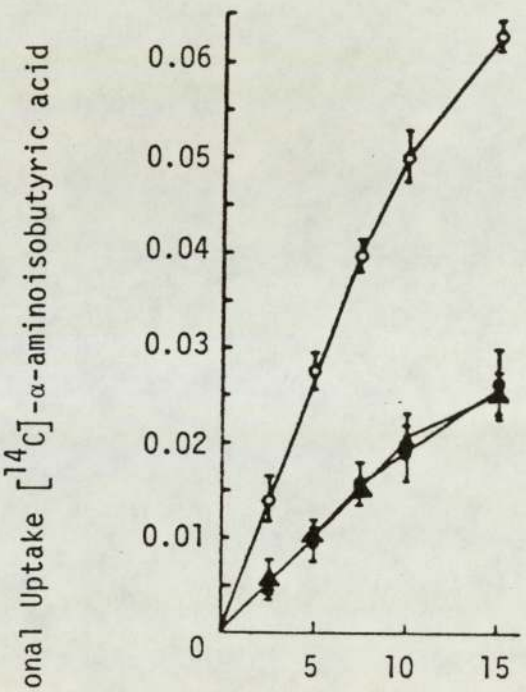
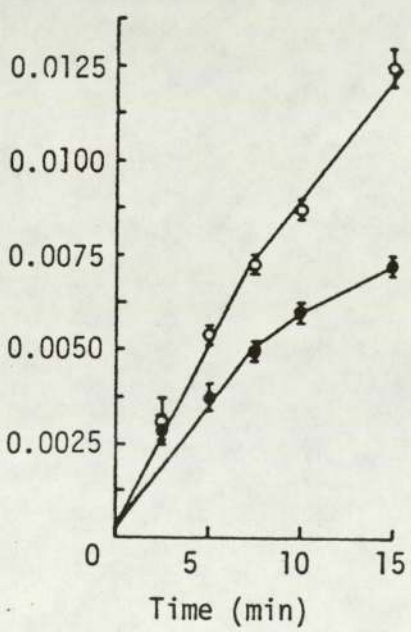


Figure 70



buffer. Figure 69 shows the accumulation of [^{14}C]-α-AIB after addition of 0.10mgml^{-1} cycloleucine, 15 minutes before, or simultaneously with, the addition of [^{14}C]-α-AIB; the initial rate of [^{14}C]-α-AIB transport was reduced by 60% in both cases. Cycloleucine was added at 0.10mgml^{-1} to mimic the leucine and isoleucine content of RPMI (Moore et al, 1967). The addition of 0.08 or 0.09mgml^{-1} cycloleucine produced very variable reduction in [^{14}C]-α-AIB uptake; the addition of 0.15 or 0.20mgml^{-1} cycloleucine reduced [^{14}C]-α-AIB uptake by little more than 60% (for clarity, this data is not shown in figure 69). In subsequent assays, L1210 cells were incubated for the required time in KR, with and without drug, then 0.1mgml^{-1} cycloleucine was added immediately prior to the addition of [^{14}C]-α-AIB. The influx of [^{14}C]-α-AIB into L1210 cells was measured as described in the methods.

4.14.1.1.3 Discussion

At least six distinct systems for the transport of neutral amino acids have been identified in the plasma membranes of mammalian cells (Shotwell et al, 1983; table 2; introduction 1.2.1). For Ehrlich ascites cells, five of these systems have deen described as follows (Christensen 1973; 1975; Shotwell et al, 1983); a Na^+-dependent, glycine specific system; the 'A' system, which requires Na^+ and metabolic energy, and has broad specificity; the 'ASC'system which requires Na^+ but not metabolic energy; the 'β' system, for transport of β-amino acids, which requires Na^+;and the 'L' system, which is Na^+-independent and has broad specificity. Additional systems exist for the transport of dibasic and diacidic amino acids (Shotwell et al, 1983). The discrimination between the transport systems is not absolute, and amino acids may be transported by multiple routes (Wallach, 1975). For example, multiple routes for the transport of

αAIB have been demonstrated in many tissues (Shotwell et al, 1983). Vistica (1979) reported that only 75% of [^{14}C]-α-AIB transport into L1210 cells was Na^+-dependent, and 13% of leucine transport was Na^+-dependent. αAIB was transported into Ehrlich acites cells through the activites of the 'A', 'ASC' and 'L' systems, with estimated relative rates of 2:3:2 respectively (Wallach,1975). In the same cells, leucine was transported by the 'L' and 'A' systems with relative rates of 4:1 respectively (Christensen, 1973; 1975; Wallach, 1975). There appear to be tissue-specific differences in the specificity of the transport systems. For example; Na^+ is a requirement for the transport of cycloleucine into some cell types but not others (Wallach, 1975).

Further measurements of of [^{14}C]-α-AIB transport into L1210 cells were made in the presence of unlabelled cycloleucine. Unlabelled αAIB was present when transport of [^{14}C]-cycloleucine was measured. The transport systems of the L1210 cells used in the present study probably were not absolutely specific for the transport of αAIB and cycloleucine by Na^+-dependent and Na^+-independent mechanisms respectively (Vistica 1979); therefore, the rates of transport of [^{14}C]-α-AIB and [^{14}C]-cycloleucine were not absolute measurements of the activites of the 'A' and 'ASC', and 'L' systems. It was reasoned that the differences in rates of accumulation of the two amino acids by L1210 cells indicated that different transport systems were preferred, and that the addition of the complementary non-radiolabelled substrate might improve the specificity of the assay for each of the transport systems.

Further definition of the amino acid transport systems in L1210 cells may be achieved by examination of the dependence of transport on extracellular Na^+ and intracellular ATP. The transport systems

were not dissected in greater detail for the present study, since the inhibition of amino acid transport by HN2 was not thought to contribute to its cytotoxicity (results 4.14.1.2.3)

4.14.1.2 The effect of nitrogen mustard on the transport of α-aminoisobutyric acid into L1210 cells from exponentially-growing cultures

4.14.1.2.1 Introduction

Nitrogen mustard inhibited the uptake of [^{14}C]-α-AIB into PC6A cells incubated in RPMI (results 4.1.1.2). The experiment was repeated with L1210 cells. The [^{14}C]-α-AIB transport into L1210 cells was further assayed as above (results 4.14.1.1) after incubation with HN2, furosemide, bumetanide and ouabain, to establish whether the inhibition of K^+ transport systems could result in inhibition of amino acid transport. [^{14}C]-α-AIB transport was measured after incubation with the Ca^{++} ionophore, A23187, or the Na^+ ionophore monensin, so as to investigate the effects of the elevation of intracellular Ca^{++} or Na^+ concentrations.

4.14.1.2.2 Results

Figure 70 shows the transport of [^{14}C]-α-AIB into L1210 cells after incubation for 4h in RPMI with and without 10^{-5}M HN2. Nitrogen mustard caused a 33% inhibition of the initial rate of transport, measured over 0 - 7.5 minutes, during which interval uptake was linear with time. For all assays described below, cells were incubated in KR with and without drug for the required time, then [^{14}C]-α-AIB transport was measured in the presence of 0.1mgml^{-1} cycloleucine.

The transport of [^{14}C]-α-AIB into L1210 cells after incubation with and without 10^{-5}M HN2 for 1,2 or 3h, is shown in figures 71, 72 and 73. [^{14}C]-α-AIB uptake was unchanged after incubation for 1h with

HN2; after 2h, HN2 caused a small (14%) inhibition in initial rate of [^{14}C]-α-AIB uptake, measured over 5 minutes, but a greater effect of HN2 (56% inhibition) was apparent after 15 minutes of transport. The initial rate of [^{14}C]-α-AIB transport into L1210 cells was reduced by 22% after incubation for 3h with 10^{-5}M HN2.

Pre-incubation for 15 minutes with 10^{-3}M furosemide, bumetanide, or HN2 had no effect on the initial rate of [^{14}C]-α-AIB transport into L1210 cells (figure 74). Incubation for 3h with 10^{-4}M bumetanide had no effect on [^{14}C]-α-AIB transport (figure 75).

Pre-incubation for 15 minutes with 10^{-3}M ouabain or 2×10^{-5}M monensin caused inhibition of [^{14}C]-α-AIB transport into L1210 cells (figure 76). The inhibition of [^{14}C]-α-AIB uptake after incubation with monensin was exacerbated in the presence of 10^{-3}M ouabain (figure 76); this observation was consistent with a further increase of intracellular Na^+ concentration after incubation with monensin in the presence of ouabain (table 23).

A 15-minute preincubation with 10^{-6}M A23187 had no significant effect on [^{14}C]-α-AIB transport into L1210 cells (figure 77).

Figure 71

The effect of preincubation for 1h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ○ control

2 ● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 72

The effect of preincubation for 2h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ○ control

2 ● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 73

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ○ control

2 ● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 74

The effect of preincubation for 15 minutes with furosemide, bumetanide or nitrogen mustard on the uptake of [^{14}C]-α-aminoisobutryic acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ○ control (n=13)

2 ● 10^{-3}M HN2 (n=3)

3 □ 10^{-4}M bumetanide (n=3)

4 ■ 10^{-3}M furosemide (n=3)

(Mean ± S.D.)

Figure 71

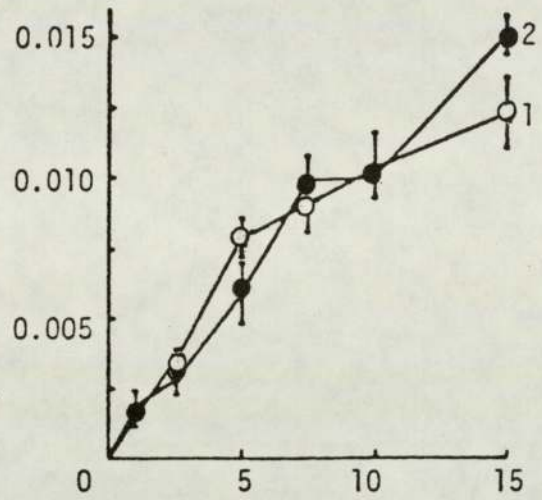


Figure 72

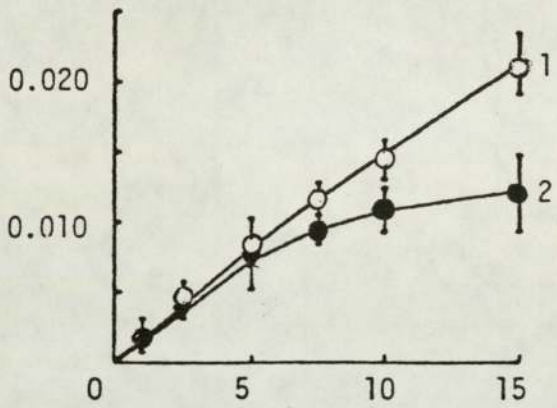


Figure 73

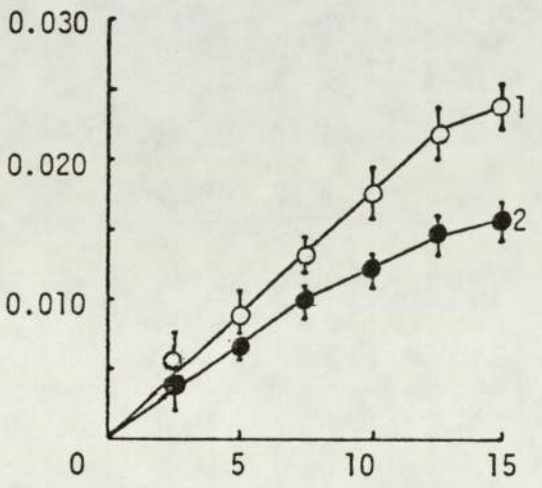
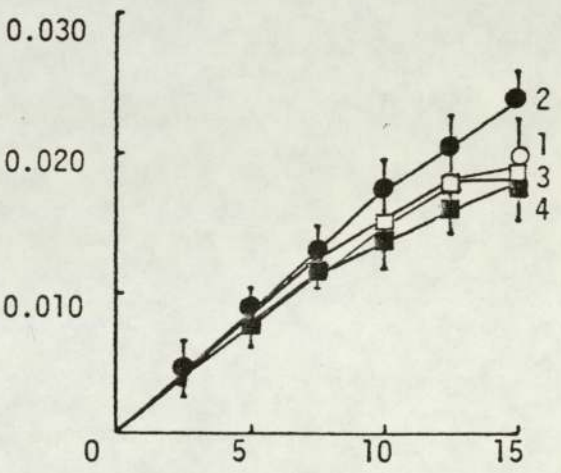


Figure 74



Fractional Uptake [^{14}C]-α-aminoisobutyric acid

Time (min)

Figure 75

The effect of preincubation for 3h with 10^{-4}M bumetanide on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1● control

2□ 10^{-4}M bumetanide (Mean ± S.D.; n=3)

Figure 76

The effect of preincubation for 15 minutes with ouabain and monensin on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1○ control (n=13)

2□ 2 x 10^{-5}M monensin (n=4)

3■ 2 x 10^{-5}M monensin + 10^{-3}M ouabain (n=3)

4● 10^{-3}M ouabain (n=6)

(Mean ± S.D.)

Figure 77

The effect of preincubation for 15 minutes with calcium ionophore A23187 on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: □ control (n=13)

● 10^{-6}M A23187 (n=3) (Mean ± S.D.)

Figure 78

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells from stationary cultures incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 75

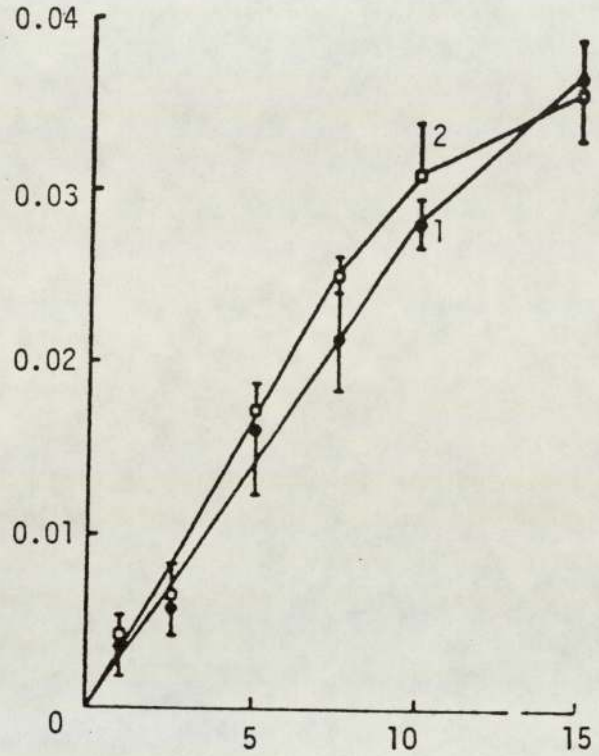


Figure 76

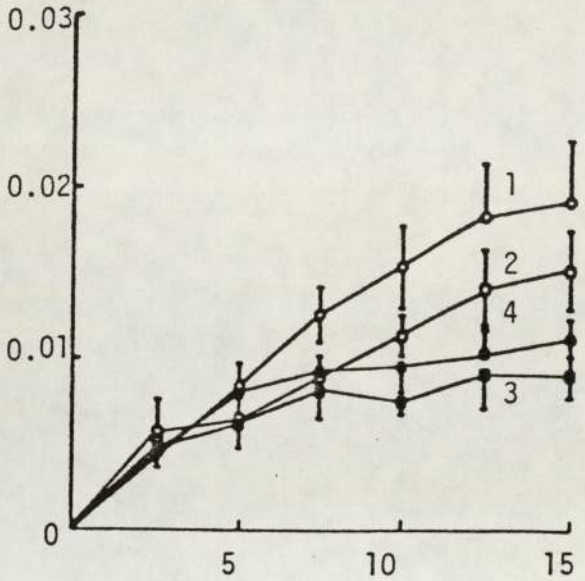


Figure 77

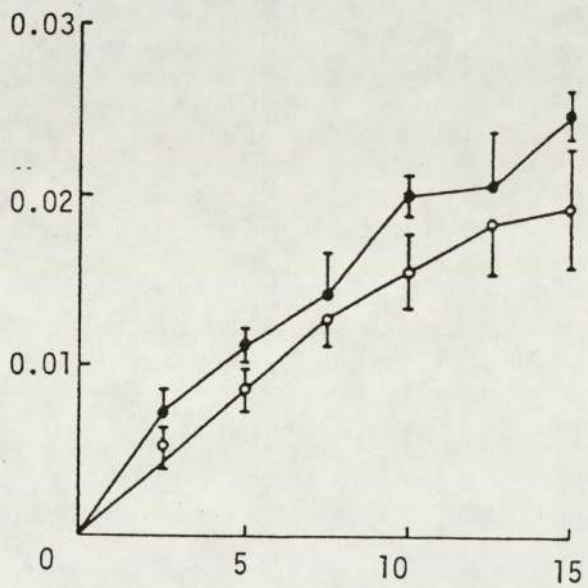
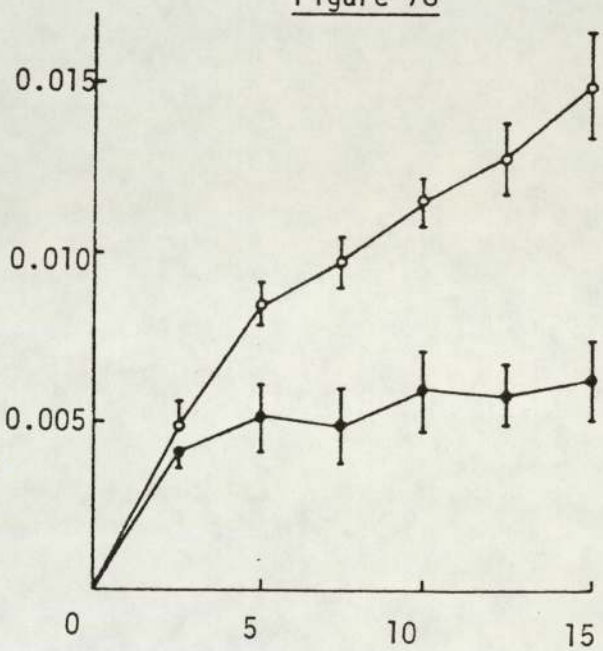


Figure 78



Fractional Uptake [^{14}C]-α-aminoisobutyric acid

Time (min)

Table 23

The effect of incubation for 15 minutes with monensin, with and without ouabain, on intracellular Na^+ concentrations of L1210 cells

Incubation	Na^+ concentration, mM
Control	23.1 ± 3.7
Monensin ($2x10^{-5}M$)	191.7 ± 13.6
Monensin ($2x10^{-5}M$) + ouabain ($10^{-3}M$)	221.1 ± 17.1

(Mean ± S.D.; n=6)

Na^+ concentration was determined by equilibrium distribution of $^{22}Na^+$ (methods 3.8.2).

4.14.1.2.3 Discussion

The initial rate of transport of [^{14}C]-α-AIB into PC6A cells was inhibited by 25% by 10^{-5}M HN2 after incubation for 4h in RPMI (results 4.1.1.2). The system(s) for AIB transport into L1210 cells was also sensitive to HN2; under the same conditions the initial rate of uptake of [^{14}C]-α-AIB was inhibited by 33%. The transport of [^{14}C]-α-AIB into L1210 cells was investigated in more detail.

The inhibition of [^{14}C]-α-AIB transport by 10^{-5}M HN2 was slow in onset; the earliest effect was observed after incubation for 2h. In contrast, there was a substantial inhibition of $^{86}Rb^+$ influx (results 4.3) and a reduction of cell volume (results 4.8) after incubation for only 1h under the same conditions. After the addition of 10^{-3}M HN2, immediate inhibition of $^{86}Rb^+$ influx was observed (results 4.1.1.4); no such immediate effect upon [^{14}C]-α-AIB influx was found. These results suggested that inhibition of [^{14}C]-α-AIB transport into L1210 cells was not a primary effect of HN2, and was

independent of the inhibition of $^{86}Rb^{+}$ influx. The possibility was considered that HN2 inhibited amino acid transport by some indirect mechanism.

Nitrogen mustard inhibited the function of the $Na^{+}K^{+}Cl^{-}$ cotransporter of L1210 cells (results 4.3.1.2). However, it was considered unlikely that inhibition of the cotransporter per se lead to the inhibition amino acid transport, since amino acid transport was unchanged after short, or more prolonged, periods of incubation with bumetanide or furosemide.

The activity of the $Na^{+}K^{+}$ATPase contributes to the maintenance of the Na^{+} gradient across the cell membrane (introduction 1.2.1). The Na^{+} gradient is exploited to provide the potential energy for the accumulation of some amino acids (introduction 1.2.1; results 4.14.1.1.3). The results described here suggest that the transport of [^{14}C]-α-AIB into L1210 cells was at least partly dependent upon Na^{+}, and the activity of the $Na^{+}K^{+}$ATPase, and are in good agreement with the findings of Vistica (1979).

The $Na^{+}K^{+}$ATPase inhibitor ouabain also inhibited [^{14}C]-α-AIB transport into L1210 cells. The elevation of intracellular Na^{+} concentration after incubation with monensin was similarly accompanied by an inhibition of [^{14}C]-α-AIB uptake. When Na^{+} extrusion through the activity of the $Na^{+}K^{+}$ATPase was blocked by 10^{-3}M ouabain, monensin caused a greater elevation of intracellular Na^{+} concentation, and, accordingly, a greater inhibition of [^{14}C]-α-AIB uptake. However, HN2 did not inhibit ouabain sensitive $^{86}Rb^{+}$ uptake (results 4.3.1.2), or cause elevations of intracellular Na^{+} concentration (results 4.7). Thus it was not feasible that these mechanisms could account for the inhibition of [^{14}C]-α-AIB uptake which was observed after incubation with HN2.

The addition of the calcium ionophore A23187 had no effect on [^{14}C]-α-AIB transport into L1210 cells. The consideration of this result together with the result after the addition of monesin, leads to the conclusion that the inhibition of amino acid transport by HN2 was not mediated by an elevation of intracellular free Ca^{++}, which in turn activated the Na^{+}/Ca^{++} exchanger to elevate the intracellular Na^{+} concentration. Changes in intracellular free Ca^{++} are thought to be involved in the hormone-induced stimulation of the 'A' system for amino acid transport (Shotwell et al, 1983; Kelley et al, 1980). The lack of effect of A23187 on [^{14}C]-α-AIB influx into L1210 cells suggested that the transport system(s) involved was not sensitive to changes in Ca^{++} concentration.

Amino acid transport systems may also be sensitive to changes in intracellular pH (see table 2). At low pH there is generally inhibition of the 'A' system, stimulation of the 'L' system, and, in some tissues, inhibition of the 'ASC' system (Shotwell et al, 1983). The lack of effect of HN2 on intracellular pH (results 4.11) suggested that the inhibition of [^{14}C]-α-AIB transport was not mediated by pH changes. In addition, HN2 did not stimulate the transport of cycloleucine (results 4.14.2.2).

The activities of the amino acid transport systems may be subject to "trans" regulation; that is, the presence of the substrate for the system on the intracellular side of the membrane may have an effect upon the transport of further substrate (Shotwell et al, 1983; table 2). Generally, the 'A' system is "trans"-inhibited, but the 'ASC' and 'L' systems are "trans"-stimulated. The reduction of cell volume of L1210 cells after incubation with HN2 (results 4.8) may give rise to an increase in the intracellular concentrations of amino acids. The possibility that this may inhibit the 'A' system for

[^{14}C]-α-AIB uptake seemed unlikely, since HN2 did not stimulate the 'L' system for cycloleucine transport (results 4.14.2.2).

The 'A' system is stimulated by amino acid deprivation (Shotwell et al, 1983). The phenomenon is demonstrated by the observation that [^{14}C]-α-AIB transport was increased with time during incubation of L1210 cells in KR in the absence of HN2 (figures 71, 72 and 73). The 'A' system of many cells appears to be sensitive to cAMP (Shotwell et al, 1983) which may have a role in the hormone-induced stimulation of amino acid transport. Although increases in cAMP generally stimulate amino acid transport (Shotwell et al, 1983), some workers have found that an elevation of cAMP caused inhibition of amino acid transport; the response to cAMP may exhibit tissue-specific heterogeneity. The alkylating agent Trenimon (2,3,5-tris(ethyleneimino)benzoquinone) elevated cAMP levels and inhibited αAIB transport in Ehrlich ascites cells (Ihlenfeldt et al, 1981). The transport of melphalan by the amino acid transport systems of L1210 cells (Vistica, 1979) was inhibited both by aminophylline, which elevated cAMP levels by inhibition of phosphodiesterase, and by HN2 (Martin et al, 1982). Nitrogen mustard has been shown to increase cellular cAMP levels (introduction 1.1.4.5) so the possibility cannot be ignored that the inhibition of AIB transport was mediated by changes in intracellular cAMP concentrations. In addition, the failure of HN2 to inhibit αAIB transport into the L1210R murine leukaemia cell line resistant to nitrogen mustard (results 4.16.3) forces further consideration to be given to this possibility; it will be discussed further in section 5. However, chlorambucil, which has been shown to elevate cAMP levels (introduction 1.1.4.5) had no effect on [^{14}C]-α-AIB transport into L1210 cells (results 4.18) or on melphalan accumulation by L1210 cells (Martin et al, 1982).

Kwock and his coworkers have shown that a membrane sulphydryl group was involved in the modulation of Na^+-dependent transport of [^{14}C]-α-AIB into rat thymocytes (Kwock *et al*, 1976; 1979) and thymocyte membrane vesicles (Kwock, 1981). It was suggested (Kwock *et al*, 1976) that transport was stimulated when the sulphydryl groups were present in disulphide form, and inhibited when the groups were reduced. Kwock (1981) showed that addition of GSH to membrane vesicles decreased [^{14}C]-α-AIB uptake, and that GSSG stimulated transport. It was proposed that glutathione may maintain the oxidation state of the sulphydryl groups through non-enzymic sulphydryl-disulphide interchange (White *et al*, 1964). Na^+-dependent amino acid transport into chick embryo fibroblasts (Smith-Johannson *et al*, 1977) and *Mycobacterium* vesicles (Jacobs *et al*, 1978) was stimulated by agents which oxidised sulphydryl groups. The lack of effect of HN2 on total glutathione content of L1210 cells (results 4.13) suggested that the inhibition of [^{14}C]-α-AIB transport was not mediated by a loss of cellular glutathione. However, the effect of HN2 on the oxidation status of cellular glutathione, which may influence the sulphydryl status of proteins, has yet to be examined (results 4.13.3).

