THE IMPORTANCE OF Na^+-K^+-Cl^- COTRANSPORT IN NITROGEN MUSTARD INDUCED CELL DEATH.

ANTHONY NARDONE

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF ASTON IN BIRMINGHAM

SEPTEMBER 1990

THE UNIVERSITY OF ASTON IN BIRMINGHAM
The importance of Na^+-K^+-Cl^- cotransport in Nitrogen Mustard induced cell death.
ANTHONY NARDONE
Doctor of Philosophy, 1990.

The incubation of murine leukaemic L1210 cells in vitro for 4 hours (hr) with 10uM nitrogen mustard (HN2), a bifunctional alkylating agent, inhibited the influx of the potassium congener, rubidium+ ($^{86}Rb^+$) by the selective inhibition of the Na^+-K^+-Cl^- cotransporter. The aim of this project was to investigate the importance of this lesion in HN2-induced cytotoxicity.

$^{86}Rb^+$ uptake in human erythrocytes was inhibited by high concentrations of HN2 (2mM) and occurred in two phases. In the first hour both the Na^+/K^+ ATPase pump and the Na^+-K^+-Cl^- cotransporter were equally inhibited but after 2 hrs exposure to 2mM HN2, the Na^+-K^+-Cl^- cotransporter was significantly more inhibited than the Na^+/K^+ ATPase pump. In contrast, both potassium transport systems were equally inhibited in L1210 cells incubated for 10 minutes with 1mM HN2. The selective inhibition of the Na^+-K^+-Cl^- cotransporter, after a 3 hrs exposure to 10uM HN2, was not absolved by coincubation with 5ug/ml cycloheximide (CHX), an inhibitor of protein synthesis.

Incubation of L1210 cells with concentrations of diuretics which completely inhibited Na^+-K^+-Cl^- cotransport did not enhance the cytotoxicity of either HN2 or its monofunctional analogue 2-chloroethyldimethylamine (Me-HN1). The incubation of L1210 cells with a twice strength Rosewell Park Memorial Institute 1640 media did not enhance the toxicity of HN2. An L1210 cell line (L1210FR) was prepared which was able to grow in toxic concentrations of furosemide and exhibited a similiar sensitivity to HN2 as parental L1210 cells.

Treatment of L1210 cells with 10uM HN2 resulted in a decrease in cell volume which was concurrent with the inhibition of the Na^+-K^+-Cl^- cotransporter. This was not observed in L1210 cells treated with either 1 or 0.5uM HN2. Thus, possible differences in the cell death, in terms of necrosis and apoptosis, induced by the different concentrations of HN2 was investigated. The cell cycle of L1210 cells appeared to be blocked non-specifically by 10uM HN2 and in S and G_2/M by either 1 or 0.5uM HN2. There were no significant changes in the cytosolic calcium concentrations of L1210 cells for up to 48 hrs after exposure to the three concentrations of HN2. No protection against the toxic effects of HN2 was observed in L1210 cells incubated with 5ug/ml CHX for up to 6 hrs. Incubation for 12 or 18 hrs with a non-toxic concentration (5mM) of L-Azetidine-2- carboxylic acid (ACA) enhanced the toxicity of low concentrations (<0.5uM) of HN2.

KEYWORDS: nitrogen mustard; Na^+-K^+-Cl^- cotransport; apoptosis; necrosis; murine leukaemia.

TO MY PARENTS

WITH LOVE

"I know why there are so many people who love chopping wood. In this activity one immediately sees the results." Albert Einstein.

Acknowledgements.

I would like to express my sincere thanks to Dr. J.A. Hickman for his guidance, support and encouragement throughout this project and the production of this thesis.

I would like also to acknowledge the pratical assistance of my colleagues at Aston University especially to Caroline Dive, Jan Pageter, Eric Tang, Jackie Walling, Andrea Watson and Jackie Whatmore. I would like to thank the following at Birmingham University for their technical expertise; Derek Mills (transmission electron micrographs), Anne Milner (flow cytometry) and Lesley Tomkins (scanning electron micrography).

I would like to express my gratitude to all members of the CRC unit at Aston University for their advice, discussion and for making the years fly past.

I acknowledge the Medical Research Council for their generous financial support.

A special thanks to Jules, Linc, Sue and Carole.

LIST OF CONTENTS.