Agents which bind to sulphydryl groups have been shown to inhibit the activity of Na^+-dependent amino acid transport systems in membrane vesicles from 3T3 cells (Lever, 1977) and intestinal brush borders (Will and Hopfer, 1979). The sulphydryl binding agent, *p*-hydroxymercuribenzoate inhibited the transport of the antitumour drug melphalan into L1210 cells (Redwood and Colvin, 1980), which has been shown to be mediated by partly Na^+-dependent amino acid transport systems (Vistica, 1979). The sulphydryl groups involved in Na^+-dependent amino acid transport may be located in the transport

systems or in the proteins which regulate ion gradients (Kwock, 1981; Jacobs et al, 1978; Lever, 1977). Such groups may present a target for the action of HN2, which readily reacts with sulphydryl groups (Wheeler, 1962; 1967; introduction 1.1.4.1). Reaction with only one of a pair of sulphydryl groups would presumably prevent disulphide formation. However, the monofunctional analogue of HN2 had no effect on [^{14}C]-α-AIB uptake into L1210 cells (results 4.17) which suggested that a cross-linking reaction was crucial. The slow onset of inhibition of amino acid transport also argued against a direct interaction of HN2 with the transport system (but see also results 4.16.3.3).

It has been suggested that the action of hormones to stimulate the activity of the 'A' system for amino acid transport may be mediated by an increase in the membrane potential. Whilst it is not established whether all of the Na^+-dependent amino acid transport systems would be sensitive to membrane potential, it is difficult to explain the selective effect of hormones on the 'A' system (reviewed by Shotwell et al, 1983). The inhibition of [^{14}C]-α-AIB uptake after incubation with HN2 did not appear to be caused by membrane depolarisation (results 4.10).

Nitrogen mustard and some of its analogues depress the incorporation of a variety of amino acids into several cell types in vivo and in vitro (Wheeler, 1962; 1967). Ankel et al (1982) showed that HN2 inhibited the transport of αAIB into rat L5178Y cells, but the inhibition did not appear to be dose-dependent. Ihlenfeldt et al (1981) showed that the alkylating antitumour agent Trenimon inhibited the influx of [^{14}C]-α-AIB and [^{14}C]-cycloleucine into Ehrlich ascites cells. HN2 also depressed the accumulation of melphalan by L1210 cells (see above).

The inhibition of amino acid transport by HN2 may have no biological significance, since protein synthesis may continue for prolonged periods after incubation with HN2 (introduction 1.1.4.2). For example, Brewer et al (1961) reported that when mouse fibroblasts were incubated with HN2, protein synthesis continued for several days, even after formation of "giant" cells. Levis et al (1963) showed that protein synthesis was unchanged for the first 24h after incubation of guinea pig kidney cells with HN2. Ihlenfeldt et al (1981) showed that Trenimon inhibited amino acid transport into Ehrlich cells, but it had no effect on non-histone protein synthesis over the same period (Wolf et al, 1973). These results may be interpreted to suggest that the rate of amino acid transport is not the limiting step for the rate of protein synthesis. This does not support the suggestion of Shank and Smith (1976) that small changes in amino acid transport may have rapid consequences for cell metabolism. In addition, there was only incomplete inhibition of [^{14}C]-α-AIB influx into L1210 cells incubated in the simple salt solution, KR buffer; the inhibition is likely to be even smaller under physiological conditions, when the presence of extracellular amino acids, proteins and thiols may reduce the effective concentration of HN2 to which the tumour cells are exposed. The proposal that this lesion may be causal to the cytotoxicity of HN2 would not account for the increased sensitivity of dividing cells to this agent (introduction 1.1.2). In summary, it was considered unlikely that inhibition of amino acid transport alone by HN2 would result in the death of the cells.

4.14.1.3 The effect of nitrogen mustard on the transport of [^{14}C]-α-aminoisobutyric acid into L1210 cells from stationary cultures.

4.14.1.3.1 Introduction

Nitrogen mustard is selectively cytotoxic to dividing cells (introduction 1.1.2) and caused a greater inhibition of $^{86}Rb^{+}$ influx into L1210 cells from exponentially-growing cultures in comparison to those from stationary cultures (results 4.3). The activities of amino acid transport systems in animal cells have been shown to be affected by the presence of growth factors, cell density and position in the cell cycle (Shotwell et al, 1983). If the inhibition of [^{14}C]-α-AIB influx into exponentially-growing L1210 cells (results 4.14.1.2) is related to the inhibition of $^{86}Rb^{+}$ influx, it would be expected that the [^{14}C]-α-AIB influx into stationary cells would be inhibited to a lesser extent, as was the $^{86}Rb^{+}$ influx. This hypothesis was investigated by the measurement of [^{14}C]-α-AIB influx into stationary L1210 cells incubated in KR with and without 10^{-5}M HN2.

4.14.1.3.2 Results

Figure 78 shows the influx of [^{14}C]-α-AIB into stationary L1210 cells after incubation for 3h in KR with and without 10^{-5}M HN2. The initial rate of [^{14}C]-α-AIB transport into stationary cells was lower than the rate of transport into exponentially-growing cells (see figures 74 and 78). The initial rate of [^{14}C]-α-AIB transport into stationary L1210 cells, measured over the 0-5 minute interval, was reduced by 40% after incubation with HN2.

4.14.1.3.3 Discussion

The decline in the rate of amino acid transport into cultured cells as confluency is achieved has been reported before (e.g.

Weber et al, 1984; Wallach 1975; Foster and Pardee, 1969) and is well-illustrated by the present results. The systems for AIB transport into exponentially-growing L1210 cells were stimulated by incubation in amino acid-free medium (figures 76, 77 and 78). These two observations indicate that the depletion of amino acids in the culture medium was not rate-limiting for the growth of L1210 cells in culture as stationary phase was entered.

Nitrogen mustard caused a greater inhibition of [^{14}C]-α-AIB transport into stationary cells than into exponentially-growing cells. Since the stationary culture would contain fewer cycling cells (results 4.1.3.4), and would therefore be less sensitive to the cytotoxicity of HN2, the inhibition of amino acid transport does not appear to be related to the cytotoxic mechanism.

The results described above provide further evidence that the inhibition of amino acid transport by HN2 was unrelated to the inhibition of $^{86}Rb^{+}$ transport (results 4.14.1.2.3). The $^{86}Rb^{+}$ influx into confluent cells was less sensitive to inhibition by HN2 in comparision to influx into exponential cells (results 4.3), yet amino acid transport was more sensitive to HN2.

4.14.2 THE EFFECT OF NITROGEN MUSTARD ON THE TRANSPORT OF [^{14}C]-CYCLOLEUCINE

4.14.2.1 The influx of [^{14}C]-cycloleucine into L1210 cells incubated in RPMI or Krebs-Ringer buffer

4.14.2.1.1 Introduction

Results showed that the transport of [^{14}C]-cycloleucine into PC6A cells was unaffected by incubation for 4h with 10^{-5}M HN2 in RPMI (results 4.1.1.2). This result was confirmed for L1210 cells. For the reasons discussed in section 4.14.1, above, it was desirable to measure the effect of HN2 on [^{14}C]-cycloleucine transport into L1210 cells incubated in amino acid-free medium. The rationale applied to

Figure 79

The uptake of [^{14}C]-cycloleucine into L1210 cells incubated in RPMI or Krebs-Ringer buffer.

Key: ○ RPMI

● Krebs-Ringer buffer (Mean ± S.D.; n=3)

Figure 80

The uptake of [^{14}C]-cycloleucine into L1210 cells incubated in Krebs-Ringer buffer in the presence or absence of α-aminoisobutyric acid.

Key: ○ control

● 15 minute preincubation with 0.3mgml^{-1} α-AIB

(Mean ± S.D.; n=3)

Figure 79

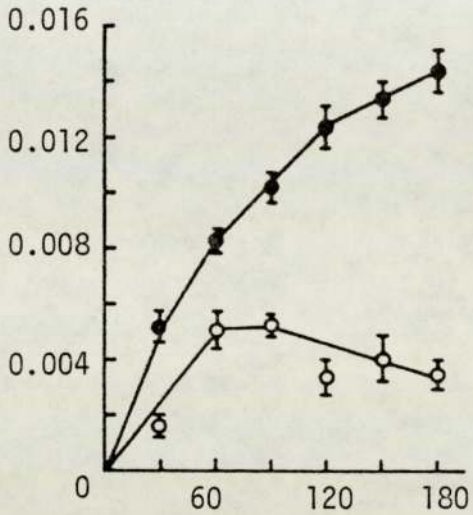
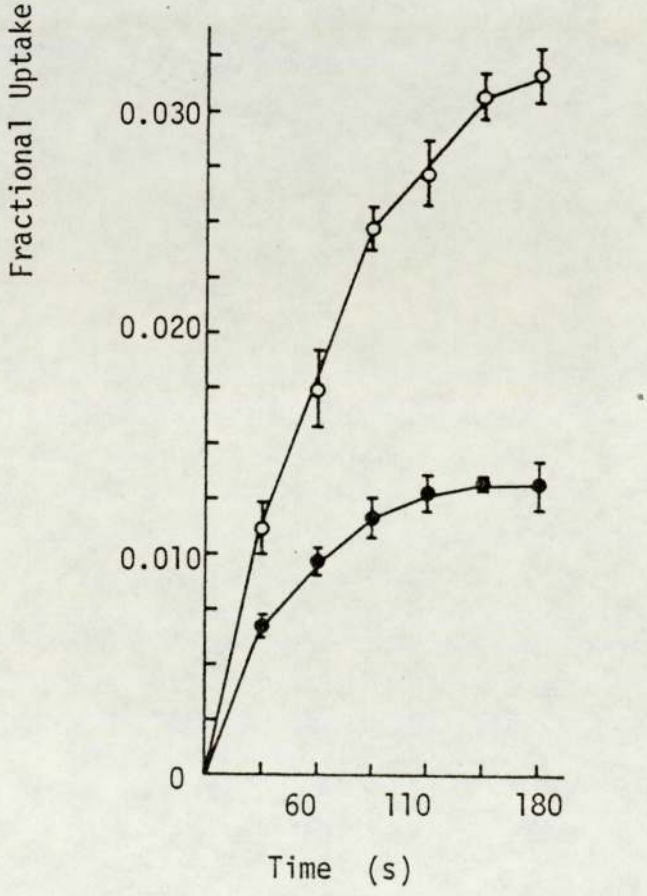


Figure 80



the improvement of the specificity of the assay for [^{14}C]-αAIB transport (results 4.14.1.1) was continued here; the transport of [^{14}C]-cycloleucine was measured in the presence of unlabelled αAIB.

4.14.2.1.2 Results

Figure 79 shows the transport of [^{14}C]-cycloleucine into L1210 cells incubated in RPMI or KR; consistent with the absence of competing amino acids, the influx of [^{14}C]-cycloleucine was greater when cells were incubated in KR rather than RPMI. Incubation time was limited to 3h, during which interval there was no significant increase in the number of cells permeable to trypan blue in comparison to controls (table 5, results 4.1.3.3). Figure 80 shows the accumulation of [^{14}C]-cycloleucine by L1210 cells incubated in KR in the absence and presence of 0.3mgml^{-1} αAIB; the initial rate of transport of [^{14}C]-cycloleucine, measured over 60s, was consistently reduced by 45% in the presence of 0.3 mgml^{-1} αAIB. The addition of 0.2 or 0.1mgml^{-1} αAIB caused very variable reduction of [^{14}C]-cycloleucine transport. For clarity, this data is not shown in figure 80.

4.14.2.1.3 Discussion

Cycloleucine has been used as a marker of the Na^+-independent 'L' system for amino acid transport (Van den Berg and Betel, 1973; Baxter et al, 1982; Ihlenfeldt et al, 1981; Wallach, 1975, and others) but it may not be transported by this system exclusively (see results 4.14.1.1.3). Vistica (1979) showed that 13% of leucine transport into L1210 cells was Na^+-dependent. The very different rates of accumulation of [^{14}C]-cycloleucine and [^{14}C]-αAIB into L1210 cells imply that different transport systems are preferred by these two amino acids (see also results 4.1.1.2). It was reasoned that the specificity of the transport of [^{14}C]-cycloleucine for the 'L' system

might be improved by the addition of unlabelled ∝AIB. Further assays of [^{14}C]-cycloleucine transport were carried out in the presence of 0.3mgml^{-1}∝AIB. Further definition of this system in L1210 cells was not attempted, since the inhibition of amino acid transport by HN2 was not thought to be causal to the cytotoxicity of the drug (results 4.14.1).

4.14.2.2 The effect of nitrogen mustard on the transport of [^{14}C]-cycloleucine into L1210 cells

4.14.2.2.1 Introduction

Nitrogen mustard (10^{-5}M) had no effect on the transport of [^{14}C]-cycloleucine into PC6A cells after incubation for 4h in RPMI (results 4.1.1.2). This experiment was repeated under the same conditions with L1210 cells. Further assays for [^{14}C]-cycloleucine accumulation were carried out for L1210 cells incubated in KR, and 0.3mgml^{-1} ∝AIB was added immediately prior to the addition of [^{14}C]-cycloleucine. The [^{14}C]-cycloleucine transport into L1210 cells was assayed after incubation with HN2, furosemide or ouabain, to examine the consequences of inhibition of K^+ transport upon Na^+-independent amino acid transport.

4.14.2.2.2 Results

Figure 81 shows the transport of [^{14}C]-cycloleucine into L1210 cells after incubation for 4h in RPMI with and without 10^{-5}M HN2. Nitrogen mustard caused a small (7%) inhibition in the transport of [^{14}C]-cycloleucine (measured after 90 seconds).

Figure 82 shows the transport of [^{14}C]-cycloleucine into L1210 cells in the presence of ∝AIB after incubation for 3h in KR with and without 10^{-5}M HN2. Nitrogen mustard had no significant effect on the transport of [^{14}C]-cycloleucine.

Figure 83 shows the transport of [^{14}C]-cycloleucine into L1210

Figure 81

The effect of preincubation for 4h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-cycloleucine into L1210 cells incubated in RPMI.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=3)

Figure 82

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-cycloleucine into L1210 cells incubated in Krebs-Ringer buffer in the presence of α-aminoisobutyric acid.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=4)

Figure 83

The effect of preincubation for 15 minutes with ouabain or furosemide on the uptake of [^{14}C]-cycloleucine into L1210 cells incubated in Krebs-Ringer buffer in the presence of α-aminoisobutyric acid.

Key: 1 ○ control

2 ● 10^{-3}M ouabain

3 ■ 10^{-3}M furosemide (Mean ± S.D.; n=3)

Figure 81

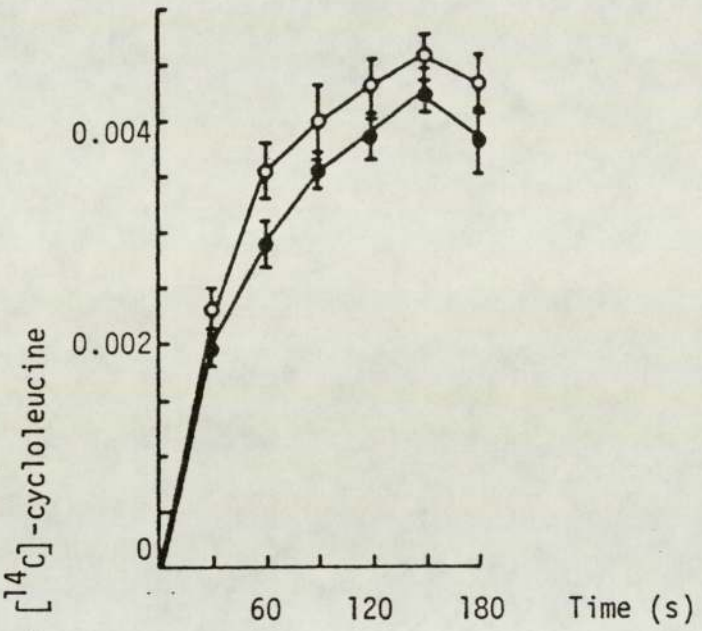


Figure 82

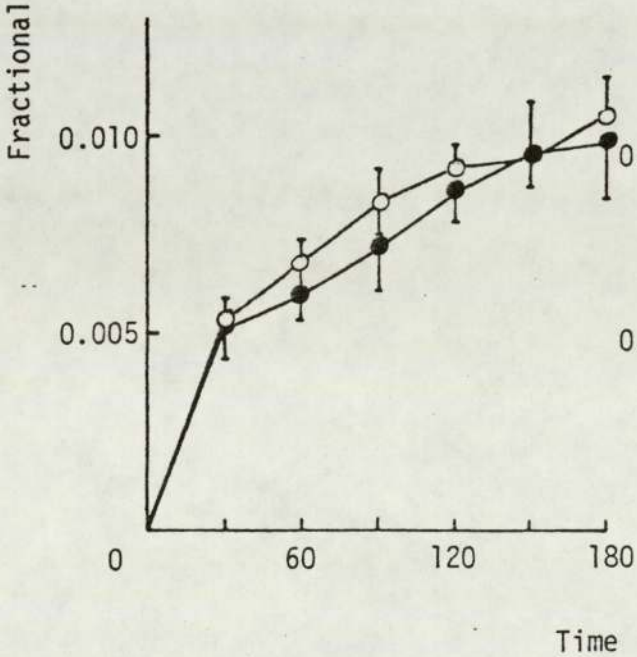
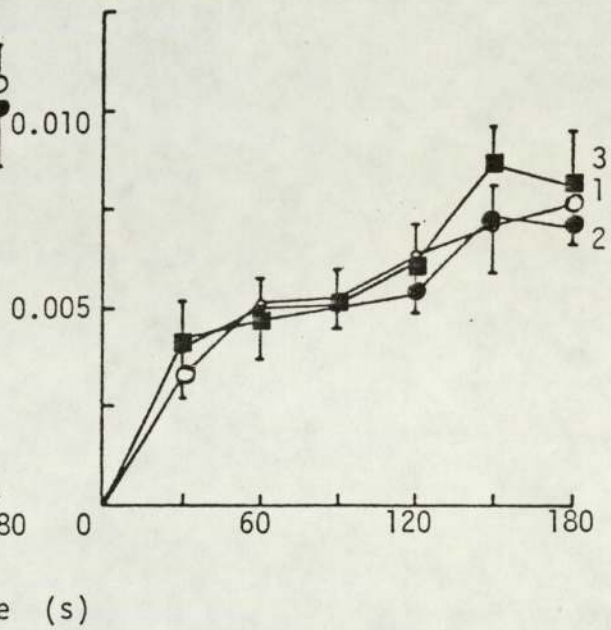


Figure 83



Time (s)

cells in the presence of ∝AIB after incubation for 15 minutes with 10^{-3}M ouabain or 10^{-3}M furosemide. Both ouabain and furosemide were without effect upon the transport of [^{14}C]-cycloleucine.

4.14.2.2.3 Discussion

Cycloleucine is transported into cells predominantly by the 'L' system for amino acid transport (see results 4.14.1.1.3). It was reasoned that the presence of unlabelled ∝AIB would improve the specificity of the measurement of [^{14}C]-cycloleucine as an assay for the activity of the 'L' system in L1210 cells. It was concluded that nitrogen mustard had no effect upon the activity of the 'L' system, and that the reduction in amino acid transport exhibited specificity for the 'A' and 'ASC' systems (results 4.14.1).

Although the 'L' system is reported to be "trans"-stimulated (table 2; see results 4.14.1.2.3), the reduction in cell volume by HN2 or furosemide (results 4.8) had no such effect upon [^{14}C]-cycloleucine transport. The addition of ouabain at a concentration which inhibited Na^+K^+ATPase (results 4.2.2), and presumably elevated intracellular Na^+ concentrations (see results 4.7.3), had no effect on [^{14}C]-cycloleucine accumulation. This observation suggested that cycloleucine transport into L1210 cells was independent of the Na^+ gradient, in contrast to the transport of ∝AIB (results 4.14.1.2). The inhibition of the $Na^+K^+Cl^-$ cotransporter by furosemide also failed to influence [^{14}C]-cycloleucine transport.

It was concluded from these studies that nitrogen mustard selectively inhibited amino acid transport via the Na^+-dependent systems, and that the mechanism for this inhibition was uncertain, but was unlikely to be causal to the cytotoxicity of HN2 to L1210 cells.

4.15 THE EFFECT OF NITROGEN MUSTARD ON ^{86} RUBIDIUM INFLUX INTO K562 HUMAN ERYTHROLEUKAEMIA CELLS

4.15.1 Introduction

Nitrogen mustard has been shown to inhibit ^{86}rubidium influx into three murine cell lines; the PC6A plasmacytoma *in vitro* (results 4.1.1.1; Baxter *et al*, 1982); the L1210 leukaemia *in vitro* (results 4.3.1; 4.3.2) and *in vivo* (results 4.3.3); and the Ehrlich ascites cell *in vitro* (Grunicke *et al*, 1985; Doppler *et al*, 1985). Since the occurrence of $Na^+K^+Cl^-$ cotransport systems is by no means limited to murine tissues (introduction 1.2.1) it was of interest to examine the effect of nitrogen mustard upon $^{86}Rb^+$ transport into cultured human cells. The human erythroleukaemia K562, maintained *in vitro*, was chosen. A toxic concentration of HN2 was determined, then the effect of this concentration upon $^{86}Rb^+$ transport was examined.

4.15.2 Results

The K562 erythroleukaemia was maintained *in vitro* as described in the methods (3.1.2.1). Cultures established at 5×10^4 cells per ml reached a stationary phase at 5×10^5 cells per ml with a mean generation time of 24h (Dr M Tisdale, personal communication).

K562 cells from exponentially-growing cultures, at a density of approximately 10^5 cells per ml, were subcultured at a density of 5×10^4 cells per ml, then incubated with a range of concentrations of HN2 for four days when the culture reached a growth plateau. The effect of HN2 on cell growth was determined as before (methods 3.3.3); the minimum concentration of HN2 which caused 99% inhibition of cell growth was found to be 10^{-5}M HN2 (table 24). K562 cells did not form colonies in soft agar (methods 3.3.4), so a more accurate estimate of the cytotoxicity of 10^{-5}M HN2 to K562 cells was not obtained for this brief study.

Figure 84

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the influx of $^{86}Rb^+$ & K^+ into K562 cells incubated in Krebs-Ringer buffer.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=3)

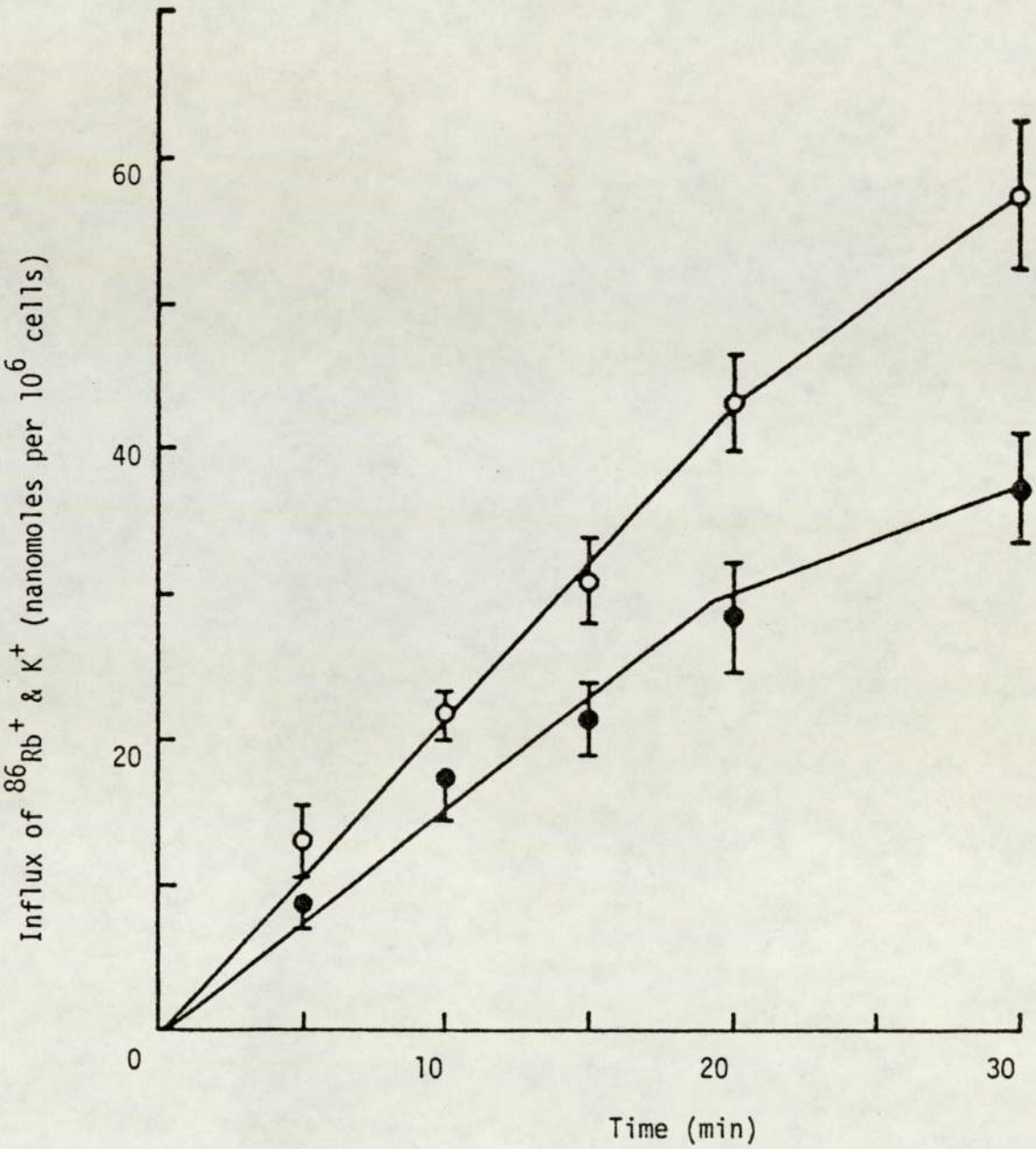


Figure 84 shows the influx of $^{86}Rb^{+}$ into 2 x $10^{6}ml^{-1}$ K562 cells after incubation, for 3h in KR with and without 10^{-5}M HN2. The initial rate of $^{86}Rb^{+}$ influx was linear for at least 10 minutes; incubation with 10^{-5} M HN2 inhibited the initial rate of $^{86}Rb^{+}$ influx by 23% of control, measured over this time interval.

There was no significant difference in the number of cells impermeable to trypan blue in control (90 ± 4%) and HN2-treated incubations (86 ± 5%; both values mean ± S.D., of 3 observations).

Table 24

The effect of continuous incubation with nitrogen mustard on the proliferation of K562 cells in vitro

[HN2] M	% inhibition of growth of control
10^{-4}	98 ± 4
10^{-5}	98 ± 2
10^{-6}	96 ± 3
10^{-7}	59 ± 12
10^{-8}	1 ± 4

Mean ± S.D.; n = 3

Initial cell number = 5 x 10^{4} per ml.

4.15.3 Discussion

The K562 erythroleukaemia cell line was established from the pleural effusion of a patient in blastic phase (Lozzio and Lozzio, 1975). The growth of K562 cells in vitro was found to be sensitive to pharmacologically relevant concentrations of HN2 (see results 4.1.3.1.3).

The aim of the experiment was to investigate whether the effect of HN2 on the $Na^+K^+Cl^-$ cotransporter (results 4.3) was confined to murine tumour cells. It was found that growth-inhibitory concentrations of HN2 inhibited total $^{86}Rb^+\&K^+$ influx into K562 cells. The initial rate of $^{86}Rb^+$ influx into K562 cells incubated in the absence of HN2 was 2.21 ± 0.05nmolmin^{-1} per 10^6 cells; this rate was similar to the rate of influx of $^{86}Rb^+\&K^+$ into L1210 cells (1.99 ± 0.22nmolmin per 10^6 cells; results, 4.2.2.3). The initial rate of $^{86}Rb^+$ influx into K562 cells was less sensitive to inhibition by 10^{-5}M HN2 (23%) than that of the L1210 cells (37% inhibition; results 4.3). The difference in sensitivity may be due to a differences in the $Na^+K^+Cl^-$cotransport activity of the two cell lines. In view of the absence of bumetanide-sensitive $^{86}Rb^+$ influx into the HN2-resistant L1210R cells, it would be of interest to determine cotransport activity in a wide range of cell types and examine the degree of correlation of this activity with the cytotoxicity of HN2 to each type, as measured by the colony forming assay.

The result described here shows that the ability of HN2 to perturb K^+ ion fluxes is not confined to effects on murine cells. The occurrence of the $Na^+K^+Cl^-$ cotransporter in normal tissues may account for the relatively poor selectivity of HN2 as an antitumour agent (introduction 1.1.2). Inhibition by HN2 of the $Na^+K^+Cl^-$ cotransporter in the nephron may account for the loss of extracellular electrolytes and intracellular K^+ and water, which are recognised as toxic side-effects of administration of HN2 to cancer patients (Gilman and Phillips, 1946).

4.16 THE EFFECT OF NITROGEN MUSTARD ON MEMBRANE TRANSPORT PROCESSES OF L1210R MURINE LEUKAEMIA CELLS RESISTANT TO NITROGEN MUSTARD

4.16.1 THE PROLIFERATION OF L1210R CELLS *in vitro*

4.16.1.1 Introduction

Attempts were made to develope a nitrogen mustard-resistant variant of L1210 cells from the parent cell line *in vitro* (methods 3.1.7). A resistant cell line, designated L1210R was obtained from Dr Neil Gibson (materials 2.3). In order to maintain the L1210R cells *in vitro*, the mean generation time and the culture densities which represented exponential and stationary phases of growth of the culture were determined in the same manner as for L1210 cells (methods 3.1.5).

4.16.1.2 Results

None of the treatments described in the methods (3.1.7) were successful in the production of an L1210 cell line with stable resistance to nitrogen mustard.

L1210R cells were routinely maintained *in vitro* (methods 3.1.3) with a mean generation time of 15h (figure 85). The minimum cell number required to initiate growth of the culture was found to be 10^4 cells per ml and a growth plateau was reached at a density of $1\text{-}2 \times 10^6$ cells per ml. Maintenance cultures were routinely initiated at 5×10^4 cells per ml and subcultured twice weekly. L1210R cells used for experiments were harvested from cultures in the exponential phase of growth, at a density of $3\text{-}7 \times 10^5$ cells per ml.

Figure 85

The proliferation of L1210R cells *in vitro*.

A typical result is shown; each point is the mean of duplicate determinations.

Figure 86

The effect of preincubation for 1h with nitrogen mustard on the formation of L1210R cell colonies in soft agar.

(Mean; n=3)

Figure 85

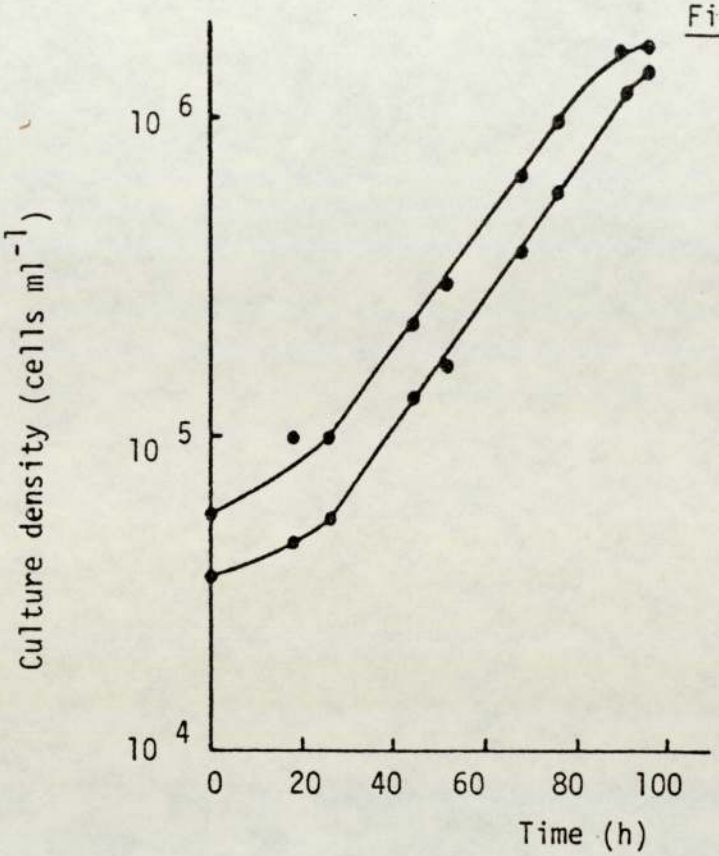
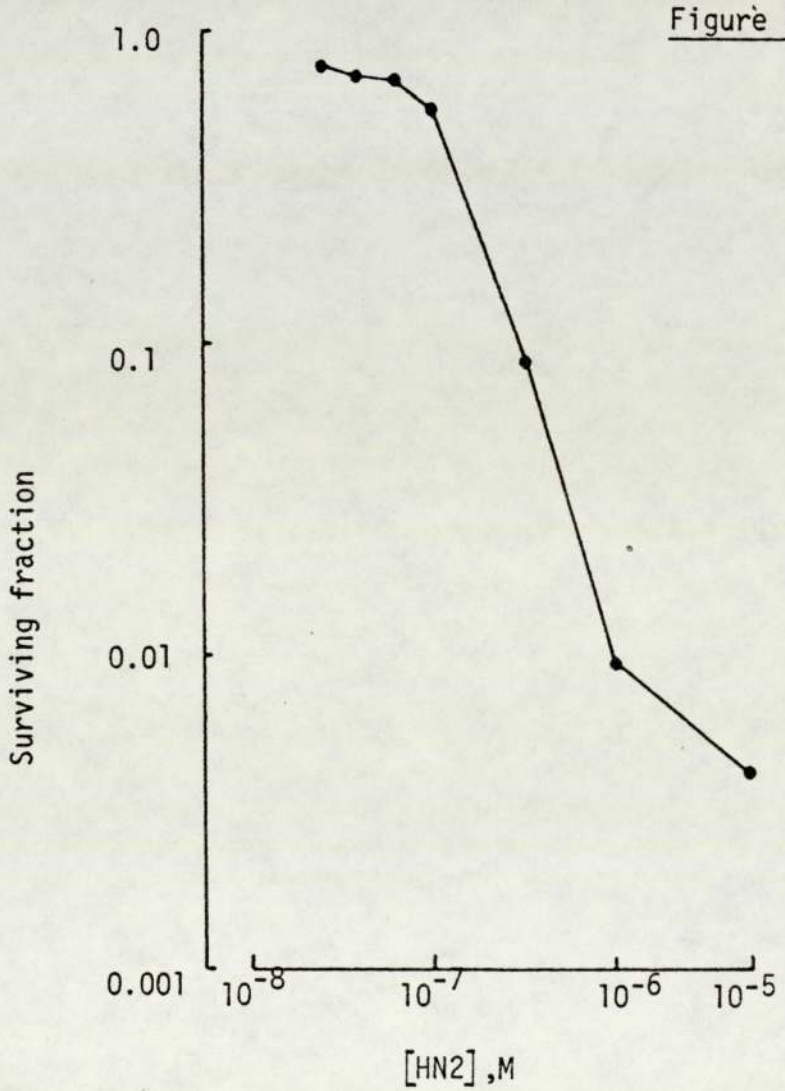


Figure 86



4.16.1.3 Discussion

The results of the experiments described above allowed a regime to be established for the routine subculture of the L1210R cell line resistant to nitrogen mustard. For the present, preliminary study, L1210R cells were harvested from cultures in the exponential phase of growth. Such cultures should contain the greatest proportion of cycling cells (results 4.1.3.4) and therefore possess the greatest potential for sensitivity to nitrogen mustard (introduction 1.1.2).

4.16.2 ESTIMATION OF THE CYTOTOXICITY OF NITROGEN MUSTARD TO L1210R CELLS *in vitro*

4.16.2.1 Introduction

The cell line designated L1210R was maintained *in vitro*, in the absence of HN2 (methods 3.1.3). In order to determine the degree of resistance to HN2, the effect of HN2 on the ability of LI2IOR cells to form colonies in soft agar was examined. L1210R cells (5×10^6 cells per ml) were incubated in RPMI medium with or without various concentrations of HN2 for 1h at 37^{o}, then incubated in soft agar (methods 3.3.4.3).

4.16.2.2 Results

The mean plating efficiency of L1210R cells incubated in the absence of drug was 52 ± 9% (mean ± S.D.; n = 3). Figure 86 shows the plot of the logarithm of the surviving fraction (methods 3.3.4.1) against concentration of nitrogen mustard. Incubation with 10^{-5}M HN2 killed between 2-3 logarithms of LI2IOR cells, thus the L1210R cell line was approximately 100 fold resistant to 10^{-5}M HN2 in comparison with the L1210 cell line (compare figures 86 and 10). The L1210R cell line was approximately 6-fold resistant to the cytotoxic affects of 10^{-2}M furosemide in comparison with the L1210 cell line (figure 58).

4.16.2.3 Discussion

It has been proposed that resistance to alkylating agents may arise from one, or a combination of more than one, of the following mechanisms: alterations in drug influx or efflux, increased cellular concentrations of some protective component (usually a sulphydryl-containing compound); increased capacity for repair of a damaged component (usually DNA); or, alterations in, or decreased concentrations of, some susceptible target (introduction 1.1).

The cell line designated L1210R was found to be approximately 100 fold more resistant than the L1210 cell line to the cytotoxicity of 10^{-5}M HN2 in a colony forming assay. The two cell lines L1210 and L1210R are suitable for comparative studies of the effects of HN2 on ion transport, which may elucidate the mechanism(s) of cytotoxicity of HN2 and mechanism(s) of resistance to HN2.

The L1210R cell line was maintained in the absence of HN2. However, the resistance to HN2 appeared to be stable over the period of time during which these experiments were performed, i.e., approximately 12 weeks. This conclusion was supported by the reproducibility of results during this period. For further investigations, the long-term stability of the resistant phenotype must be established.

Examination of the relationship between the surviving fractions of L1210 (figure 10) and L1210R cells (figure 86) in the colony forming assay and the concentration of HN2 reveals that the graphs describe different slopes; this observation is suggestive of different mechanisms of cytotoxicity of HN2. The L1210R cell line was also approximately 6 times more resistant than the L1210 cell line to the cytotoxicity of 10^{-2}M furosemide; again, the plots of surviving fraction displayed different slopes, suggestive of

different cytotoxic mechanisms (figure 58). On the basis of these observations, attention was turned to the possibility that resistance to HN2 in the L1210R cell line arose from changes in a sensitive cellular target, with particular emphasis on the possibility that the target may be involved in K^+ transport processes.

4.16.3 THE EFFECTS OF NITROGEN MUSTARD ON MEMBRANE TRANSPORT PROCESSES OF L1210R CELLS

4.16.3.1 Introduction

Nitrogen mustard caused a concentration- and time-dependent inhibition of $^{86}Rb^+$ influx into LI2I0 cells, through interference with the function of a $Na^+K^+Cl^-$ cotransport system (results 4.3). The cell line designated LI2I0R was 100 fold more resistant than the L1210 cell line to the cytotoxic effects of 10^{-5}M HN2. It was thus of interest to examine the activities of the K^+ transport processes in the resistant cells, and the sensitivity of these processes to 10^{-5}M HN2; if the perturbation of cotransport is important for the cytotoxicity of HN2, it may be expected that such perturbation is reduced or absent in resistant cells.

Incubation for 3h with 10^{-5}M HN2 selectively inhibited the Na^+-dependent transport of amino acids into L1210 cells (results 4.14); the effect of HN2 upon amino acid transport into LI2I0R cells was subsequently examined.

4.16.3.2 Results

Figure 87 shows the influx of $^{86}Rb^+$ into 5 x 10^6 L1210R cells per ml after incubation for 3h in KR with and without 10^{-5}M HN2. Trypan blue was excluded by 89 ± 6% and 80 ± 9% of cells after incubation in the absence and presence, respectively, of HN2 (means ± S.D.; n = 5). The initial rates of transport were measured over the first 10 minutes when transport was linear with time. Both the

initial rate of transport, and the rate at a later (30 minute) time point, were lower in the L1210R line than in the L1210 line. Incubation with 10^{-5}M HN2 had no effect on the initial rate of $^{86}Rb^{+}\&K^{+}$ transport into LI2IOR cells (figures 87 and 88). Neither 10^{-3}M furosemide nor 10^{-4}M bumetanide inhibited the $^{86}Rb^{+}$ & K^{+} influx into L1210R cells after preincubation for 15 minutes (figure 88).

The total $^{86}Rb^{+}\&K^{+}$ influx into L1210R cells was approximately 52±13% ouabain-sensitive and 48±6% ouabain and furosemide-insensitive. Thus the $^{86}Rb^{+}\&K^{+}$ influx into L1210R cells appeared to have no furosemide or bumetanide-sensitive component. Further evidence to support this conclusion was provided by the results from measurement of $^{86}Rb^{+}$ efflux from preloaded L1210R cells incubated in K^{+}-replete medium. Bumetanide (10^{-4}M) had no effect on K^{+} efflux or cell volume of L1210R cells (results 4.4.3; figures 46 and 47; see discussion below).

After incubation for 3h in KR with and without 10^{-5}M HN2, the mean volume of 10^{6} L1210R cells was 0.742 ± 0.064 ul and 0.770 ± 0.072 ul respectively (Mean ± S.D.; n = 3).

Figure 89 shows the influx of $[^{14}C]$-α-AIB in the presence of cycloleucine into L1210R cells after incubation for 3h in KR with and without 10^{-5}M HN2. Nitrogen mustard had no effect upon $[^{14}C]$-α-AIB transport into L1210R cells.

Figure 87

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the influx of $^{86}Rb^{+}$ & K^{+} into L1210R cells incubated in Krebs-Ringer buffer.

Key: ○ control

● 10^{-5}M HN2 (Mean ± S.D.; n=5)

Figure 88

The initial rate of influx of $^{86}Rb^{+}$ & K^{+} into L1210R cells, measured in the presence of inhibitors of K^{+} transport, after preincubation for 3h with and without 10^{-5}M nitrogen mustard.

Key: 1 no inhibitor

2 10^{-3}M furosemide

3 10^{-4}M bumetanide

4 10^{-3}M ouabain

5 10^{-3}M ouabain + 10^{-3}M furosemide

(Mean + S.D.; n=3)

Figure 89

The effect of preincubation for 3h with 10^{-5}M nitrogen mustard on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210R cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○ control

● 10^{-5}M HN2

(Results are the mean of triplicate determinations of a single experiment)

Figure 87

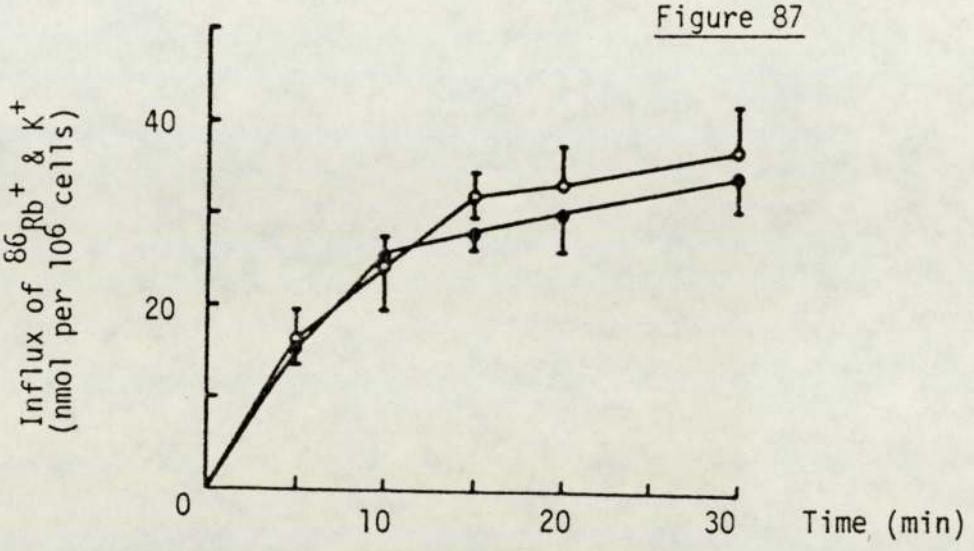


Figure 88

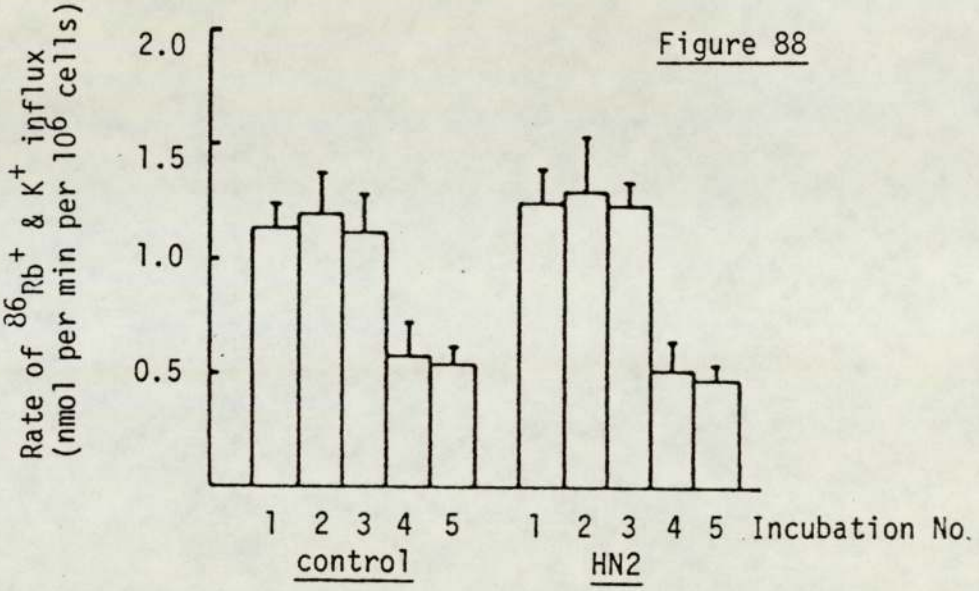
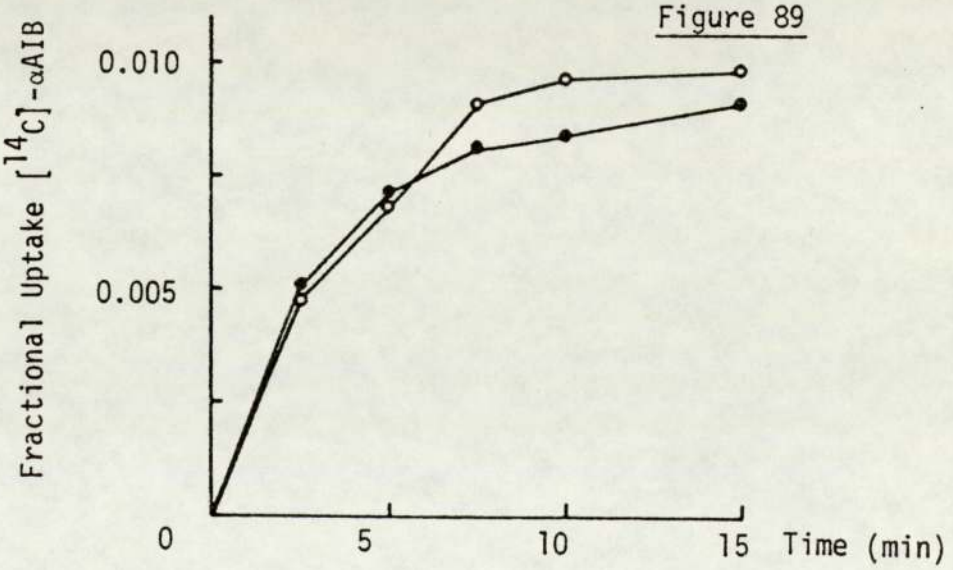


Figure 89



4.16.3.3 Discussion

Nitrogen mustard (10^{-5}M) had no effect upon the total $^{86}Rb^{+}\&K^{+}$ influx into L1210R cells, which appeared to lack a diuretic-sensitive component, suggesting that a $Na^{+}K^{+}Cl^{-}$ cotransport system was absent, inoperative, or resistant to inhibition by the diuretics. The direction of the net flux through the cotransporter has been the subject of several investigations (see result 4.4, and references quoted therein). However, the possibility that a cotransport system was present in L1210R cells but functioning to mediate a net $^{86}Rb^{+}\&K^{+}$ efflux, was discounted by the results of preliminary experiments to examine $^{86}Rb^{+}$ efflux (results 4.4.3; figures 46 and 47). Bumetanide caused an apparent stimulation of $^{86}Rb^{+}$ efflux from preloaded exponentially-growing L1210 cells incubated in K^{+}-replete medium, which was consistent with the blockade of re-entry of $^{86}Rb^{+}$. In contrast, $^{86}Rb^{+}$ efflux from L1210R cells was unaffected by bumetanide, which suggested that there was no bumetanide-sensitive component of $^{86}Rb^{+}$ influx or efflux. The existence of a cotransport system which is resistant to inhibition by bumetanide or furosemide may be investigated by examination of the dependence of $^{86}Rb^{+}\&K^{+}$ influx upon external Cl^{-} and Na^{+}, and analysis of the specificity of the system (see results 4.2.3).

It is possible that different pathways operate to maintain K^{+} homeostasis in L1210 and L1210R cells. There were lower rates of $^{86}Rb^{+}$ influx (compare figures 32 and 87) and $^{86}Rb^{+}$ efflux (compare data in figures 46 and 47 in the resistant cell line. The activity of the Ca^{++}-stimulated channel for K^{+} efflux (introduction 1.2.1 see results 4.4.3.3) may differ in the L1210 and L1210R cell lines. Quinine, which has been shown to inhibit the channel (Lew and Ferreira, 1978), was found to stimulate $^{86}Rb^{+}$ efflux from L1210R cells.

Two observations strongly implicated the interference of cotransport function as an important biochemical event in the cytotoxic mechanism of HN2. Firstly, the HN2-resistant L1210R cell line was also relatively resistant to furosemide. Secondly, the LI2IOR cell line lacked a component of $^{86}Rb^{+}$ influx sensitive to $10^{-3}M$ furosemide. The mechanisms by which cytotoxicity may arise are considered in the general discussion, section 5.

If the cotransport system in L1210R cells is altered or absent, it may be expected that cytotoxic concentrations of HN2 or bumetanide have no effect upon $^{86}Rb^{+}$ influx, but that they effect their toxicity *via* some other mechanism. The different slopes of the survival curves of L1210 and L1210R cells after incubation with HN2 (figures 86 and 10) or furosemide (figure 58) support this possibility. The possible lack of $Na^{+}K^{+}Cl^{-}$ cotransport activity may be probed for by measurement of the number of bumetanide binding sites and the kinetics of binding; this may be achieved by use of [^{3}H]-bumetanide (Forbush and Palfrey, 1983), or by photoaffinity labelling with bumetanide (Jorgensen *et al*, 1984). Differences in the structure of the cotransporter in L1210 and L1210R cells may be revealed as changes in the [^{3}H]-bumetanide labelling patterns after polyacrylamide gel electrophoresis of membrane proteins.

Three groups of workers have isolated mutant cell lines deficient in $Na^{+}K^{+}Cl^{-}$ cotransport (in each case, the mutation conferred upon the cells the ability to grow in K^{+}-depleted medium (Gargus and Slayman, 1980; McRoberts *et al*, 1983; Sussman and O'Brien, 1985). The absence of cotransporter function in LI2IOR cells may therefore be detected by examination of their degree of dependence on external K^{+} in comparison with L1210 cells.

There may be a mutation in the $Na^+K^+Cl^-$ cotransporter of L1210R cells which changes the affinity for Na^+, K^+ or Cl^- ions, so that Na^+ and Cl^- are transported independently of K^+. Sussman and O'Brien (1985) have demonstrated that altered cotransport activity in a 3T3 cell mutant was accompanied by changes in the affinities for K^+ and especially Na^+, but that the affinity for Cl^- was unaltered. The investigation of such a mutation in L1210R cells awaits a successful method for the measurement of Na^+ and Cl^- fluxes into L1210 and L1210R cells (results 4.5). A mutation which changed the affinity of the cotransporter for the ions may also alter the affinity for bumetanide. Forbush and Palfrey (1983) examined the kinetics of binding of [^{3}H]-bumetanide to membranes of dog kidney medulla. The diuretics act directly upon the cotransport system rather than *via* a regulatory mechanism (Palfrey *et al*, 1980); Forbush and Palfrey (1983) suggested that bumetanide binds at one of the anion binding sites, and that the inhibited complex may be cotransport system+1Na^++1K^++1Cl^-+ bumetanide. However, NO_3^-, which cannot substitute for Cl^- for K^+ transport (results 4.2.3), was found to inhibit binding of [^{3}H]-bumetanide. This result was interpreted to suggest that the anion sites involved in the stimulation and inhibition of [^{3}H]-bumetanide binding are not transport sites, or that NO_3^- is bound by the system but not transported. Studies of the kinetics of [^{3}H]-bumetanide binding to membranes of L1210 and L1210R cells may contribute towards the resolution of the nature of the mutation. However, in a more recent report, it was demonstrated that a mutant macrophage cell line deficient in adenylate cyclase was resistant to bumetanide (Bourrit *et al*, 1985); this observation suggests that mutations other than those in the cotransport system itself may confer changes in the activity of the system.

The presence of a Na^+Cl^- cotransport system in L1210R cells may be detected by its possible role in the regulation of cell volume. The function of such a system may be expected to produce an increase in the volume of cells incubated in the presence of ouabain (see results 4.8.3). However, the volume of L1210R cells under steady-state conditions was unaffected by HN2 or the diuretics suggesting that if this system is present, it is insensitive to these agents.

It was considered unlikely that an increased metabolism of HN2 in L1210R cells may account for the lack of inhibition of $^{86}Rb^+$ & K^+ influx. Although several authors have suggested that elevated concentrations of cytoplasmic sulphydryl compounds may confer resistance to alkylating agents (Suzukake et al, 1982; 1983; Endresen et al 1983), it is difficult to explain how this might protect against the inhibition of the cotransporter by the diuretics.

Nitrogen mustard inhibited the Na^+-dependent uptake of the amino acid analogue [^{14}C]-α-AIB by L1210 cells; the inhibition appeared to be unrelated to the inhibition of $^{86}Rb^+$&K^+ transport (results 4.14). However, the observation that the accumulation of [^{14}C]-α-AIB by L1210R cells was also unaffected by HN2 raises doubts about this conclusion. The result suggests that changes in some regulatory factor in HN2-treated L1210 cells may lead to interference of both $Na^+K^+Cl^-$cotransport and amino acid transport independently of each other. This possibility will be considered further in the general discussion (section 5), and may be resolved experimentally by studies with membrane vesicles prepared from L1210 and L1210R cells. Isolated membrane vesicles (methods 3.17; appendices 6.2) allow the study of the direct effects of HN2 on the ion transport systems, the activities of which may be examined by a number of methods (Hochstadt et al, 1975). Membrane vesicles may be

prepared in the presence or absence of likely regulatory factors, and the consequences to transport examined as above.

Some of the data presented in this brief study is based on observations of only one experiment, and this work should be repeated to allow statistical validation of the results. However, it is evident from the results described thus far that the K^+ transport processes of L1210 and L1210R cells differ in their activities and in their sensitivities to nitrogen mustard. These differences must surely warrant further investigation, for two reasons. Firstly, further comparative studies of the L1210 and L1210R cell lines may yield exciting and innovative results to elicit the mechanism of action of HN2. Secondly, the comparison of $Na^+K^+Cl^-$ cotransport activities in the two cell lines may reveal mechanisms by which this transporter is regulated. It has been proposed by others that the comparison of $Na^+K^+Cl^-$ cotransport in genetically different cells will eventually lead to identification of the protein(s) responsible, and an understanding of the role of the system in cell proliferation (Gargus et al, 1978; Gargus and Slayman, 1980; Jayme et al 1980; Jayme et al, 1984; Sussman and O'Brien, 1985; Sussman et al, 1985).

4.17 THE EFFECT OF 2-CHLOROETHYLDIMETHYLAMINE, A MONOFUNCTIONAL ANALOGUE OF NITROGEN MUSTARD, ON MEMBRANE TRANSPORT PROCESSES OF L1210 CELLS

4.17.1 Introduction

Nitrogen mustard inhibited the transport of $^{86}Rb^{+}$&K^{+} into L1210 cells in a concentration-dependent manner which suggested that this biochemical lesion may have a role in the cytotoxic mechanism of HN2 (results 4.3.1.1). The bifunctional alkylating agents, including HN2, possess antitumour activity which is absent from their monofunctional analogues (introduction 1.1.2; Connors 1974; Loveless, 1951). Therefore, if a specific biochemical lesion is to be considered important in the cytotoxic mechanism of HN2, it must account for the requirement of bifunctionality for biological activity. Accordingly, the effects of 2-chloroethyldimethylamine (HN2-1) upon the proliferation of L1210 cells in suspension culture and in soft agar, and upon transport of $^{86}Rb^{+}$ and amino acids were examined.

4.17.2 Results

Figure 90 shows the effect of continuous exposure to various concentrations of HN2-1 on the proliferation of L1210 cells during 72h in suspension culture; the IC_{50} was approximately $6 \times 10^{-5}M$, whilst the minimum concentration causing 99% inhibition of cell proliferation was $10^{-4}M$.

Figure 91 shows the effect of preincubation for 1h in RPMI with various concentrations of HN2-1 on the formation of colonies from L1210 cells in soft agar; the concentration which reduced colony formation by 50% was found to be approximately $5 \times 10^{-5}M$, and $10^{-4}M$ HN2-1 inhibited 98.5% of colony formation.

Figure 90

The effect of continuous incubation with 2-cholorethyldimethylamine on the proliferation of L1210 cells during 72h *in vitro*.

(Mean ± S.D.; n=4)

Figure 91

The effect of preincubation for 1h with 2-cholorethyldimethylamine on the formation of L1210 cell colonies in soft agar.

(Mean; n=3)

Figure 90

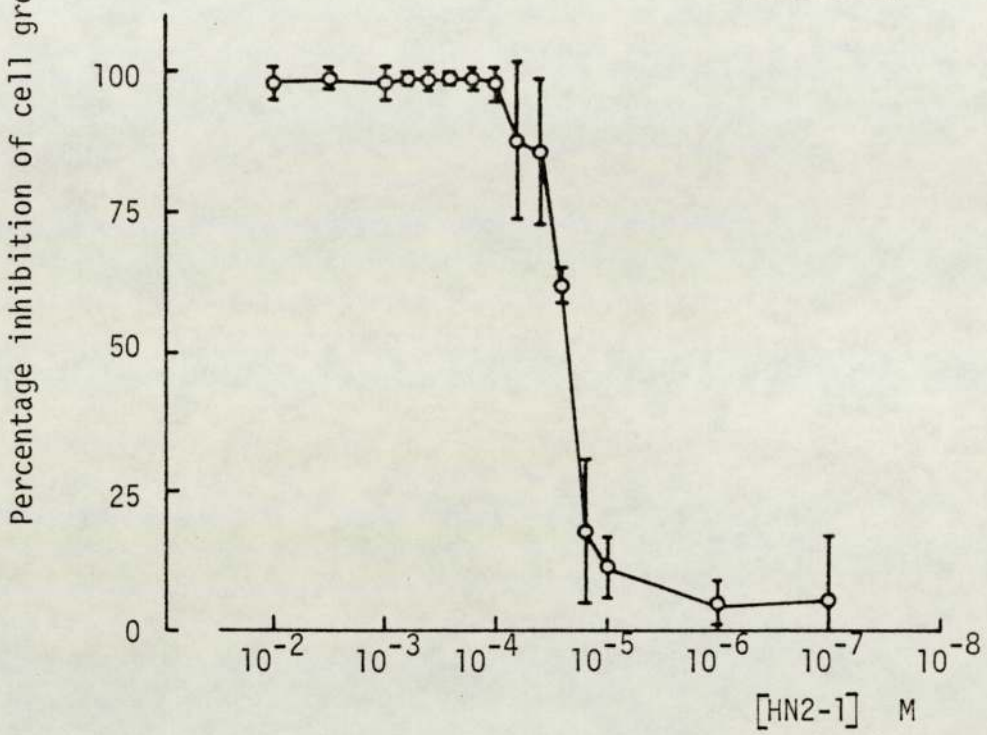
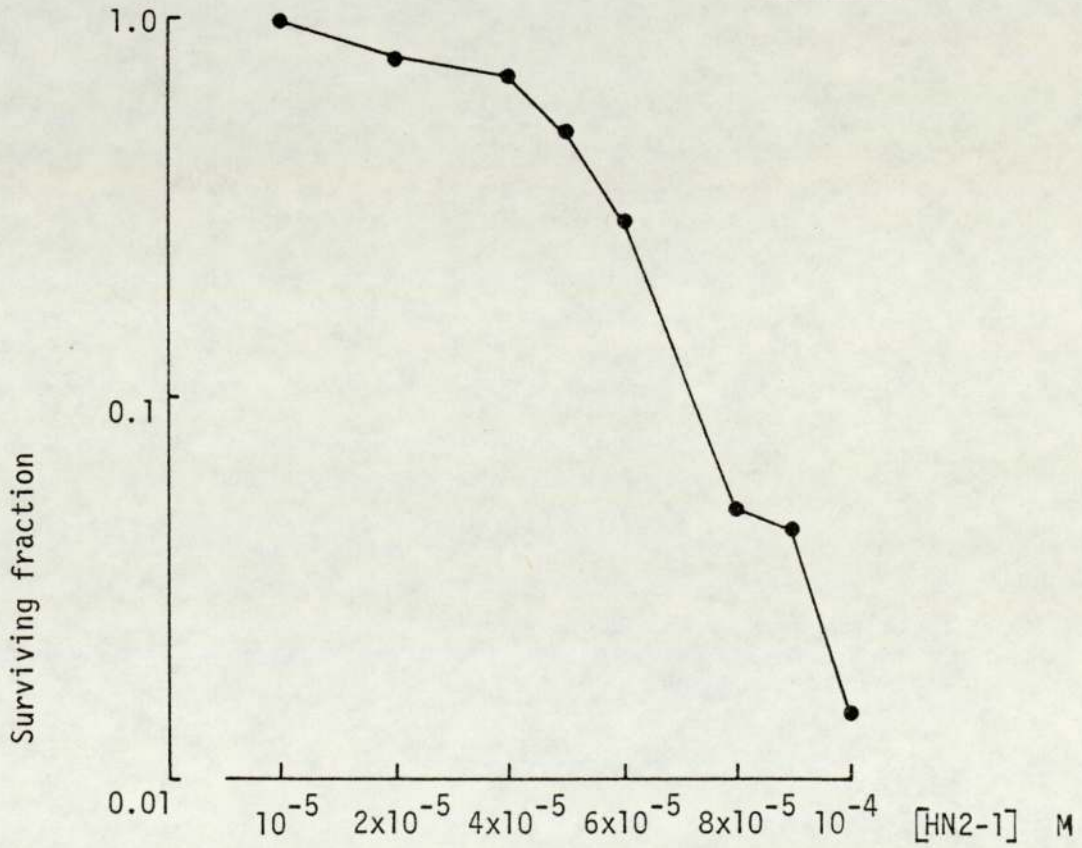


Figure 91



Preincubation of L1210 cells with 10^{-5}M HN2 caused a progressive inhibition of $^{86}Rb^+$ uptake (results 4.3.1.1) ; this concentration was found to be the minimum required to inhibit 99% of growth in suspension culture (results 4.1.3.1). On this basis an equitoxic concentration of HN2-1 was estimated to be 10^{-4}M.

Thus the effects of equimolar (10^{-5}M) and equitoxic (10^{-4}M) concentrations of HN2-1 on the influx of $^{86}Rb^+$ into L1210 cells were examined. Figure 92 shows the effect of preincubation for various times in KR buffer with and without 10^{-4}M or 10^{-5}M HN2-1, upon the initial rate of $^{86}Rb^+$ uptake into rapidly-dividing L1210 cells, measured in K^+-depleted KR buffer. Under these conditions, 10^{-5}M HN2 exerted its maximum effect upon diuretic-sensitive $^{86}Rb^+$ uptake (results 4.3.1.2). In contrast, only after preincubation for 2h with 10^{-4}M HN2-1 was the initial rate of uptake of $^{86}Rb^+$ statistically significantly different ($p < 0.05$) from controls; this difference was not observed after longer periods of preincubation with HN2-1.

Preincubation for 3h with 10^{-4}M HN2-1 was without effect upon ouabain-resistant $^{86}Rb^+$ uptake into L1210 cells incubated in KR in the presence (figure 92) or absence (figure 34) of extracellular Na^+; these observations indicated that HN2-1 did not inhibit the function of the $Na^+K^+Cl^-$ cotransporter of L1210 cells.

In common with HN2, HN2-1 was without effect upon the efflux of $^{86}Rb^+$ from preloaded L1210 cells incubated in K^+-replete (figures 40 and 41) or K^+-depleted (figure 44) media.

Preincubation with 10^{-4}M HN2-1 was without effect upon the transport of [^{14}C]-α-AIB into L1210 cells (figure 93); under the same conditions, transport of [^{14}C]-cycloleucine was also unaffected (figure 94), indicating that the plasma membrane was an intact barrier to the exterior.

Figure 92

The effect of preincubation in Krebs-Ringer buffer with 2-chloroethyldimethylamine on the initial rate of influx of $^{86}Rb^{+}$ & K^{+} into L1210 cells incubated in K^{+}-depleted Krebs-Ringer buffer.

(Mean + S.D.; n=4)

Figure 93

The effect of preincubation for 3h with 10^{-4}M 2-chloroethyl-dimethylamine on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: □ control

● 10^{-4}M HN2-1 (Mean ± S.D.; n=4)

Figure 94

The effect of preincubation for 3h with 10^{-4}M 2-chlorethyl-dimethylamine on the uptake of [^{14}C]-cycloleucine into L1210 cells incubated in Krebs-Ringer buffer in the presence of α-aminoisobutyric acid.

Key: ○ control

● 10^{-4}M HN2-1 (Mean ± S.D.; n=4)

Figure 92

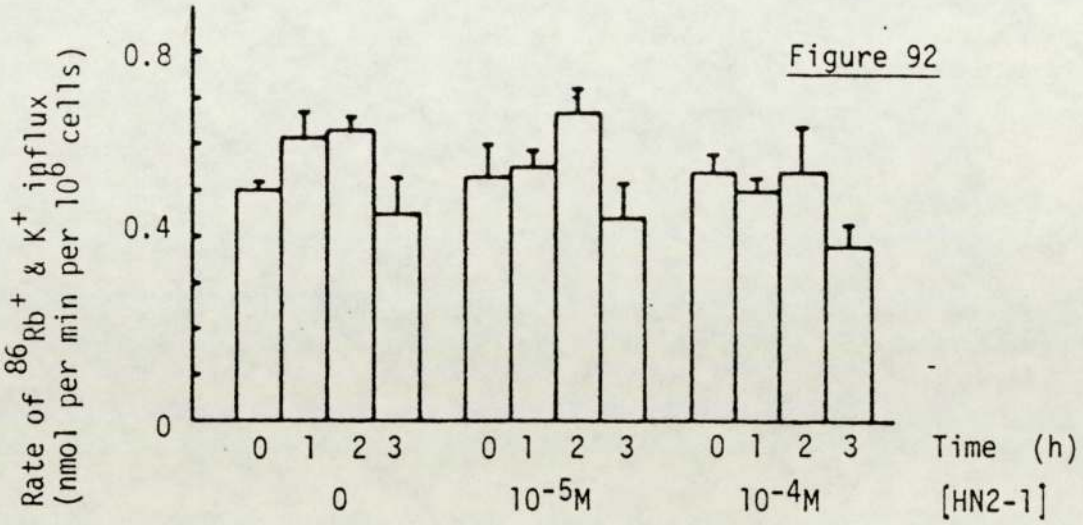


Figure 93

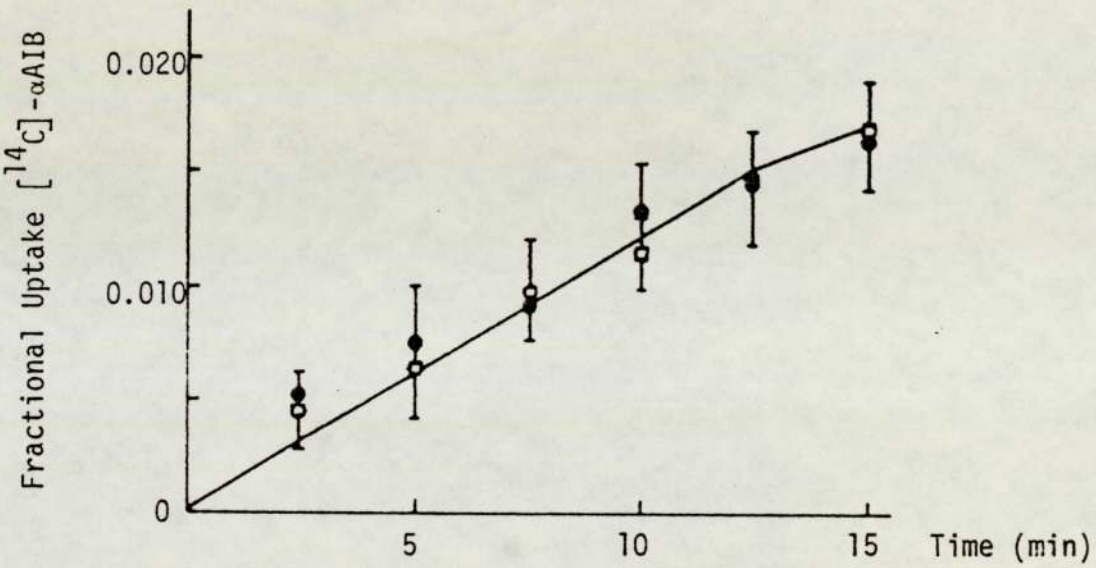
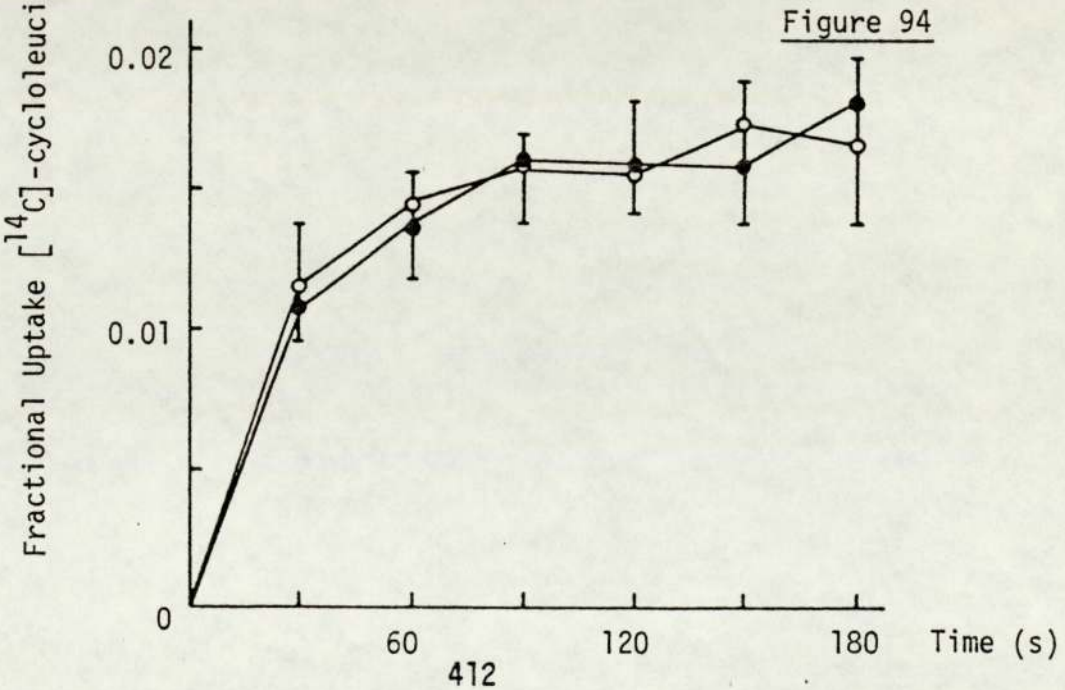


Figure 94



4.17.3 Discussion

Dimethyl 2-chloroethylamine was found to be a less effective inhibitor of L1210 cell growth in vitro than its bifunctional analogue, nitrogen mustard. The proliferation of L1210 cells in suspension culture was completely inhibited by 10^{-5}M HN2 (results 4.1.3.1; or by 10^{-4}M HN2-1 (figure 90). The difference in cytotoxic potency of HN2 and HN2-1 was expected, since both alkylating functional groups are required for biological antitumour activity (Connors, 1974; Loveless, 1951).

On the basis of these results, 10^{-4}M HN2-1 was estimated to be equitoxic with 10^{-5}M HN2 to the growth of L1210 cells in suspension culture, suggesting a 10-fold difference in potency. However, later experiments to examine the effects of HN2 and HN2-1 upon the formation of colonies from L1210 cells in soft agar revealed a much greater difference in potency. After preincubation for 1h with 10^{-5}M HN2 or HN2-1, the fractions of cells surviving to form colonies were 3.5×10^{-5} and 1.0 respectively; similarly, after incubation with 10^{-4}M HN2 or HN2-1 the surviving fractions were 2.2×10^{-5} and 1.5×10^{-2} respectively (figures 10 and 91). The disparity of the results from the growth delay assays and the clonogenic assays reiterate the limitations of the former and the advantages of the latter as techniques for the estimation of drug cytotoxicity (see also results 4.1.3.2.3).

Therefore, although the growth delay assay suggested that 10^{-5}M HN2 and 10^{-4}M HN2-1 were of equal cytotoxicity to L1210 cells, further experiments are required to determine a concentration of HN2-1 which is truly equitoxic to 10^{-5}M HN2.

Nitrogen mustard, at concentrations which were inhibitory to the growth of L1210 cells, selectively inhibited the function of $Na^+K^+Cl^-$

cotransport (results 4.3). In contrast, HN2-1 was without effect upon total $^{86}Rb^{+}$ transport (figure 92), or upon the ouabain-resistant diuretic-sensitive, Na^{+}-dependent $^{86}Rb^{+}$ transport (figures 34), either at equimolar ($10^{-5}M$) concentrations or at concentrations which completely inhibited proliferation of L1210 cells in suspension culture. In common with its bifunctional analogue, HN2-1 was without effect upon $^{86}Rb^{+}$ efflux from L1210 cells (results 4.3), indicating that the cell membrane was an intact barrier to the exterior.

Spurgin (1981) reported that approximately 10-fold concentrations of HN2-1 were required to produce inhibition of $^{86}Rb^{+}$ transport into PC6A cells, equivalent to that produced by a given concentration of HN2; in contrast, the results of the present study suggest that the difference in potency of inhibition of $^{86}Rb^{+}$ influx into L1210 cells was greater than 10-fold.

This result suggests that bifunctionality is required for the inhibitory effect of HN2 upon $^{86}Rb^{+}$&K^{+} transport into L1210 cells, and thus this lesion is compatible with the requirement of bifunctionality for biological activity. In addition, the lack of effect of a toxic concentration ($10^{-4}M$) of HN2-1 upon $^{86}Rb^{+}$ transport into L1210 cells supports the conclusion that such inhibition is a specific effect of HN2 rather than a common event in cells destined to die.

Spurgin (1981) examined the effect of various agents upon the activity of $Na^{+}K^{+}$ATPase in a broken cell preparation of PC6A plasmacytoma cells. Ouabain-sensitive phosphatase activity was completely inhibited by $10^{-10}M$ HN2, or $10^{-5}M$ HN2-1, by $10^{-1}M$ iodoacetic acid (IAA) or by $10^{-5}M$ N-ethylmaleimide (NEM) or $10^{-6}M$ parachloromercuribenzoic acid (pcmb); in addition, $10^{-4}M$ HN2-1 inhibited ouabain-insensitive phosphatase activity. It was suggested

that inhibition by monofunctional alkylating agents (HN2-1, IAA) and sulphydryl-binding agents (NEM, pcmb) arise from random collision of these molecules with sensitive sulphydryl groups located within the enzyme molecule. However, no evidence was found for the inhibition of Na^+K^+ATPase in intact L1210 cells by HN2 (results 4.3.1.2) or HN2-1, despite the presence of sulphydryl groups in the active site (Patzelt-Wenczler et al, 1975; Hart and Titus, 1973). NEM has been shown to stimulate furosemide-sensitive, but Na^+-independent K^+Cl^- influx into human erythrocytes (Wiater and Dunham, 1983) and genetically K^+ deficient sheep erythrocytes (Lauf, 1984). HN2 inhibited only the Na^+-dependent $^{86}Rb^+\&K^+$ influx into L1210 cells (results 4.3.1.3); in human erythrocytes this flux was insensitive to NEM, suggesting that the inhibition of $Na^+K^+Cl^-$ cotransport by HN2 does not involve reaction with sulphydryl groups (Wiater and Dunham, 1983).

If the inhibition of $^{86}Rb^+$ transport by HN2 required reaction with sensitive sulphydryl groups, it would be expected that monofunctional agents were also inhibitors of at least equivalent potency (Tisdale, 1974). However, the reaction of HN2 with sulphydryl groups may be subject to stearic restrictions, allowing a degree of selectivity; for example, if two sensitive sulphydryl groups are closely adjacent, and both must be blocked to effect inhibition, the bifunctional molecule may be more potent than two monofunctional molecules which may suffer stearic hindrance.

The results of the present study suggest that inhibition of $^{86}Rb^+\&K^+$ transport has no role in the cytotoxic mechanism of HN2-1, although as noted above, the effects of higher (10^{-4}M) concentrations of HN2-1 need to be examined to confirm or refute this possibility. It appears that a cross-linking event, perhaps

preventing conformational changes during activity, is essential for the inhibition of $^{86}Rb^{+}\&K^{+}$ transport by HN2. Similarly, the inhibition of Na^{+}-dependent amino acid transport into L1210 cells incubated with HN2 also appears to require a cross-linking event (results 4.14). Sodium-dependent amino acid transport is subject to regulation by sulphydryl groups (Kwok et al, 1976; 1979; Kwock, 1981) which may be located in proteins which function to regulate ion transport (Jacobs et al, 1978). However, inhibition of [^{14}C]-α-AIB transport in HN2-treated L1210 cells does not appear to arise from alterations in the function of $Na^{+}K^{+}$ATPase or $NaK^{+}C^{+}1^{-}$ cotransporter (results 4.14.1.2.3). Therefore, whilst both the inhibition of $^{86}Rb^{+}\&K^{+}$ and amino acid transport into L1210 cells by HN2 appears to depend upon cross-linking reactions, further work is required to establish whether these reactions are at the same susceptible cellular target.

4.18 THE EFFECT OF CHLORAMBUCIL ON MEMBRANE TRANSPORT PROCESSES OF L1210 CELLS

4.18.1 Introduction

Concentrations of nitrogen mustard which were inhibitory to the proliferation of L1210 cells in vitro (results 4.1.3.1) were found to inhibit $^{86}Rb^{+}\&K^{+}$ transport in these cells in a manner which was concentration and time dependent (results 4.3.1.1), satisfied the requirement of bifunctionality (results 4.17) and was selective for the function of $Na^{+}K^{+}Cl^{-}$ cotransport (results 4.3). This particular interference with membrane function was also specific to nitrogen mustard; cytotoxic concentrations of 2-chloroethyldimethylamine (results 4.17), cisplatin (results 4.19), adriamycin (Chahwala and Hickman, 1985) and the new antitumour agent mitozolamide (Dr S Chahwala, personal communication) were without effect on $^{86}Rb^{+}\&K^{+}$ transport into L1210 cells. None of these agents possess the bifunctional alkylating ligands of HN2; however, chlorambucil is a substituted bis-2-chloroethylamine (see figure 2), so it was of interest to examine the effects of this agent on $^{86}Rb^{+}\&K^{+}$ transport.

4.18.2 Results

Table 25 shows the effect of continuous incubation with various concentrations of chlorambucil on the proliferation of L1210 cells during 72h in culture. A concentration of 10^{-5}M chlorambucil was of similar toxicity to 10^{-5}M HN2 (results 4.1.3.1) to the growth of L1210 cells in vitro.

Table 25

The effect of continuous incubation with chlorambucil on proliferation of L1210 cells.

Concentration of chlorambucil, M	Percent inhibition of growth of control
10^{-4}	96 ± 3
10^{-5}	97 ± 2
10^{-6}	37 ± 3
10^{-7}	6 ± 4
10^{-8}	9 ± 7

Mean ± S. D.; n = 3

Figure 95 shows the effect of preincubation for 3h with 10^{-5}M chlorambucil on the initial rate of $^{86}Rb^{+}\&K^{+}$ influx into exponentially-growing L1210 cells in the presence of inhibitors of potassium transport. Influx of $^{86}Rb^{+}\&K^{+}$ into cells in control incubations was 38 ± 11% diuretic-sensitive, 49 ± 11% ouabain-sensitive, and 21 ± 11 % diuretic-and ouabain-insensitive; these results were similar to those obtained from other experiments (results 4.2.2; figures 18 and 19). Influx of $^{86}Rb^{+}\&K^{+}$ into chlorambucil-treated cells was 30 ± 8% diuretic-sensitive, 40 ± 15% ouabain-sensitive, and 21 ± 12% diuretic-and ouabain-insensitive; the total $^{86}Rb^{+}\&K^{+}$ influx into these cells was 108 ± 16% of controls. Thus the $^{86}Rb^{+}\&K^{+}$ fluxes into chlorambucil-treated cells were not significantly different ($p > 0.05$) from those in controls.

Figure 96 shows the effect of preincubation for 3h with 10^{-5}M chlorambucil on the influx of [^{14}C]-α-aminoisobutyric acid into exponentially-growing L1210 cells, incubated in KR buffer in the presence of cycloleucine; chlorambucil was without significant effect.

Figure 95

The initial rate of influx of $^{86}Rb^+$ & K^+ into L1210 cells, measured in the presence of inhibitors of K^+ transport, after preincubation for 3h in the presence and absence of 10^{-5}M chlorambucil.

Key: 1 no inhibitor

2 10^{-3}M furosemide

3 10^{-4}M bumetanide

4 10^{-3}M ouabain

5 10^{-3}M ouabain + 10^{-3}M furosemide (Mean + S.D.; n=3)

Figure 96

The effect of preincubation for 3h with 10^{-5}M chlorambucil on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○ control

● 10^{-5}M chlorambucil (Mean ± S.D.; n=3)

Figure 95

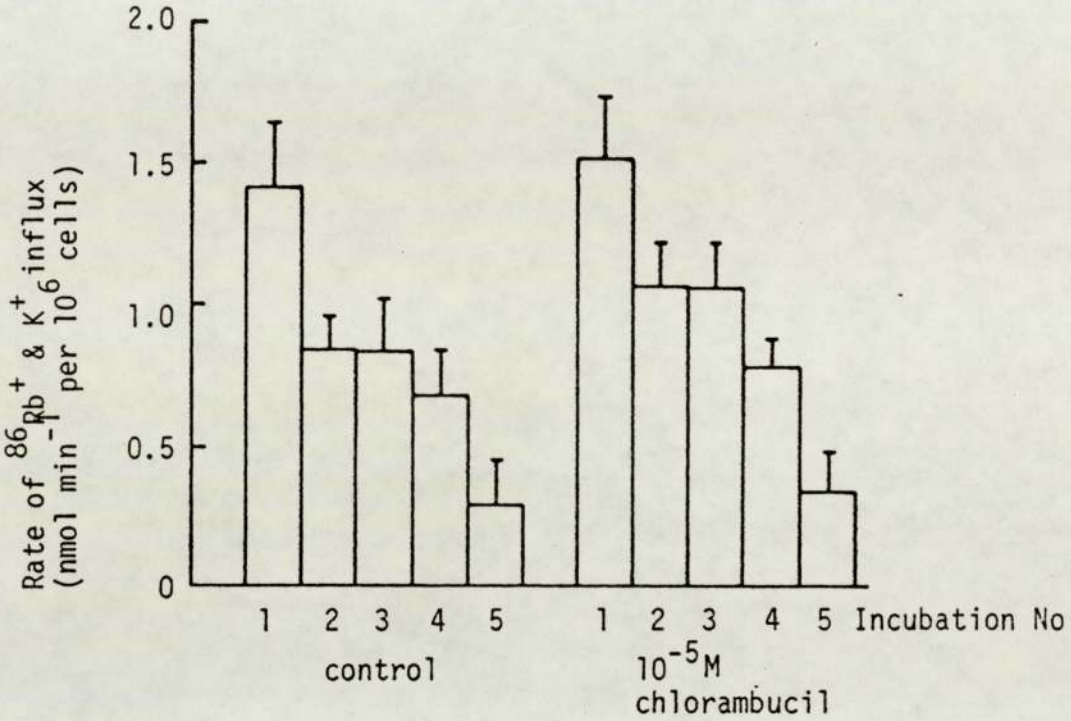
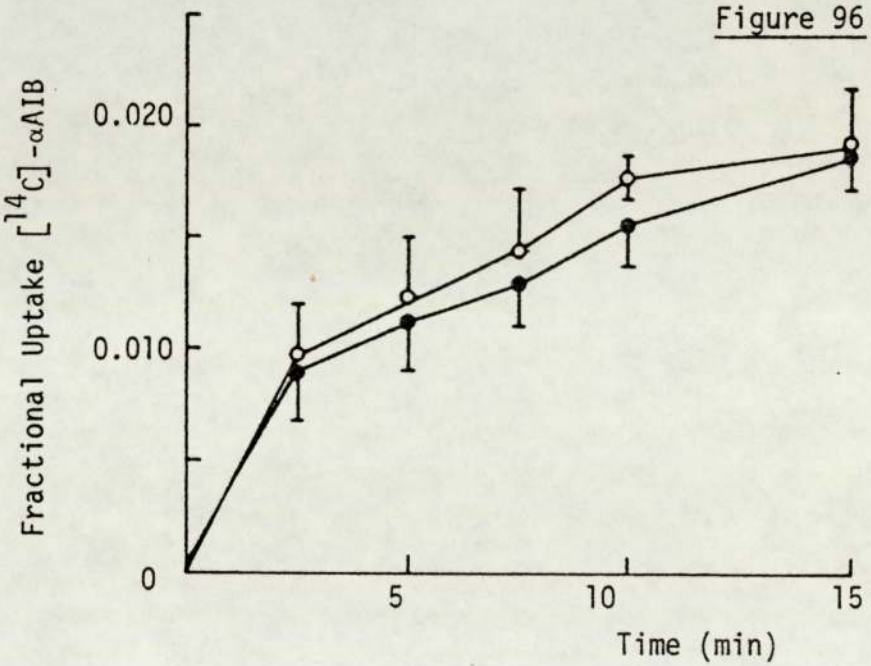


Figure 96



4.18.3 Discussion

Since the first clinical trial of nitrogen mustard four decades ago (Gilman and Phillips, 1947) many analogues based upon the bis-2-chloroethylamine structure have been synthesised (Ochoa and Hirschburg 1977). Chlorambucil was chosen for the present study since it represented a member of the group of complex alkylating agents and its effect upon cellular cAMP levels have been investigated in some detail (Tisdale, 1974; 1975. Tisdale and Phillips, 1975a; 1975b; 1975c; 1976a; 1976b; 1976c). Changes in the intracellular concentrations of cAMP may regulate the activity of the $Na^+K^+Cl^-$ cotransporter in certain cells (introduction 1.2.1).

It was shown by Tisdale and Phillips that chlorambucil inhibited the membrane-bound, low K_m form of the enzyme cAMP phosphodiesterase, and thereby caused prolonged elevation of cellular cAMP concentration (Tisdale and Phillips, 1975b; 1975c). An identical concentration of the monofunctional analogue was without effect (Tisdale, 1974; Tisdale and Phillips, 1975b). The effects of the alkylating agents were emulated by the addition of dibutyryl cAMP, the synthetic cAMP analogue (Tisdale and Phillips, 1975b).

Nitrogen mustard was also shown to elevate intracellular cAMP levels in Walker carcinoma cells (Tisdale and Phillips, 1975c) and thus it was of interest to investigate the possibility that the inhibition of the $Na^+K^+Cl^-$ cotransport of L1210 cells was mediated by an elevation of intracellular cAMP concentrations (results 4.2.5). The stimulation of $Na^+K^+Cl^-$ cotransport in avian erythrocytes by β-adrenergic hormones is mediated by changes in cAMP levels (McManus and Schmidt, 1978).

The work of Spurgin (1981) suggested that chlorambucil may inhibit $^{86}Rb^{+}$ transport across the cell membrane. The ouabain-sensitive $Na^{+}K^{+}$ATPase activity of a suspension of broken PC6A cells was completely inhibited by 10^{-11}M chlorambucil, or by 10^{-7}M of the N-ethyl monofunctional analogue. Both of these agents were of greater potency in this assay than their nitrogen mustard counterparts; $Na^{+}K^{+}$ATPase activity was completely inhibited by 10^{-10}M HN2 or by 10^{-5}M 2-chloroethyldimethylamine. Further, it appears that $Na^{+}K^{+}$ATPase activity can be affected by changes in cAMP concentrations (Luly et al 1972). It has also been suggested that the enzymes adenyl cyclase and $Na^{+}K^{+}$ATPase are linked in some way in the membrane, sharing a common pool of ATP and capable of regulating the activity of each other (Lelievre, 1977; Hadden et al, 1972). Chlorambucil has been shown to affect the membranes of human red blood cells and Ehrlich ascites cells (Linford et al, 1963). One report has suggested that chlorambucil may exert cytotoxic effects upon Ehrlich ascites cells, even when it is prevented from penetrating the membrane (Grunicke et al, 1979; 1980).

However, it was found that whilst nitrogen mustard did indeed inhibit $^{86}Rb^{+}$ transport into PC6A cells (results 4.1.1.1; Baxter et al, 1982; Spurgin, 1981) and L1210 cells (results 4.14; 4.3), the ouabain-sensitive flux was unaffected, and the locus for the inhibition was the $Na^{+}K^{+}Cl^{-}$ cotransporter (results 4.3).

In contrast, chlorambucil, at concentrations which inhibited L1210 cell proliferation, was without effect on $^{86}Rb^{+}$ flux. This result suggested that the inhibition of $Na^{+}K^{+}Cl^{-}$ cotransport, which appeared to be important in the cytotoxic mechanism of HN2, was not involved in the process by which chlorambucil exerted its cytotoxicity.

The alkylating agent Trenimon was shown to inhibit $^{86}Rb^{+}$ transport into Ehrlich ascites cells; it also elevated intracellular cAMP concentrations, but there was no effect on adenyl cyclase or cAMP phosphodiesterase (Ihlenfeldt et al, 1981). This result suggested that the alkylating agent exerted its cytotoxicity by a mechanism independent of inhibition of cAMP phosphodiesterase. The addition of dibutyryl cAMP has been shown to mimic the effects of elevated cAMP concentrations on $Na^{+}K^{+}Cl^{-}$ cotransport in avian red cells (McManus and Schmidt, 1978), and on protein kinase activity in Walker carcinoma cells (Tisdale and Phillips, 1976b).

Although the $Na^{+}K^{+}Cl^{-}$ cotransport system may be regulated by changes in activity of a protein kinase (Sussman et al, 1985; O'Brien and Krzeminski, 1983), the addition of dbcAMP was without effect on $^{86}Rb^{+}\&K^{+}$ influx into L1210 cells (results 4.2.5). In conclusion, the elevation of cAMP concentrations appears to bear little relationship to alterations in $^{86}Rb^{+}$ transport in cells treated with alkylating agents.

Chlorambucil fulfilled the requirement of bifunctionality for inhibition of $^{86}Rb^{+}$ transport, but was without effect. Thus it appeared that nitrogen mustard and chlorambucil did not share a common mechanism of cytotoxicity. It would be of interest to compare the binding of these two agents to the membranes of L1210 cells, and to investigate whether the L1210R cell line resistant to HN2 was cross-resistant to chlorambucil.

The inhibition of $Na^{+}K^{+}Cl^{-}$ cotransport by HN2 did not appear to be common to the family of bifunctional alkylating agents, but may be specific for HN2 alone. The lack of effect of chlorambucil may be due to stearic hindrance, the sensitive sites in the cotransporter proteins being inaccessible to the larger molecule. A range of

analogues of HN2 of increasing size may be of use to determine the stearic relationship of the two sites which appeared to be crosslinked by HN2.

4.19 THE EFFECTS OF cis-AND trans-DIAMINODICHLOROPLATINUM ON MEMBRANE TRANSPORT PROCESSES OF LI2IO CELLS

4.19.1 Introduction

Cisplatin (cis-diaminodichloroplatinum(II); CDDP) is a widely-used antitumour drug (Prestyko et al, 1980; Einhorn, 1981), whereas the trans isomer (TDDP) lacks antitumour activity (Lippard, 1982; Macquet and Butour, 1983). The strict relationship between stereochemistry and activity has been the subject of many studies to elicit the mechanism of action of cisplatin (Drobnik, 1983), and most, but not all, authors on the subject attempt to account for the different toxicities of CDDP and TDDP. Antitumour activity of cisplatin also depends upon the presence of two leaving groups (Drobnik, 1983), and is usually ascribed to a cross-linking reaction with cellular DNA (Roberts and Thomson, 1979). These observations have led to many comparisons of the antitumour activity of cisplatin with that of the alkylating agents such as HN2. Also, in common with HN2, the specific nature of the cytotoxic lesion caused by CDDP has yet to be resolved.

Studies with short deoxyribonucleosides have shown that platination may occur at the following sites: N7 of guanine, N3 of cytosine, N1 or N7 of adenosine; between N7 and N7 of adjacent adenosine residues; N7 and N7 of adjacent guanosines; N1 and N7 of adjacent adenosines; between N7 of guanosine and N1 of adenosine; and N7 of adenosine and N7 of guanosine (Eastman, 1983, 1985). The primary binding site appears to be N7 of guanosine (Marzilli et al, 1980; Goodgame et al, 1975); this is supported by studies of cisplatin binding to DNA and to short oligonucleotides (Lippard et al, 1983; Marcelis et al, 1981; 1983), which seem to confirm the proposal that the chloride ions of cisplatin are replaced

by N7 of adjacent or closely adjacent guanosine groups to form DNA intrastrand cross-links. Other observations support this possibility (Brouwer et al, 1981; Royer-Pakora et al, 1981; Tullius and Lippard, 1981; Cohen et al, 1980), and recently some evidence has been found for the formation of intrastrand crosslinks in DNA in vivo.

There is evidence that both CDDP and TDDP produce covalent cross-links between DNA and histones, and between DNA and non-histone proteins (Zwelling et al, 1979; 1981; Lippard, 1982; Fornace and Seres, 1982; Fornace and Little, 1980; Filipski et al, 1980; 1983; Banjar et al, 1983; 1984; Simpkins and Pearlman, 1984; Olinski and Wojtkowiak, 1985; Roberts and Thomson, 1979). Throughout the literature there is controversy over whether it is cisplatin or transplatin which produces the greater number of DNA-protein cross-links, and a coherent proposal to relate this reaction to cytotoxicity has yet to emerge.

The alkaline elution of DNA from irradiated cells (Kohn and Ewig, 1979) has been used to demonstrate that cisplatin produces DNA-protein and DNA-DNA interstrand cross-linking in vivo (Zwelling et al, 1979; 1981; Roberts and Thomson, 1979). The extent of DNA interstrand cross-linking produced by cisplatin correlated with sensitivity to the drug for some, but by no means at all, of the cells which have been studied (Zwelling et al, 1981; Ducore et al, 1982; Strandberg et al, 1982; Micetich et al, 1983; Ward and Hill, 1984).

The inhibition of DNA synthesis by cisplatin (Howle and Gale, 1970) is generally thought to arise from the formation of crosslinks, and to correlate with antitumour activity (Roberts and Thomson, 1979). However, TDDP also inhibits DNA synthesis in several cell types (Alazard et al, 1982; Kohl et al, 1980; Plooy and Lohman, 1980;

Salles et al, 1983). A non-antitumoural platinum salt, K_2 [Pt Cl_4], has also been shown to inhibit DNA synthesis (Hurwitz et al, 1982). Salles et al (1980) concluded that inhibition of DNA synthesis could not account for the antitumour activity of cisplatin.

Cisplatin may react with a variety of cellular constituents (Rosenberg, 1980) through reaction with nucleophilic nitrogen and especially sulphur atoms, particularly in amino acids and proteins (Morris and Gale, 1973), Cisplatin may also react with cellular thiol compounds such as glutathione (Belluco, 1974) and metallothionein (Sharma and Edwards, 1983). After administration of CDDP to rats, the majority of platinum in kidney and liver was associated with cytosolic, and not nuclear, fractions (Sharma and Edwards, 1983).

There is evidence that cisplatin interferes with enzyme activity (Aull et al, 1979) and damages mitochondria to inhibit oxidative phosphorylation (Aggarwal et al, 1980). Inhibition of such processes may have lethal consequences for cell metabolism. Cell metabolism may also be affected by the elevation of cAMP caused by cisplatin (Tisdale and Phillips, 1974; Hall et al, 1980). The elevation of cAMP also results in a decrease in histone phosphorylation (Hall et al 1980).

There is evidence that cisplatin may cause changes in the plasma membrane. For example, cellular cAMP may regulate certain elements of the cytoskeleton (Howard et al, 1980; Margolis and Wilson, 1979). Further, cisplatin changed the electron-spin resonance of spin-labelled membranes from human erythrocytes and mastocytoma cells (Sinha and Chignell, 1979) which suggested that membrane proteins had suffered conformational changes. Such changes may modify the activities of membrane proteins (VanStevenink et al, 1965; Rega et al, 1967; Stoneberg, 1969). The capping of lymphocytes in response

to concanavalin A, which is mediated by the cytoskeleton and dependent on cell metabolism, was found to be inhibited by cisplatin (Tsokos and Choie, 1980). Cisplatin caused structural changes in the microfilaments (Aggarwal et al, 1980) and removed cell surface nucleic acids (Juckett and Rosenberg, 1982) of Sarcoma 180 cells. Kleinerman et al (1980) showed that cisplatin stimulated the membrane-dependent process of spontaneous monocyte-mediated cytotoxicity.

The dose-limiting nephrotoxicity of cisplatin may be due to the interaction with kidney membranes (Guarino et al, 1979; Levi et al 1980; Walker and Gale, 1981). Cisplatin has been found in microsomal fractions of rat kidneys after injection with the drug (Sharma and Edwards, 1983), and binds to kidney slices (Safirstein et al, 1982) and kidney tubules (Slater et al, 1977; Dobyan et al, 1980). Cisplatin inhibited Na^+K^+ATPase and Ca^{++} ATPase and interfered with ATP production in isolated kidney tubules (Dobyan et al, 1980); it inhibited Na^+K^+ATPase in flounder and rat kidneys (Guarino et al, 1979) and in homogenates of plasmacytoma cells (Spurgin, 1981). Inhibition of membrane-bound enzymes by cisplatin has been reported by others (Aull et al, 1979; Aggarwal and Niroomand-Rad, 1983); such inhibition may affect membrane transport processes. Cisplatin was reported to inhibit $^{86}Rb^+$ transport into PC6A cells (Tritton and Hickman, 1984) and increased the permeability to Na^+ of frog skin epithelia (Van den Berg et al, 1981).

The experiments described in this section were prompted by the observations of Scanlon and coworkers (Scanlon et al, 1983) that cisplatin inhibited the Na^+-dependent transport of methionine into L1210 cells. The authors proposed that this inhibition might be toxic to L1210 cells, which are auxotrophic for methionine, in common

with many other tumours (Hoffman, 1982). The mechanism by which this inhibition occurred was not investigated. In the light of reports which suggested that cisplatin inhibited Na^+K^+ATPase, it was decided to examine the effect of cisplatin on $^{86}Rb^+$ influx into L1210 cells. During the course of these experiments it was found that the L1210 cell line maintained in vitro in our laboratory was relatively resistant to cisplatin, and attempts were made to cultivate L1210 cells with increased sensitivity to cisplatin (see discussion below).

4.19.2 Results

Figure 97 shows the effect of continous incubation with a range of concentrations of CDDP and CDDP on the growth of L1210 cells during 72h in culture. A 50% inhibition of cell multiplication was produced by approximately 10^{-5}M CDDP or approximately 8×10^{-5}M TDDP. Therefore, in common with many other cell types, the L1210 cell line was differently sensitive to the two isomers (see section 4.19.1 above).

Figure 98 shows the initial rate (measured over 5 minutes) of [^{14}C]- α-AIB transport into L1210 cells incubated in KR containing different concentrations of methionine. [^{14}C]- α-AIB transport was blocked by methionine, suggesting some common route(s) of transport into L1210 cells.

[^{14}C]- α -AIB transport was measured in the presence of cycloleucine so that the assay might more accurately reflect the activity of the 'A' and 'ASC' systems for amino acid transport (results 4.14.1.1). The same rationale was applied for the measurement of [^{3}H]-methionine transport, and cycloleucine was added to the assay buffer (methods 3.16.3)

[^{3}H]-Methionine transport into L1210 cells incubated at 37^o was very rapid (figure 99). In order to obtain a linear initial rate of

transport, [^{3}H]-methionine transport was measured in cells incubated at 25^o (methods 3.16.3; figure 100).

Transport of [^{3}H]-methionine into L1210 cells was unchanged after incubation for 30 minutes with 10^{-5}M (figure 100) or 10^{-4}M (figure 101) CDDP, added from a freshly prepared solution of the drug (methods 3.2.1.3); in contrast, 10^{-4}M TDDP produced a small (9%) inhibition of the initial rate of [^{3}H]-methionine transport (figure 101).

The initial rate of [^{14}C]-α-AIB transport into L1210 cells was unaffected by 10^{-5}M CDDP or 10^{-5}M TDDP after a 30 minute preincubation for 30 minutes (figure 102); however, after a longer period (30 min) of influx, the transport of [^{14}C]-α-AIB was impaired by 10^{-5}M TDDP only (figure 102). Preincubation for 30 minutes with 10^{-4}M TDDP produced a greater inhibition (63%) of [^{14}C]-α-AIB transport than the same concentration of CDDP (23% inhibition; Figure 103); however no inhibition of [^{14}C]-αAIB transport was detected after preincubation for 1 h with 10^{-4}M CDDP or 10^{-4}M TDDP (figure 104). This result was suggestive of repair of some lesion for the restoration of amino acid transport. Preincubation for 30 minutes with either 10^{-3}M CDDP or 10^{-3}M TDDP inhibited the initial rate of amino acid transport by 60% (figure 105).

The initial rate of $^{86}Rb^+$ influx into L1210 cells was unchanged by preincubation for 30 minutes with CDDP or TDDP at concentrations of 10^{-5}M (figure 106) or 10^{-4}M (figure 107).

[^{3}H]-Methionine transport into L1210 cells was unaffected by a preincubation for 30 minutes with 10^{-5}M (total Pt) "CDDP" or "TDDP" (figure 108) added from solutions which had been prepared 24h previously (methods 3.2.1.3); however, in some cases, the 24h-old drug solutions had different effects on amino acid transport compared

to fresh solutions. [^{3}H]-Methionine transport was impaired by 10^{-4}M "CDDP" or 10^{-4}M "TDDP" from old solutions (figure 109), in contrast to the result obtained with fresh solutions (figure 101). [^{14}C]-∝-AIB transport was reduced by 30% after preincubation with 10^{-4}M "CDDP" or 10^{-4}M "TDDP" added from 24h old solutions (figure 110).

The monoaquated derivatives (methods 3.2.1.4) of CDDP (figure 111) or TDDP (figure 112) had no effect on the transport of [^{14}C]-∝-AIB incubated for 30 minutes with 10^{-5}M total platinum concentration; transport was almost completely inhibited in the presence of the diaquated derivatives (figures 111 and 112).

The aquated derivatives of CDDP were less cytotoxic than intact CDDP to the growth of L1210 cells in vitro. In contrast, the aquated derivatives of TDDP were more cytotoxic than intact TDDP (table 26, compared with figure 97).

L1210C murine leukaemia cells nominally sensitive to cisplatin (materials 2.3) were maintained in Fischers medium supplemented with 10% horse serum. The culture entered stationary phase at a density of 1-2x10^{6} cells per ml, with a mean generation time of 12h (figure 113). Figure 114 shows the effect of continuous incubation with CDDP or TDDP on the growth of L1210C cells during 72h in culture (methods 3.3.3). A 50% inhibition of cell growth was produced by approximately 6 x 10^{-5}M CDDP or approximately 4 x 10^{-4}M TDDP. Therefore the L1210C cell line was less sensitive than the L1210 line to the cytotoxicity of the platinum compounds.

Figure 97

The effect of continuous incubation with *cis*- or *trans*-diaminodichloroplatinum on the proliferation of L1210 cells during 72h *in vitro*.

Key: ■ CDDP

▲ TDDP (Mean ± S.D.; n=7)

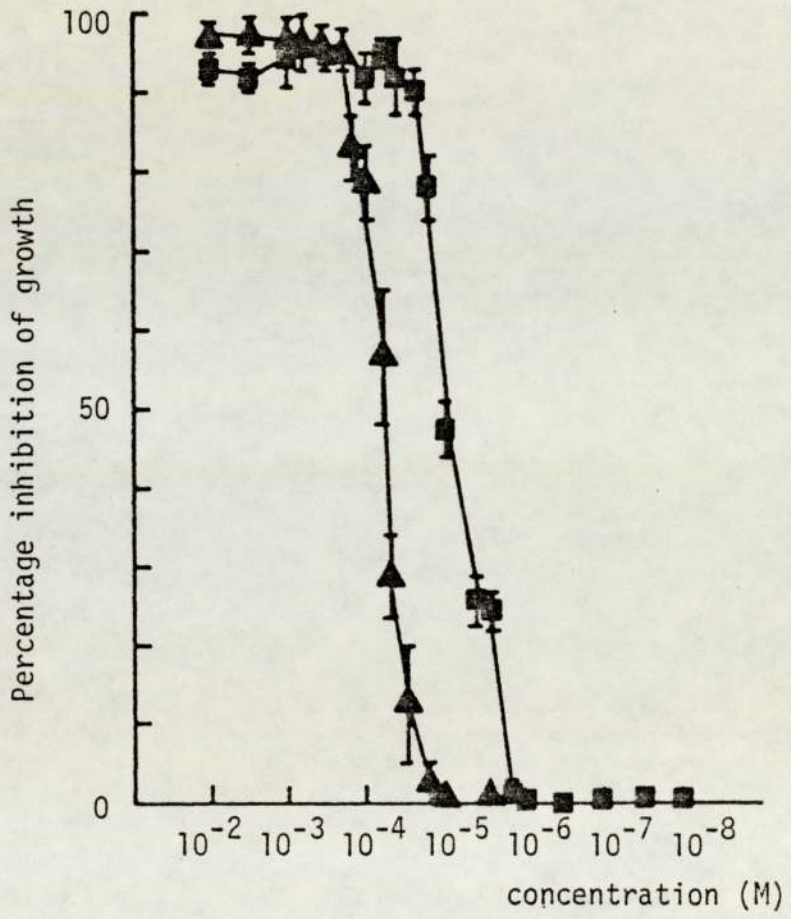


Figure 98

The effect of methionine on the uptake of[^{14}C]-α-aminoisobutyric acid into L1210 cells.

(Mean ± S.D.; n=3)

Figure 99

The effect of preincubation for 30 minutes with 10^{-5}M cis-diaminodichloroplatinum, added from freshly-made solution, on the uptake of [^{3}H]-methionine into L1210 cells incubated at 37° in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ● control

2 ■ 10^{-5}M CDDP (Mean ± S.D.; n=3)

Figure 100

The effect of preincubation for 30 minutes with 10^{-5}M cis-diaminodichloroplatinum, added from freshly-made solution, on the uptake of [^{3}H]-methionine into L1210 cells incubated at 25° in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ● control

2 ■ 10^{-5}M CDDP (Mean ± S.D.; n=3)

Figure 101

The effect of preincubation for 30 minutes with 10^{-4}M cis- or trans-diaminodichloroplatinum, added from freshly-made solutions, on the uptake of [^{3}H]-methionine into L1210 cells incubated at 25° in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ● control

2 ■ 10^{-4}M CDDP

3 ▲ 10^{-4}M TDDP (Mean ± S.D.; n=6)

Figure 98

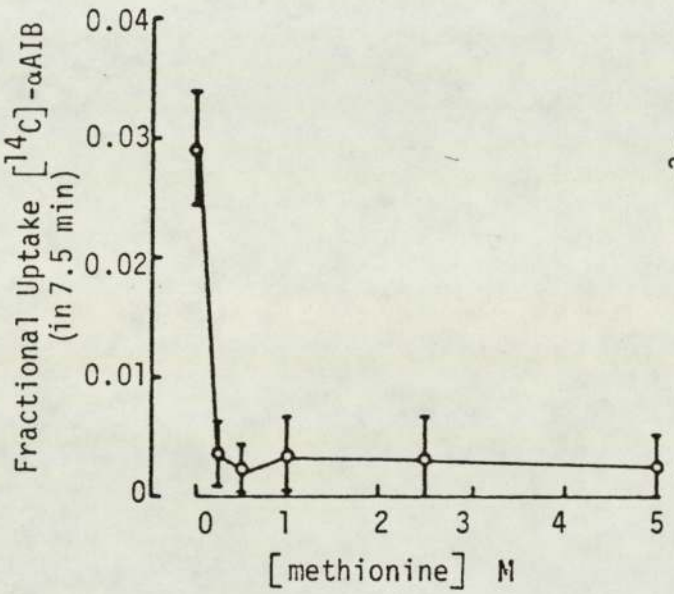


Figure 99

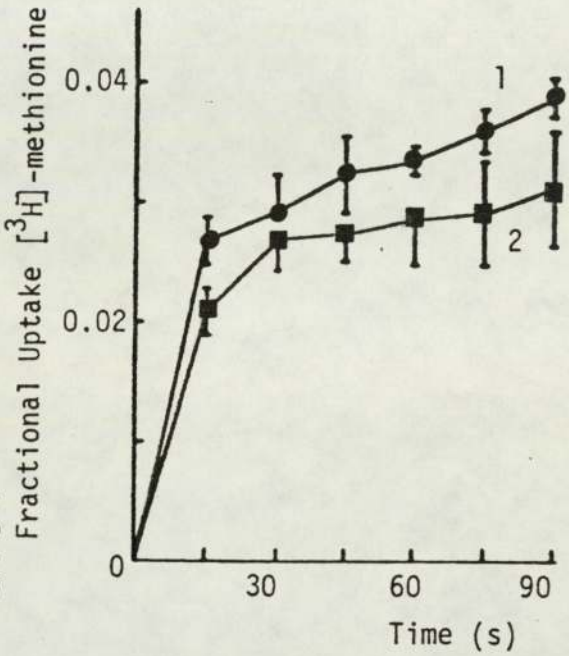


Figure 100

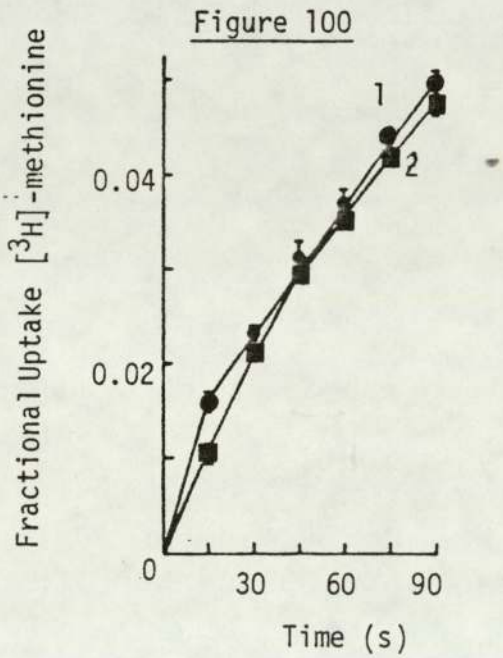


Figure 101

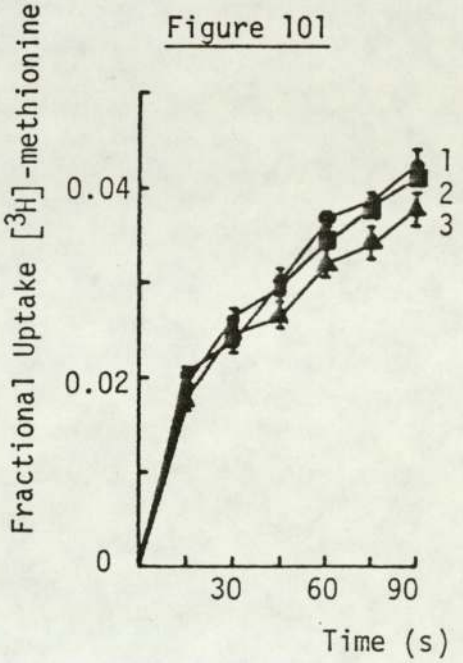


Figure 102

The effect of preincubation for 30 minutes with 10^{-5}M cis- or trans-diaminodichloroplatinum added from freshly-made solutions, on the uptake of [^{14}C]-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○control ■ 10^{-5}M CDDP ▲ 10^{-5}M TDDP

(Mean ± S.D.; n=5)

Figure 103

The effect of preincubation for 30 minutes with 10^{-4}M cis- or trans-diaminodichloroplatinum, added from freshly-made solutions, on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○control ■ 10^{-4}M CDDP ▲ 10^{-4}M TDDP

(Mean ± S.D.; n=5)

Figure 104

The effect of preincubation for 1h with 10^{-4}M cis- or trans-diaminodichloroplatinum added from freshly-made solutions, on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○control ■ 10^{-4}M CDDP ▲ 10^{-4}M TDDP

(Mean ± S.D.; n=4)

Figure 105

The effect of preincubation for 30 minutes with 10^{-3}M cis- or trans-diaminodichloroplatinum, added from freshly-made solutions, on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○control ■ 10^{-3}M CDDP ▲ 10^{-3}M TDDP

(Mean ± S.D.; n=3)

Figure 102

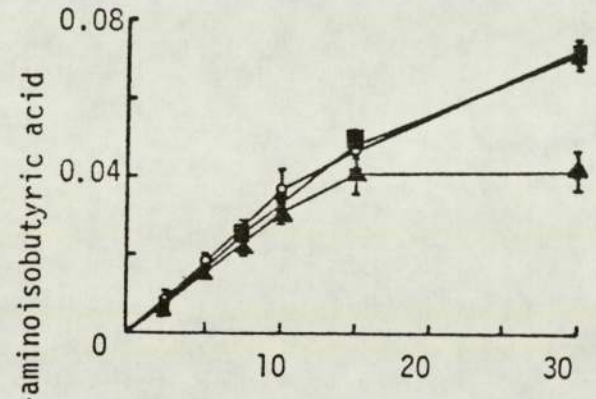


Figure 103

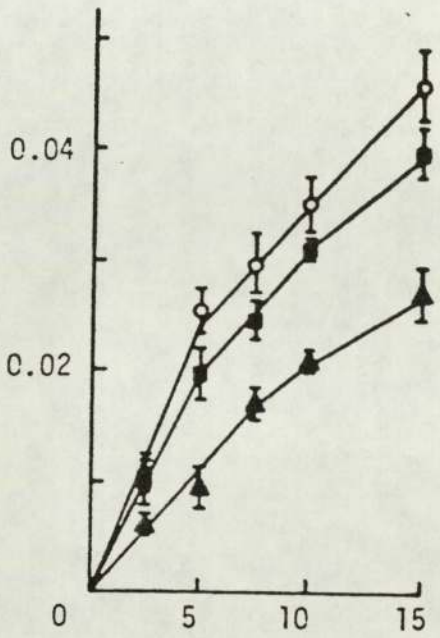


Figure 104

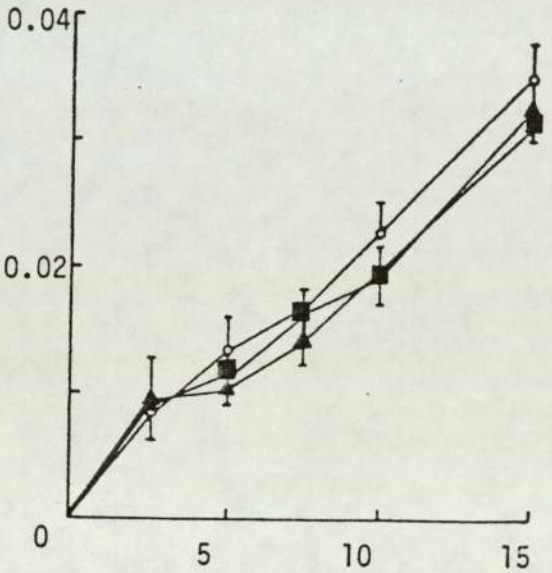
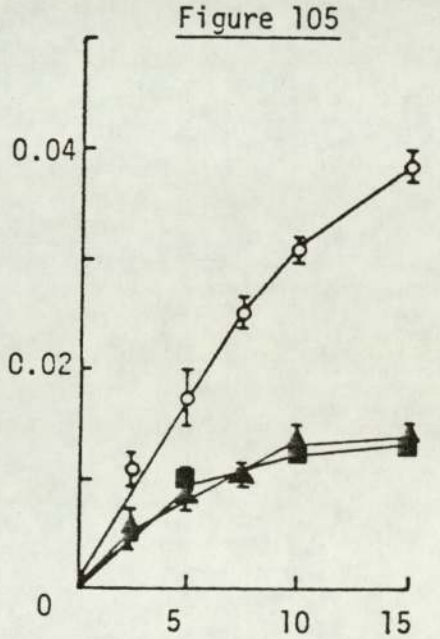


Figure 105



Fractional Uptake [^{14}C]-α-aminoisobutyric acid

Time (min)

Figure 106

The effect of preincubation for 30 minutes with 10^{-5}M cis- or trans-diaminodichloroplatinum, added from freshly-made solutions, on the influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in Krebs-Ringer buffer.

Key: ○ control
■ 10^{-5}M CDDP
▲ 10^{-5}M TDDP (Mean ± S.D.; n=3)

Figure 107

The effect of preincubation for 30 minutes with 10^{-4}M cis- or trans-diaminodichloroplatinum, added from freshly-made solutions, on the influx of $^{86}Rb^+$ & K^+ into L1210 cells incubated in Krebs-Ringer buffer.

Key: ○ control
■ 10^{-4}M CDDP
▲ 10^{-4}M TDDP (Mean ± S.D.; n=3)

Figure 106

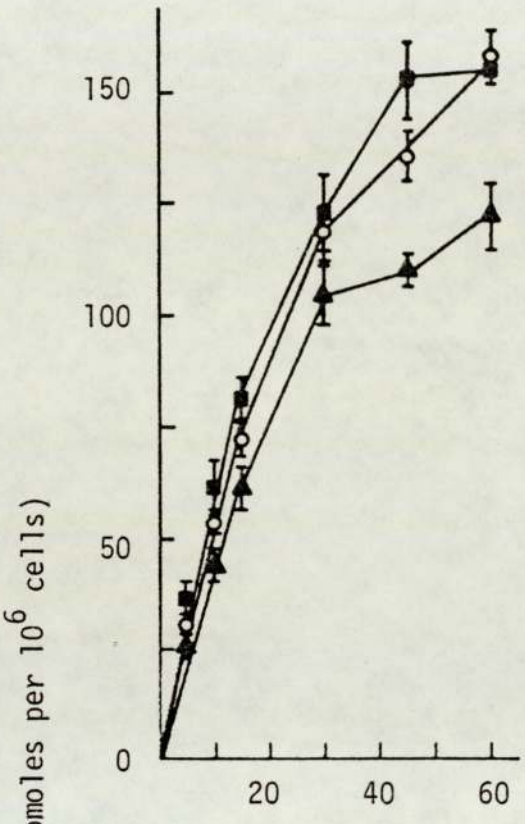


Figure 107

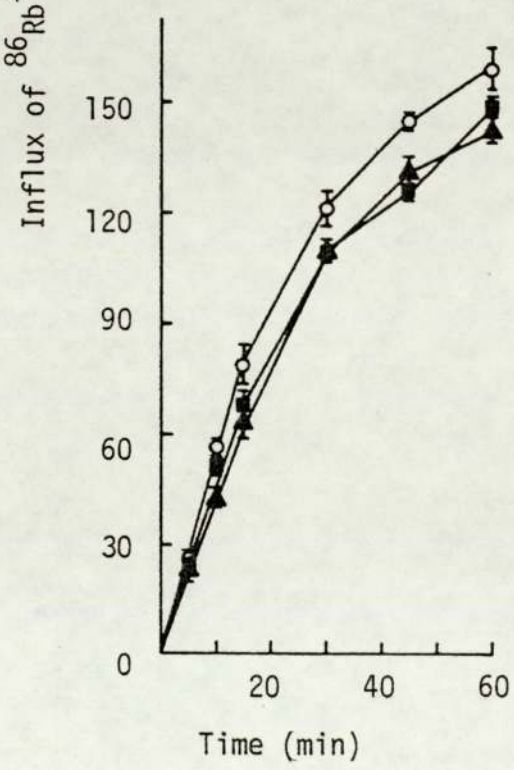


Figure 108

The effect of preincubation for 30 minutes with 10^{-5}M *cis*- or *trans*-diaminodichloroplatinum, added from 24h old solutions, on the uptake of [^{3}H]-methionine into L1210 cells incubated at 25° in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ● control
2 ■ 10^{-5}M CDDP
3 ▲ 10^{-5}M TDDP (Mean ± S.D.; n=3)

Figure 109

The effect of preincubation for 30 minutes with 10^{-4}M *cis*- or *trans*-diaminodichloroplatinum, added from 24h old solutions on the uptake of [^{3}H]-methionine into L1210 cells incubated at 25° in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ● control
2 ■ 10^{-4}M CDDP
3 ▲ 10^{-4}M TDDP (Mean ± S.D.; n=3)

Figure 110

The effect of preincubation for 30 minutes with 10^{-4}M *cis*- or *trans*-diaminodichloroplatinum, added from 24h old solutions, on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: 1 ● control
2 ■ 10^{-4}M CDDP
3 ▲ 10^{-4}M TDDP (Mean ± S.D.; n=4)

Figure 108

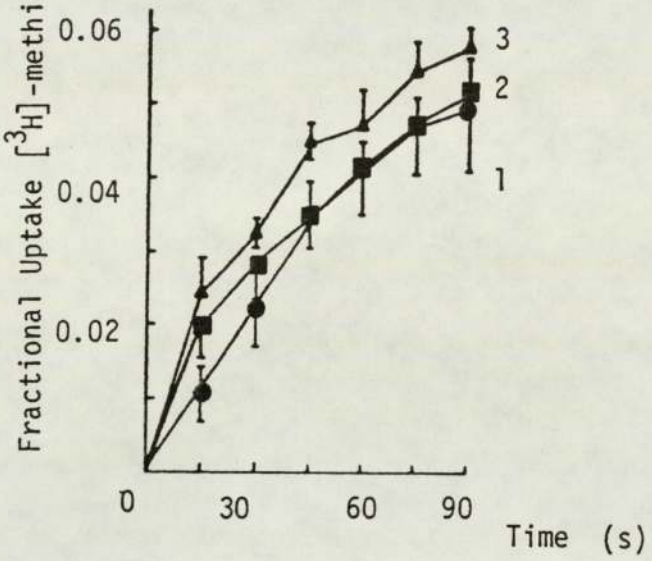


Figure 109

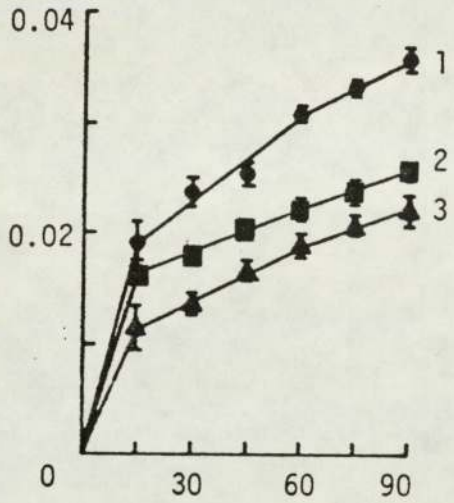


Figure 110

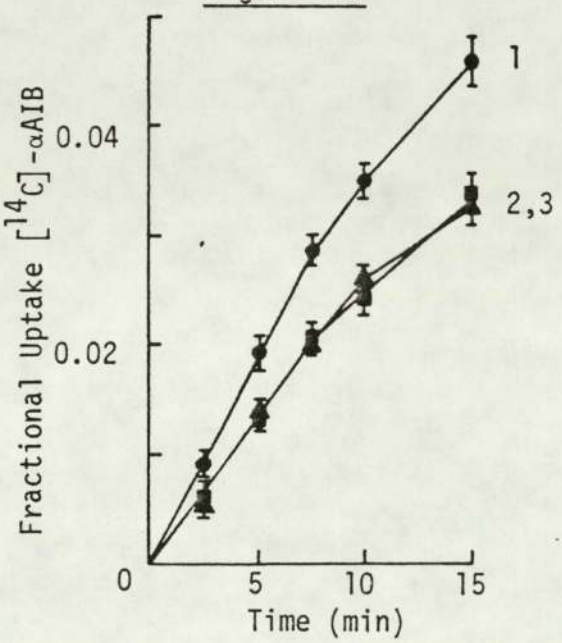


Figure 111

The effect of preincubation for 30 minutes with 10^{-5}M [Pt] mono- and di-aquated derivatives of cis-diaminodichloroplatinum, on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○ control

◪ mono-aquated CDDP

□ di-aquated CDDP (Mean ± S.D.; n=3)

Figure 112

The effect of preincubation for 30 minutes with 10^{-5}M [Pt] mono- and di-aquated derivatives of trans-diaminodichloroplatinum on the uptake of [^{14}C]-α-aminoisobutyric acid into L1210 cells incubated in Krebs-Ringer buffer in the presence of cycloleucine.

Key: ○ control

◭ mono-aquated TDDP

△ di-aquated TDDP (Mean ± S.D.; n=3)

Figure 111

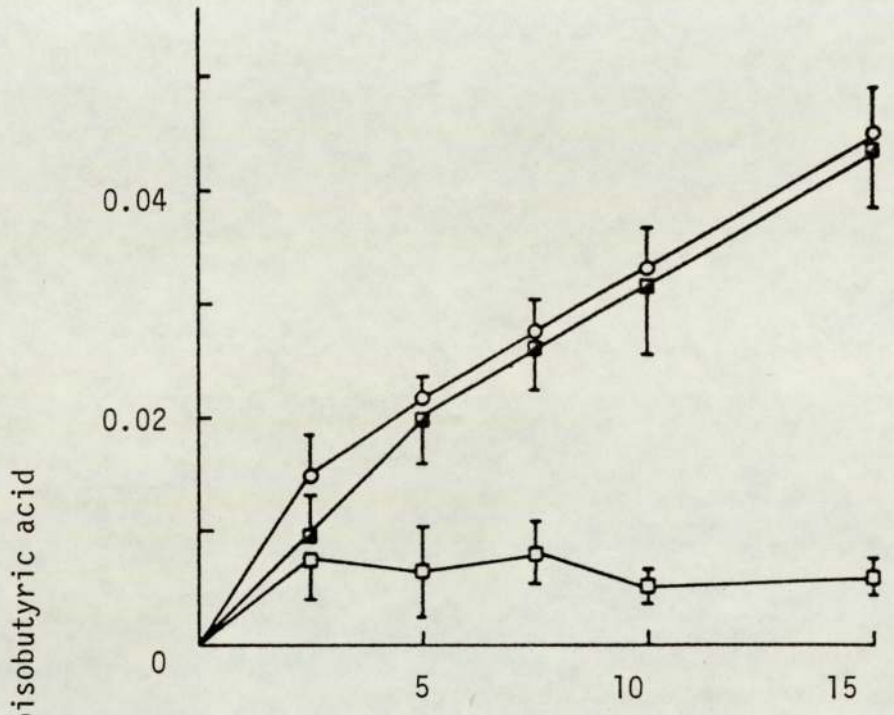


Figure 112

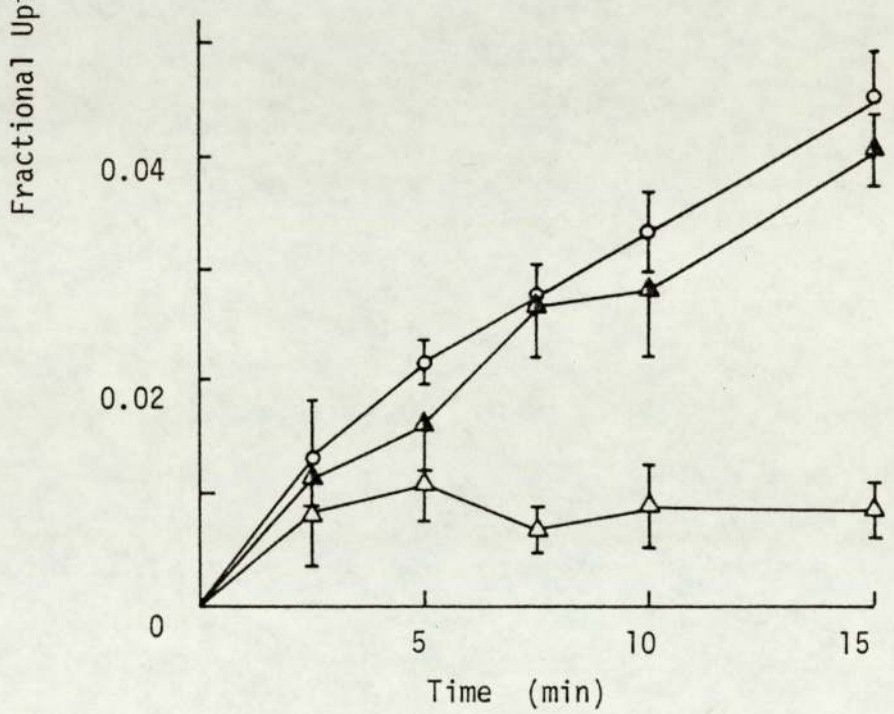


Figure 113

The proliferation of L1210C cells *in vitro*.

Cultures were established at different densities.

A typical result is shown.

Figure 114

The effect of continuous incubation with various concentrations of *cis*- and *trans*-diaminodichloroplatinum on the proliferation of L1210C cells during 72h *in vitro*.

Key: ■ CDDP

▲ TDDP (Mean ± S.D.; n=8)

Figure 113

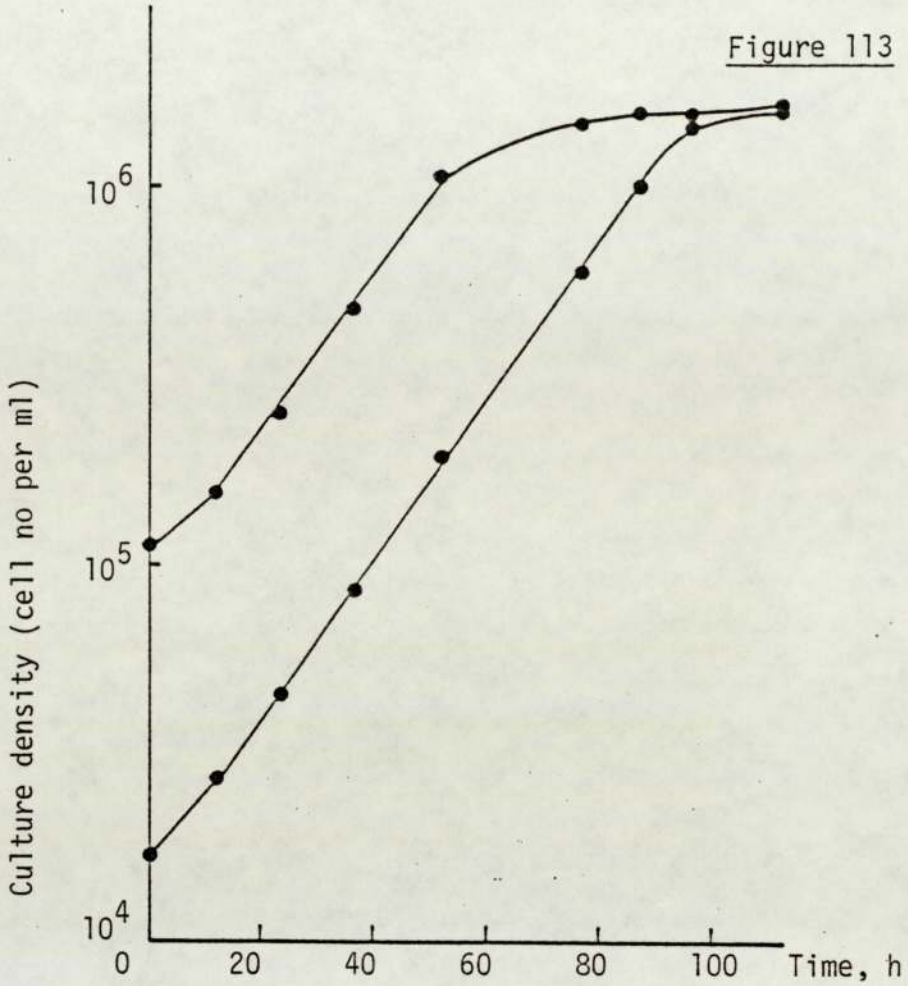


Figure 114

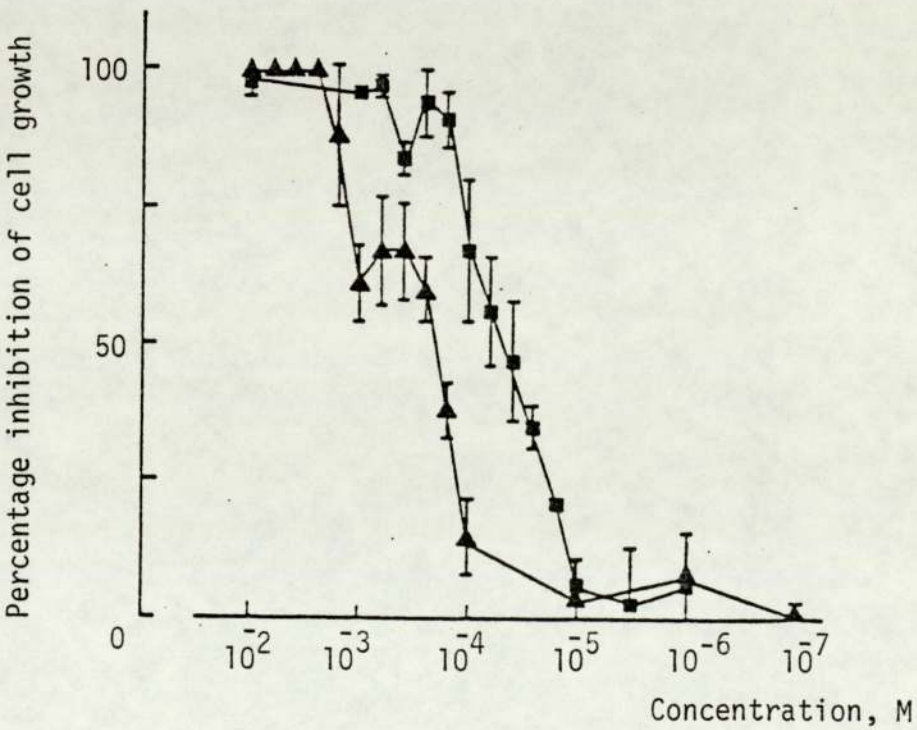


Table 26

The effect of continuous incubation with aquated derivatives of CDDP and TDDP on the proliferation of L1210 cells.

Pt concentration M	Species mono-(Aq) or di-(Aq_2)-aquated	% inhibition of growth of control
10^{-5}	cis-$Pt(NH_2)_2Cl$-Aq	34 ± 3
10^{-5}	cis-$Pt(NH_2)_2$-Aq_2	14 ± 7
10^{-7}	cis-$Pt(NH_2)_2Cl$-Aq	1 ± 4
10^{-7}	cis-$Pt(NH_2)_2$-Aq_2	2 ± 2
10^{-5}	trans-$Pt(NH_2)_2Cl$-Aq	10 ± 5
10^{-5}	trans-$Pt(NH_2)_2$-Aq_2	16 ± 7
10^{-7}	trans-$Pt(NH_2)_2$-Cl_{Aq}	5 ± 4
10^{-7}	trans-$Pt(NH_2)_2$-Aq_2	1 ± 3

(Mean ± SD, n = 3)

4.19.3 Discussion

Cisplatin and some of its newer analogues are effective for the chemotherapy of human tumours (Prestyko et al, 1980). Attempts to explain its cytotoxicity and antitumour activity are mostly founded on its ability to react with DNA, but in several different cell types the sensitivity to cisplatin cannot be attributed entirely to such reactions (section 4.19.1). Therefore, alternative mechanisms of action of CDDP are worthy of investigation. The present study was provoked by the observations of Scanlon and his coworkers (Scanlon et al, 1983) that cisplatin interfered with Na^+-dependent amino acid

transport into L1210 cells, and that this action might contribute to the cytotoxicity of the drug. It was suggested that inhibition of amino acid transport was mediated by the monoaquated derivative of cisplatin (Scanlon et al, 1983), but no attempt was made to quantitatively correlate this action with the cytotoxicity of the drug. The effects of the trans-isomer were not examined, yet any proposal for the mechanism of action of cisplatin must account for the ineffectiveness of the trans-isomer as an antitumour agent. The present study addressed both points, and found no evidence to support the hypothesis that interference with amino acid transport might contribute to the cytotoxicity of cisplatin.

Scanlon et al (1983) had reported that preincubation for 30 minutes with 10^{-5}M CDDP inhibited the Na^+ dependent transport of AIB and methionine into L1210 cells. For the L1210 cells used in the present study, 10^{-5}M CDDP was the ID_{50} value, yet had no effect on transport of AIB or methionine after 30 minutes. The transport of AIB and methionine was affected differently by higher concentrations of CDDP; preincubation for 30 minutes with 10^{-4}M CDDP reduced AIB uptake by 23% but did not reduce methionine uptake. This observation suggested that AIB and methionine preferred different routes for transport into the L1210 cells, although some common route(s) was suggested by the ability of methionine to block [^{14}C]-α-AIB influx (figure 98). The same conclusion is evident from the data of Scanlon et al (1983) who suggested common routes for AIB and methionine transport into L1210 cells; the rates of transport of the two amino acids were very different, a larger fraction of methionine uptake was Na^+-dependent, and different concentrations of each amino acid were required to produce the same inhibition of transport of the other in competition experiments. Scanlon et al (1983) presented data which

showed that the initial rate of AIB transport was inhibited by a greater amount than that of methionine after incubation for 30 minutes with 10^{-5}M CDDP.

Scanlon et al (1983) reported that the addition of cisplatin had no immediate effect on amino acid transport, but that a maximum inhibition was produced after preincubation for 30 minutes. If inhibition was due to direct interaction of the drug with the amino acid transport system, high concentrations of cisplatin may be expected to produce an immediate effect. Cisplatin has the potential to react with sulphydryl groups (section 4.19.1) located at important sites in the amino acid transport system (Kwock, 1979), but, for the reasons discussed in detail in section 4.14.1.3, this lesion may not account for the requirement of bifunctionality for activity of the platinum complexes. In the same study, Scanlon and coworkers reported that the addition of cisplatin at concentrations above 10^{-5}M had little further effect upon amino acid transport. Thus, the inhibition of transport was by no means complete, and it is difficult to account for the cytotoxicity of cisplatin on this basis. The authors cited the requirement of L1210 cells for exogenous methionine (Hoffman, 1982), but did not attempt to define the methionine requirement of their own L1210 cell line or quantitatively assess the cytotoxicity arising from loss of methionine transport; no mention was made of the cytotoxicity of cisplatin to the L1210 cell line (Scanlon et al, 1983).

If, however, it is assumed that the L1210 cells were of the same sensitivity to cisplatin as the "L1210S" cell line described by the same authors elsewhere (Gross et al, 1983), the data may be re-examined. A 50% inhibition of cell growth was produced by 0.045 μg ml^{-1} (or 1.5 x 10^{-7}M) CDDP; thus, the cells were very much

more sensitive to cisplatin than the L1210 cell line used for the present study (IC_{50} = 10^{-5}M). A concentration of 10^{-5}M CDDP thus represents approximately 67 times the IC_{50} for the "L1210S" line; in the present study, cisplatin at a concentration of 10^{-3}M, or 100 times the IC_{50} for the L1210 cells, was found to inhibit AIB transport. Scanlon et al (1983) did not relate the inhibition of cell growth to inhibition of amino acid transport, but concentrations of CDDP which produced some inhibition of growth may be expected to produce some inhibition of transport, if the two effects are causally related. The IC_{50} of CDDP for the L1210 cells used in the present study produced no inhibition of transport of AIB or methionine. The results of Gross et al (1983) suggest that CDDP cytotoxicity may be related in some way to the activity of the amino acid transport systems; a study of L1210 cells sensitive and resistant to CDDP revealed that the resistant cell line was deficient in the Na^+-dependent transport of certain amino acids, including methionine, and had a reduced growth requirement for exogenous methionine. The Na^+-independent transport of at least one amino acid, threonine, was increased in the resistant cell line, which suggested that the cells met amino acid requirements by transport through other routes. The effects of TDDP on amino acid transport were described as minimal, but the concentrations used and the data obtained were not shown.

The L1210 cell line described by Scanlon's group (Scanlon et al, 1983) was maintained in Fischers medium containing 100mgL^{-1} methionine; the relatively resistant L1210 cell line used in the present study was maintained in RPMI 1640 medium, containing 15mgL^{-1} methionine. If cisplatin sensitivity was related to methionine dependence, it might be expected that the cells grown in RPMI would be less dependent on exogenous methionine for growth, and therefore

less sensitive to cisplatin. The L1210C cell line, nominally sensitive to cisplatin, was maintained in the methionine-rich Fischers medium, but was not found to have greater sensitivity to cisplatin.

Scanlon et al (1983) used chromatography to examine the aquated derivatives of CDDP inside L1210 cells. It was proposed that inhibition of amino acid transport was mediated by the monoaquated derivative of the drug, and that the preincubation time required for the onset of inhibition corresponded to the time taken for formation of this species. A preincubation of only 2 minutes with 10^{-5}M "aquated cisplatin" inhibited AIB and methionine transport. The "aquated" CDDP consisted of about 0.3% monoaquated derivative. For the present study, monoaquated derivative of CDDP was prepared by the method (section 3.2.1.4) which produced 45-50% monoaquated derivative (Scanlon et al, 1983). Preincubation for 30 minutes with 10^{-5}M (total Pt) of the product failed to inhibit AIB uptake. The diaquated derivative produced almost complete inhibition of AIB transport, but this may have been due to the nitrate used in its production (section 3.2.1.4; Scanlon et al, 1983).

However, care must be taken in the interpretation of these results. Scanlon et al (1983) showed that monoaquated CDDP was formed inside cells, but that when efflux of drug was monitored, only the parent molecule was detected in the extracellular medium; they recognised the possibility that other forms may have effluxed from the cell, only to be reconverted back to intact drug in the presence of the higher extracellular chloride concentration. No mention was made of this possibility in the discussion of the effect of aquated derivatives on amino acid transport, but the cells were exposed to the compounds in a buffer of similarly high chloride concentration.

The results of the present study provide some evidence that the parent drug was not the only form present in such incubations; methionine transport was inhibited by 10^{-4}M CDDP added from 24h old, but not from freshly made solutions of the drug. However, the aquated derivatives of CDDP were less toxic to the growth of L1210 cells than the parent drug.

Re-examination of the results of Scanlon et al (1983) together with the results of the present study lead to the conclusion that inhibition of amino acid transport is unrelated to the cytotoxic mechanism of cisplatin.

Section 5

GENERAL DISCUSSION

The antitumour alkylating agents have been shown to exert many diverse effects on the biochemical pathways of both tumour and normal cells. A number of objections may be raised against the widely-held acceptance that DNA is the primary cellular target of such agents, and their mechanism of action is likely to be multimodal (Tritton and Hickman, 1984; Hickman, 1982; introduction 1.1.4). Since the early suggestion of Peters (1947) that alkylating agents might exert their cytotoxicity through an interaction with the plasma membrane, nitrogen mustard has been shown to perturb the physical and functional properties of membranes (introduction, 1.1.4.6; 1.3).

Nitrogen mustard was shown to inhibit processes of transmembrane ion transport. Since the control of ion transport is implicated as having a cruicial role in the control of cell division, the sensitivity to nitrogen mustard may contribute towards the cytotoxicity of the agent.

Spurgin (1981) showed that HN2 inhibited ouabain-sensitive phosphatase activity in broken plasmacytoma cell preparations and homogenates. Subsequently, Baxter et al (1982) showed that the influx of ^{86}rubidium, a congener for K^+, into plasmacytoma cells was inhibited by cytotoxic concentrations of nitrogen mustard, which were also shown to inhibit $^{86}Rb^+$ transport in Ehrlich ascites tumour cells (Grunicke et al, 1982). Baxter et al (1982) suggested that the inhibition of $^{86}Rb^+$ transport may arise from crosslinkage of the membrane-bound enzyme, Na^+K^+ATPase, but only indirect evidence was presented to support the suggestion. Against this background the present study was undertaken to identify the cellular locus or loci for the inhibition of $^{86}Rb^+$ transport by HN2; further, to examine the consequence of the lesion upon cellular ion homeostasis; and finally, to investigate whether the inhibition of ion transport contributed to the cytotoxicity of HN2.

Firstly, nitrogen mustard was shown to inhibit influx of $^{86}Rb^{+}$ and a Na^{+}-dependent amino acid into PC6A murine plasmacytoma cells (results 4.1.1.1 and 4.1.1.2), thus confirming the results of Baxter et al, (1982). It was shown that the effects were not due to cell death or to generalised membrane leakage (results 4.1.1.3).

The L1210 murine leukaemia cell line maintained in vitro was employed for further study. The characteristics of proliferation of L1210 cells in vitro were identified, allowing experiments to be conducted with populations exhibiting different distributions throughout the cell cycle. The proliferation of L1210 cells in suspension culture (results 4.1.3.1) and soft agar (results 4.1.3.2) was found to be susceptible to the cytotoxicity of pharmacological concentrations of nitrogen mustard. The interaction of HN2 with L1210 cells which resulted in cytotoxicity appeared to be rapid and irreversible (results 4.1.3.1). However, when the large majority of cells were destined to die, they were still able to exclude trypan blue for some time after exposure to HN2, demonstrating that the effects of HN2 on membrane function were not preceeded by cell death or loss of membrane integrity (results 4.1.3.3).

^{86}Rubidium was shown to be a true congener of K^{+} in L1210 cells (results 4.2.2.2), and was subsequently used as a tracer of K^{+} movement. The influx of $^{86}Rb^{+}$ into L1210 cells was shown to be sensitive to cytotoxic concentrations of HN2 (results 4.1.4; 4.3.1), and the nature of the inhibition was investigated in greater detail.

Three components of $^{86}Rb^{+}$ & K^{+} influx into rapidly-dividing L1210 cells were identified pharmacologically; approximately 30-40% of total $^{86}Rb^{+}$ & K^{+} transport was sensitive to ouabain, and so was presumed to represent the activity of $Na^{+}K^{+}$ATPase; 30-40% was sensitive to the loop diuretics, bumetanide and furosemide; and a

further 10-20% was insensitive to both ouabain and the diuretics (results 4.2.2).

The diuretic-sensitive component of $^{86}Rb^+$ & K^+ influx into L1210 cells was dependent upon external Na^+ and Cl^- ions, and the specificity for these ions was characteristic of $Na^+K^+Cl^-$cotransport systems described in other cells. It was not possible to confirm whether the Na^+ and Cl^- ions were cotransported into the cell with Rb^+ & K^+, or whether they were required only to be bound at the extracellular surface of the membrane to allow the transport of $^{86}Rb^+$ & K^+ (results 4.2.3; 4.5).

The cotransporter of different cell types may serve as an ion exchanger, or, it may mediate net ion fluxes in either direction, under physiological conditions, or in response to changes in ion concentrations on either side of the membrane (Palfrey and Greengard, 1981; Atlan et al, 1984; Chipperfield, 1986; Geck and Heinz, 1986). However, in the L1210 cell line used in the present study, no evidence was found for the operation of a diuretic-sensitive $^{86}Rb^+$ efflux, either under conditions of physiological ion concentration, or in the absence of external Na^+, K^+ and Cl^- (results 4.4). It was considered possible that the operation of the system in certain modes was dependent upon the presence of serum, as has been shown in other murine cells (Amsler et al, 1985). With the exception of the lack of reversibility, the properties of the diuretic-sensitive $^{86}Rb^+$ & K^+ influx into L1210 cells suggested that the flux represented the activity of a $Na^+K^+Cl^-$ cotransporter similar to the systems described in other cells (introduction 1.2.1; Chipperfield, 1986; Geck and Heinz, 1986).

Concentrations of nitrogen mustard which were cytotoxic to the proliferation of L1210 cells in vitro were shown to inhibit $^{86}Rb^+$ &

K^+ transport by a selective inhibition of the $Na^+K^+Cl^-$ cotransporter (results 4.3.1.2). There was no effect upon the membrane potential (results 4.10), passive $^{86}Rb^+$ & K^+ influx, ouabain-sensitive $^{86}Rb^+$ & K^+ influx (results 4.3.1.2), $^{86}Rb^+$ & K^+ efflux (results 4.4), cycloleucine transport (results 4.14.2) or permeability to trypan blue (results 4.1.3.3); therefore, inhibition did not arise from generalised membrane damage or from cell death. Further, the inhibition of $^{86}Rb^+$ & K^+ transport is not a universal phenomenon of exposure to cytotoxic agents (results 4.17; 4.18; 4.19; Chahwala and Hickman, (1985); indeed, it is not common to all alkylating agents (see results 4.1.3.3, 4.18). The effects of HN2 on $^{86}Rb^+$ & K^+ transport are not confined to murine cells (results 4.15).

The onset of inhibition of $^{86}Rb^+$ & K^+ transport by pharmacological concentrations of HN2 was relatively slow; the cells were destined to die before an effect on $^{86}Rb^+$ & K^+ transport was detected (results 4.1.3; 4.3). Since a number of factors may regulate $Na^+K^+Cl^-$cotransport (Chipperfield, 1986; Geck and Heinz, 1986), the possibility that HN2 had an indirect effect on $^{86}Rb^+$ & K^+ transport was investigated. The inhibition of $Na^+K^+Cl^-$cotransport by HN2 was not caused by depletion of intracellular ATP (results 4.12), changes in intracellular pH (results 4.11) or changes in intracellular sulphydryl levels (results 4.13). No evidence was found for an effect of HN2 on cellular Ca^{++} levels (results 4.9). High concentrations ($10^{-3}M$ or $10^{-2}M$) of HN2 immediately inhibited $^{86}Rb^+$ transport (results 4.1.4), suggesting a direct effect of HN2 upon the transporter; the slow onset of inhibition by $10^{-5}M$ HN2 may represent the time taken for the formation of an essential crosslinkage.

The proposal that inhibition of $Na^+K^+Cl^-$transport may be a primary target for the cytotoxic action of HN2 is supported by a number of observations. The inhibition was concentration - and time-dependent, and only those concentrations of HN2 which were cytotoxic to L1210 cell growth were effective (results 4.3.1). Inhibition was achieved at pharmacological concentrations of HN2 (results 4.1.3; 4.3.1), and was detected in L1210 cells treated with equivalent concentrations of HN2 *in vivo* (results 4.3.3). The sensitivity of proliferating cells to HN2 was accounted for, since cotransport activity was elevated in dividing cells compared to their quiescent counterparts (results 4.2.2.3). The requirement of two functional groups for pharmacological acitivty of HN2 was also satisfied; a monofunctional analogue of HN2 did not inhibit $^{86}Rb^+$ transport at equimolar or equitoxic concentrations (results 4.17). The proposal also seemed to account for a mechanism of resistance to HN2.

Preliminary experiments showed that the growth of mutant cell line, L1210R, which was ten-fold resistant to HN2, was also resistant to furosemide (results 4.16.2). Furthermore, $^{86}Rb^+$ & K^+ transport in L1210R cells was resistant to $10^{-3}M$ furosemide (results 4.16.3), providing strong evidence that the $Na^+K^+Cl^-$ cotransporter of L1210 cells was sensitive to HN2, and that the inhibition of cotransport may be important for cytotoxicity.

Hans Grunicke and his colleagues have previously suggested that the Na^+K^+ATPase of Ehrlich ascites tumour cells was sensitive to alkylating agents (Grunicke *et al*, 1982; 1983; Ihlenfeldt *et al*, 1981; introduction 1.3). More recently, however, they have concluded that the Na^+K^+ATPase is inhibited by HN2 only at concentrations greater that $10^{-5}M$, and that the inhibition is not correlated with the inhibition of cell growth (Doppler *et al*, 1985; Grunicke *et al*,

1985). In apparent contradiction to their earlier studies, which showed the $^{86}Rb^+$ uptake to be wholly sensitive to $5x10^{-4}M$ ouabain (Ihlenfeldt et al, 1981), they report that a diuretic-sensitive component of $^{86}Rb^+$ influx is the major target for HN2 at pharmacological concentrations (Doppler et al, 1985; Grunicke et al, 1985). The response of the furosemide-sensitive $^{86}Rb^+$ uptake to various concentrations of HN2 was found to parallel the inhibition of cell proliferation in vitro. In addition, the furosemide-sensitive $^{86}Rb^+$ uptake into an HN2-resistant Ehrlich cell line was less sensitive to HN2. However, the authors concluded that inhibition of the furosemide-sensitive system alone is not sufficient to explain the antitumour activity of HN2.

Their conclusion was drawn from a comparison of the effects of HN2 and furosemide on cell proliferation. Furosemide-sensitive $^{86}Rb^+$ influx into Ehrlich ascites cells in vitro was inhibited by 50% by $2x10^{-6}M$ HN2, after incubation for 4h; cell multiplication, measured 48h later, was inhibited by 80%. In contrast, incubation with $5x10^{-6}M$ furosemide, which also inhibited furosemide-sensitive $^{86}Rb^+$ influx by 50%, was without effect on cell growth. Incubation with $10^{-3}M$ furosemide maximally inhibited ouabain-resistant $^{86}Rb^+$ uptake and cell proliferation, but maximal inhibition of $^{86}Rb^+$ uptake was also caused by lower concentrations of furosemide, which were without effect on cell multiplication. It was concluded that inhibition of $Na^+K^+Cl^-$ cotransport was not cytotoxic to Ehrlich cells (Grunicke et al, 1985; Doppler et al, 1985). Indeed, a number of mutants deficient in $Na^+K^+Cl^-$ cotransport have been grown in culture (Gargus and Slayman, 1980; McRoberts et al, 1983; Sussman and O'Brien, 1985).

The results of the present study also reveal differences in the cytotoxicity of the inhibition of $^{86}Rb^+$ transport by furosemide and

HN2. Incubation of L1210 cells with 10^{-5}M HN2 resulted in complete inhibition of diuretic-sensitive $^{86}Rb^+$ & K^+ influx (results 4.3.1.2) and >99% inhibition of cell proliferation in suspension culture (results 4.1.3.1); in contrast, 10^{-3}M furosemide completely inhibited diuretic-sensitive $^{86}Rb^+$ & K^+ influx, but inhibited cell proliferation by only 50% (results 4.16).

The reason for the difference in cytotoxicity of furosemide and HN2 is unknown. Clearly, these agents inhibit the function of cotransport by different mechanisms. There is no structural homology (figure 2). Furosemide is a competitive inhibitor, binding at one of the anion sites on the cotransporter proteins(s) (Forbush and Palfrey, 1983), whilst HN2 covalently cross-links some component (results 4.3.1.1.3). One possible explanation may be derived from the results of Jayme et al (1981), who noted that diuretic-sensitive transport is increased in cells which are exposed to diuretics for prolonged periods. It may be that cells survive the inhibition of $^{86}Rb^+$ uptake by furosemide because they subsequently up-regulate the activity of the cotransporter; it may be envisaged that some of the other effects of HN2 on cell metabolism (introduction 1.1.4) prevent such up-regulation in response to inhibition.

Doppler et al (1985) examined the recovery of furosemide-sensitive $^{86}Rb^+$ uptake into Ehrlich ascites cells after incubation with HN2, and its relationship to the repair of crosslinks in DNA. The cells were incubated with $2x10^{-6}$M HN2 for 4h, after which time the exposure was terminated by the addition of thiourea. Furosemide-sensitive $^{86}Rb^+$ uptake was reduced by 50% in Ehrlich ascites cells, but by only 20% in an HN2-resistant mutant. Although it was noted that $^{86}Rb^+$ influx into the mutant was less sensitive to HN2, no mention was made of its sensitivity to furosemide; the HN2-resistant

L1210R cell line was also found to be resistant to furosemide (results 4.16.2). This possibility may throw some doubts on the validity of the measurement of furosemide-sensitive $^{86}Rb^+$ uptake in the HN2-resistant Ehrlich ascites cell line (Doppler et al, 1985).

The authors report that the repair of crosslinks in the DNA of Ehrlich ascites cells began immediately upon removal of HN2 with thiourea. DNA repair in HN2-resistant cells was not investigated. Since HN2 has a very short half-life in aqueous media (introduction 1.1.3), it would be expected that very little intact HN2 remained in the medium after 4h; thus, it is difficult to understand why the removal of this residual amount of HN2 would allow initiation of repair of DNA (Doppler et al, 1985). The recovery of $^{86}Rb^+$ influx into both sensitive and resistant Ehrlich ascites cells displayed an initial lag, but 4h after removal of HN2 it began to rise, reaching control levels after 8h. Despite the recovery, cell multiplication during the 48h after exposure to the drug was impaired by 50%.

The difference in cell survival after inhibition of $^{86}Rb^+$ transport by HN2 and furosemide may suggest that the HN2 interferes with $Na^+K^+Cl^-$ cotransport via an indirect mechanism, in contrast to furosemide. HN2 is known to affect the cytoskeleton (introduction 1.1.4.6), which one report has linked to components of the $Na^+K^+Cl^-$ cotransporter (Jorgensen et al, 1984); it may also have an effect on membrane fluidity (introduction 1.1.4.6), which may regulate the activity of membrane proteins (Pang et al, 1979);or, it may affect some process by which the cotransport is regulated.

It is known that $Na^+K^+Cl^-$cotransporters of certain cells may be regulated by intracellular cAMP (see results 4.2.5.3; Chipperfield, 1986; Geck and Heinz, 1986). The most frequently-studied examples are avian erythrocytes (Palfrey and Rao, 1983; Saier and Boyden,

1984; Kregenow, 1981; Palfrey and Greengard, 1981), in which any agent which elevates cAMP stimulates $Na^+K^+Cl^-$ cotransport; the physiological significance is unknown. The $Na^+K^+Cl^-$ cotransport system of L1210 cells failed to respond to an analogue of cAMP (results 4.2.5); this result suggested that, although HN2 was known to interfere with cellular cAMP levels (introduction 1.1.4.5), its effects on cotransport were not mediated by cAMP.

A later experiment with the HN2-resistant L1210R cell line produced an unexpected result. It was known that HN2 inhibited the Na^+-dependent transport of an amino acid analogue, α-AIB (results 4.1.1.2; 4.14.1; introduction 1.3; Baxter et al, 1982); the inhibition was not thought to be related to the inhibition of $Na^+K^+Cl^-$ cotransport in L1210 cells (results 4.14.1.2.3), and did not account for the increased toxicity to dividing cells (results 4.14.1.3). Thus it was thought that HN2 may react directly with the protein(s) responsible for α-AIB transport (results 4.14.1.2.3). However, in the L1210R cell line, 10^{-5}M HN2 failed to inhibit both $Na^+K^+Cl^-$ cotransport and α-AIB uptake, suggesting that both processes may be susceptible to a common lesion in HN2-treated L1210 cells (results 4.16.3). Amino acid transport may also be regulated by intracellular cAMP (Shotwell et al, 1983). Further studies are required to examine the effects of HN2 on cAMP levels and cAMP phosphodiesterase in L1210 and L1210R cells and to examine their relationship to the effects of HN2 on $Na^+K^+Cl^-$cotransport. A second indirect mechanism by which HN2 may interfere with $Na^+K^+Cl^-$ cotransport may involve the protein kinase C. Phorbol esters, which activate protein kinase C (Castagna et al, 1982), have been shown to modify $Na^+K^+Cl^-$cotransport activity (O'Brien and Krzeminski, 1983; Sussman et al, 1985; Owen, 1985; Paris and Pouyssegur, 1986). The

phorbol ester TPA was found to inhibit $Na^+K^+Cl^-$cotransport in vascular smooth muscle cells (Owen, 1985) and 3T3 cells (O'Brien and Krzeminski, 1983; Sussman et al, 1985; see also introduction 1.2.2). In Balb/c 3T3 cells, TPA inhibited cotransport, reduced cell volume and elevated cellular K^+ content, which induced the gene ornithine decarboxylase. In a mutant cell line deficient in $Na^+K^+Cl^-$cotransport, TPA failed to induce the enzyme (Sussman et al, 1985). TPA has also been shown to induce cellular oncogenes in 3T3 cells (Kelly et al, 1983; Greenberg and Ziff, 1984), and it is tempting to speculate that the inhibition of K^+ transport by HN2 may contribute to its mutagenic properties.

Activation of protein kinase C is also involved in the response of cells to growth factors (De Laat et al, 1985). The binding of growth factors to membrane receptors stimulates the tyrosine kinase activity of receptor and the breakdown of phosphatidyl inositol 4,5-bisphosphate to produce inositol triphosphate and 1,2diacyclglycerol. Inositol triphosphate mobilises intracellular Ca^{++}, and diacyclglycerol activates protein kinase C. The structural homology to diacyclglycerol allows TPA to similarly activate protein kinase C. This enzyme phosphorylates the Na^+/H^+ antiporter, which alters its affinity for the ions. The activity of Na^+/H^+ exchange is stimulated, thus elevating intracellular Na^+ and intracellular pH. The intracellular Na^+ stimulates the activity of Na^+K^+ATPase. In this mechanism, $Na^+K^+Cl^-$cotransport may potentially respond to the signals of elevated Na^+, changes in pH, elevated Ca^{++}, or protein kinase C (Chipperfield, 1986).

Paris and Pouyssegur (1986) studied the effect of growth factors on $Na^+K^+Cl^-$ cotransport in hamster fibroblasts. The mitogen αthrombin was shown to stimulate bumetanide-sensitive $^{86}Rb^+$ uptake,

even in a mutant which lacked the Na^+/H^+ antiport. It was concluded that the response of $Na^+K^+Cl^-$ cotransport to mitogens was independent of the stimulation of Na^+/H^+ antiport activity, although the authors speculated that in normal cells, the change in intracellular pH due to antiport activity may contribute to the activation of the $Na^+K^+Cl^-$ cotransporter. Epidermal growth factor stimulates cotransport in hamster fibroblasts (Paris and Pouyssegur, 1986) Balb/c 3T3 cells (O'Brien and Krzeminski, 1983) and vascular smooth muscle cells (Owen, 1985).

Paris and Pouyssegur (1986) have attempted to explain the parodoxical effects of phorbol esters and growth factors on the activity of the $Na^+K^+Cl^-$ cotransporter. They found that both thrombin and TPA stimulated $Na^+K^+Cl^-$ cotransport into hamster fibroblasts. However, if cells were first preincubated with TPA, the stimulatory effect of thrombin was inhibited (see also Owen, 1985). The authors interpreted this result to suggest that activated protein kinase C exerted a feedback inhibitory control. The maximum stimulation of cotransport which was caused by TPA was much less than the maximum stimulation caused by thrombin, suggesting that part of the stimulatory effect of growth factors on $Na^+K^+Cl^-$ cotransport was independent of protein kinase C.

The precise role of $Na^+K^+Cl^-$ cotransport in mitogenesis is unknown. Although it is rapidly stimulated by growth factors, the complete inhibition of the system by bumetanide does not completely inhibit the initiation of DNA synthesis upon mitogenesis (Paris and Pouyssegur, 1986; Amsler et al, 1985). There was some evidence that serum activated $Na^+K^+Cl^-$ cotransport in quiescent L1210 cells, although inconsistent results were obtained (results 4.2.2.3). Ambiguous responses of $Na^+K^+Cl^-$ cotransport to serum have been

observed by others (results 4.2.2.3.3; Owen and Prastein, 1985). Clearly, phorbol esters and purified growth factors will be of use in elucidating the role of $Na^+K^+Cl^-$ cotransport in mitogenesis of L1210 cells; the availability of L1210R, the mutant apparently deficient in cotransport, will also be of value. Such information will allow a more precise consideration of the role of inhibition of $Na^+K^+Cl^-$cotransport in the cytotoxicity of HN2 to dividing cells.

A major role of the $Na^+K^+Cl^-$ cotransporter appears to be the regulation of cell volume under isosmotic and anisosmotic conditions (Chipperfield, 1986; Geck and Pfeiffer, 1985, see results 4.8.3). Complete inhibition of $Na^+K^+Cl^-$ cotransport in L1210 cells by furosemide or HN2 caused a reduction in cell volume, suggesting that the cotransporter contributed to the maintenance of L1210 cell volume under steady-state conditions (results 4.8). The volume of L1210R cells was unaffected by HN2 (results 4.16.3). In contrast to the results of the present study, Doppler et al (1985) found no effect of HN2 or furosemide on the volume of Ehrlich ascites tumour cells, when the diuretic-sensitive $^{86}Rb^+$ uptake was reduced by 50%. The effects of complete inhibition were not investigated. It was found that the volume of HN2-treated cells increased after 12h, but this was associated with the formation of giant cells typically seen after exposure to alkylating agents (Brewer et al, 1961).

The authors did not speculate on the consequences of inhibition of K^+ transport on the cellular K^+ homeostasis, in the absence of any effect on cell volume (Doppler et al, 1985; Grunicke et al, 1985). In the present study, it was found that the reduction of cell volume upon exposure to HN2 allowed the maintenance of intracellular K^+ concentration in the face of decreased K^+ content (results 4.7). The different responses of the volume of L1210 and Ehrlich cells may

represent different functions of the $Na^+K^+Cl^-$ cotransporters of the two cell types. The cotransporter of L1210 cells appeared to mediate only the influx of $^{86}Rb^+$ & K^+ (results 4.4), but the cotransporter of Ehrlich cells has been shown to mediate a component of $^{86}Rb^+$ & K^+ efflux (Tupper, 1975; Bakker-Grunwald, 1978). If the cotransporter of the Ehrlich cells in Doppler's study (Doppler et al, 1985) was acting as a K^+/K^+ exchanger, inhibition of the exchange by HN2 or furosemide would be expected to have little effect on K^+ and water contents. However, neither the $^{86}Rb^+$ efflux or the K^+ concentrations were measured, so no firm conclusion regarding this possibility may be drawn.

Nitrogen mustard (10^{-5}M) produced a gradual inhibition of $^{86}Rb^+$ & K^+ uptake; inhibition of $Na^+K^+Cl^-$cotransport into L1210 cells was complete after 3h (results 4.3.1.2). However, the reduction in L1210 cell volume was maximum within 30 minutes of incubation with HN2 (results 4.8). This result suggests that inhibition of $Na^+K^+Cl^-$cotransport alone cannot wholly explain the volume change, and it is possible that HN2 interfered with one (or more) other unknown volume-regulatory process.

It is possible that the inhibition of cell volume regulation by HN2 is toxic to growing cells. Again, the cotransport-deficient L1210R cell line will be of use in elucidating the role of volume changes in toxicity of HN2. A second explanation of the role of inhibition of $Na^+K^+Cl^-$ cotransport in the toxicity of HN2 may come from the report of van Ankeren and Wheeler (1985), who investigated the effect of hypertonic media on the repair of radiation-induced damage to DNA.

Rat brain tumour cells were exposed to potentially lethal doses of X-irradiation, then to isotonic or hypertonic buffers. The repair

of DNA damage was examined in relationship to the survival of cells in soft agar. Hypertonic treatment of unirradiated cells was without effect on DNA or cell survival. In contrast, hypertonic treatment after radiation was found to inhibit repair of DNA damage and reduce cell survival. Similar results were obtained by Raaphorst and Dewell (1979), Utsumi and Elkind (1979) and Ben-Hur et al (1980).

Cells exposed to hypertonic media initially shrink then undergo a regulatory volume increase, which may involve activation of the $Na^+K^+Cl^-$ cotransporter in certain cell types (Geck and Pfeiffer, 1985; McManus and Schmidt, 1978; see results 4.8.3). The effects of HN2 on DNA are well-documented and DNA has been accepted to be the principal target for the toxicity of the agent (introduction 1.1.4.2). It is possible that the damage to DNA and the effect on cell volume may both contribute to the toxicity of nitrogen mustard.

Section 6

APPENDICES

6.1 APPENDIX I: EVALUATION OF THE OIL-BARRIER METHOD FOR THE MEASUREMENT OF RADIOISOTOPE FLUXES IN L1210 CELLS; DISTRIBUTION OF [^{14}C]-INULIN CARBOXYLIC ACID AND ^{51}CHROMIUM IN CELLULAR AND SUPERNATANT FRACTIONS.

6.1.1 Introduction

The oil barrier technique (methods 3.4.1; results, 4.1.1) for the separation of cells from unabsorbed radiolabel was modified by Spurgin (1981) and used to measure the influx of $^{86}Rb^{+}$ into PC6A cells. The experiments described in this section were carried out to establish whether the method described by Spurgin (1981; see also Baxter et al, 1982) might be applied for similar experiments with L1210 cells.

6.1.2 Results

L1210 cells incubated for 4h in RPMI with $^{51}Cr^{3+}$ with and without 10^{-5}M HN2 were washed (methods 3.5.1.2) and layered above oil barriers in microfuge tubes (methods 3.4.1). After centrifugation the formic acid layer in the microfuge tube (methods 3.4.1) contained 92.4% of the total $^{51}Cr^{3+}$; the formic acid layer obtained after centrifugation of HN2-treated cells contained 90 ± 4% of total $^{51}Cr^{3+}$ (both values are mean ± SD; n=4; $p > 0.1$).

Washed, $^{51}Cr^{3+}$-labelled L1210 cells (methods 3.5.1.2) were incubated for 4h in RPMI with and without 10^{-5}M HN2 and layered above oil barriers in microfuge tubes. After centrifugation the aqueous layer above the oil contained 10 ± 5% of the total $^{51}Cr^{3+}$; the aqueous layer obtained after centrifugation of HN2-treated cells contained 12 ± 3% of total $^{51}Cr^{3+}$ (both values are mean ± S.D.; n=4;$p>0.5$).

L1210 cells which had been incubated for 4h in RPMI with and without 10^{-5}M HN2 were incubated with [^{14}C]-inulin carboxylic acid for 15 minutes (methods 3.5.1.3) and layered above oil barriers in microfuge tubes. Upon centrifugation the formic acid layer in the microfuge tube (methods 3.4.1) contained 0.038 ± 0.007% of the total ^{14}C; the acid layer obtained after centrifugation of HN2-treated cells contained 0.040 ± 0.008% of total ^{14}C (both values mean ± S.D.; n=6, $p>0.5$).

6.1.3 Discussion

The separation of cells from supernatant by centrifugation through an oil layer was described by Kepner and Tosteson (1972) and Averdunk and Lauf (1979). The technique was later modified by Finkelstein and Adelberg (1977) and Gargus and Slayman (1980) for use with microfuge tubes. The mixture of corn oil and silicon oil was varied by Spurgin (1981) to obtain a suitable density for the separation of PC6A cells from supernatant. The final assay allowed 92.4% of ^{51}chromium-labelled PC6A cells to pass into the acid layer accompanied by less than 0.2% of label from the supernatant. The method allowed the separation of cells from unabsorbed label and eliminated the need to wash the cells and correct for consequent efflux of radiolabel (L'Allemain et al, 1984).

The oil barrier technique may be considered suitable for the measurement of ^{86}rubidium transport into L1210 cells if the following conditions are fulfilled:-

(i) the oil layer is of the appropriate density to allow the majority of the L1210 cells to pass through into the acid layer during centrifugation.

(ii) unabsorbed radiolabel remains in the aqueous supernatant above the oil layer and does not pass into the acid layer during centrifugation

(iii) a minimal amount of extracellular water, containing unabsorbed radiolabel, is trapped by the cells and carried into the acid layer

^{51}Chromium was used as a marker for the movement of the L1210 cells through the oil layer upon centrifugation; the release of $^{51}Cr^{3+}$ from washed, prelabelled cells was taken as a marker of cell lysis. The release of $^{51}Cr^{3+}$ was first used to indicate lysis of cells after incubation with immune lymphocytes (Berke et al, 1969; Wigzell, 1965; Goodman, 1961) and has been widely used to assess cell damage independent of proliferative capacity (Faanes et al, 1973). Radiolabelled chromate is bound by small organic cations and cell organelles (Sanderson, 1976) and is reduced on binding (Berke and Amos, 1973). In comparison with trypan blue permeability, as an indicator of cell death, $^{51}Cr^{3+}$ release more closely correlated with the number of dead cells (Wigzell, 1965).

The values for the percentage of $^{51}Cr^{3+}$ released from L1210 cells in the present study are similar to the percentages of cells permeable to trypan blue after incubation in RPMI with and without 10^{-5}M HN2 (results, 4.1.3.3); these observations further support the conclusion that perturbations of $^{86}Rb^{+}$ transport into HN2-treated L1210 cells (results, 4.1.4 & 4.3) are not due to general leakiness of the membrane (results, 4.3). The majority of the $^{51}Cr^{3+}$ was found in the formic acid layer after centrifugation of cells incubated for 4h in RPMI with $^{51}Cr^{3+}$ in the presence and absence of 10^{-5}M HN2; this observation indicates that the majority of the cells had passed through the oil layer. There was no significant difference ($p>0.1$) in the percentage of total $^{51}Cr^{3+}$ carried into the oil layer after centrifugation of cells incubated with or without HN2. It was concluded from this result that the observed differences in the fractional uptake of $^{86}Rb^{+}$ into L1210 cells incubated with and without HN2 (results, 4.1.4) could not be ascribed to the different movement of cells into the formic acid layer in the microfuge tube.

[^{14}C]-Inulin carboxylic acid is a heterogenous polysaccharide (see Phelps, 1965) which does not enter cells and has been extensively used as a marker of extracellular space and renal filtration rates. It was used in the present study as a marker of unabsorbed radiolabel and of extracellular water carried through the oil layer with the cells during centrifugation. Most of the [^{14}C]-inulin carboxylic acid remained in the aqueous supernatant layer above the oil layer in the microfuge tube, suggesting that unabsorbed radiolabel from the medium did not pass into the formic acid layer. There was no significant difference in the percentage of total [^{14}C]-inulin carboxylic acid trapped in extracellular water by L1210 cells incubated in the absence or presence of HN2. It was concluded that the differences in accumulation of $^{86}Rb^{+}$ by L1210 cells incubated with and without HN2 (results 4.1.4 & 4.3) did not arise from differences in the amount of extracellular label carried through the oil layer. Such differences may arise if an agent causes gross changes in the surface morphology of cells. The biconcave shape of the human erythrocyte may account for the very variable results obtained when $^{86}Rb^{+}$ fluxes in these cells were examined by use of the oil barrier technique (Dr P Feig, personal communication).

The results described in this section show that the oil barrier technique fulfils the criteria listed above, and is suitable for the measurement of $^{86}Rb^{+}$ transport into L1210 cells incubated with or without HN2.

6.2 APPENDIX II: THE ISOLATION OF L1210 CELL MEMBRANES

6.2.1 Introduction

The production of plasma membrane vesicles from L1210 cells was desirable for two reasons: firstly, to enable examination of the membrane proteins by polyacrylamide gel electrophoresis in the presence of SDS, and so to investigate the effects of HN2 upon them; and, secondly, to produce intact vesicles in which to examine the direct effects of HN2 upon membrane transport processes in the absence of any cytoplasmic interaction. As a preliminary investigation, the yield of plasma membrane protein produced by two different methods (methods 3.17) was evaluated.

6.2.2 Results

Attempts were made to disrupt L1210 cells by repeated passage through a fine needle and syringe, but examination of a portion of the cell suspension in the presence of trypan blue suggested that the cells were resistant to this treatment. Similarly, after repeated Dounce homogenisation, many cells remained intact. In contrast, sonication (3x2 seconds on full power, with 20 seconds rest intervals, on ice) completely disrupted the cells but after centrifugation (method 2, methods 3.17.2), very little material was recovered, suggesting that the lipid bilayer had been destroyed. Optimal cell disruption was achieved by repeated slow freezing and thawing, interspersed with Dounce homogenisation in the presence of hexylene glycol (methods 3.17.2).

The method based on a two-phase polymer separation (methods 3.17.1) gave a yield of 370ng per 10^6 cells. The second method, based on the separation by centrifugation (methods 3.17.2) gave a yield of 660 ± 190ng protein per 10^6 cells (n=3; mean ± S.D.).

6.2.3 Discussion

The L1210 murine leukaemia maintained *in vitro* is resistant to cell rupture by mechanical means; an essential step in the preparation of membranes from L1210 cells is to ensure optimum cell disruption. This was achieved by repeated freezing and thawing together with homogenisation in the presence of hexylene glycol which increases membrane rigidity (Yang *et al*, 1979).

The method of Yang *et al* (1979), after Tsai *et al* (1975), using density gradient centrifugation (methods 3.17.2) provided an improved yield of membrane protein compared to the method relying upon the two polymer phases (methods 3.17.1; Hourani *et al*,1973). However, large number of cells (10^9) necessitating large volumes of culture suspension (1000-1500ml), were required to produce sufficient material for experimentation. To this end, cells were harvested and stored at -70°, then pooled before use, or else maintained in 300ml portions in 500ml flasks on a roller; the latter procedure allowed a greater cell density to be reached before the culture entered stationary phase (data not shown).

Since only a small amount of material was available, it was not assayed for the presence of plasma membrane marker enzymes, or evaluated for contamination by other cellular membrane fractions. However, these assays were carried out by the authors of the original paper (Yang *et al*, 1979; Tsai *et al*, 1975). Similarly, no attempt was made to assess what portion of the vesicles were closed, or to determine their sidedness, although under the phase contrast microscope, small discrete vesicles were seen. If the vesicles are to be used in further studies of membrane transport systems (Hochstadt *et al*, 1975, Garty *et al*, 1983), the maximum amount of intact sealed vesicles will be required.

A preliminary study was carried out with the plasma membrane

proteins subjected to polyacrylamide gel electrophoresis in the presence of SDS, by the methods of Laemmli (1971) (data not shown). Vesicles were prepared from L1210 cells which had been incubated for 3h in the absence and presence of 10^{-5}M HN2; vesicles prepared from L1210 cells were also incubated for fifteen minutes in the presence and absence of 10^{-3}M HN2. In all cases, the preparation was boiled under reductive and denaturing conditions, then loaded on to 5% acrylamide gels. In all cases a complex pattern of bands was obtained, but no marked differences were seen between controls and HN2-treated samples.

In conclusion, the method for isolation of L1210 cell membranes by density gradient centrifugation provides suitable material for the examination of membrane proteins by gel electrophoretic techniques. Further experiments are required to confirm whether it is suitable for production of vesicles for use in transport experiments. Both types of study will be of interest to examine the direct effects of HN2 upon the cell membrane.

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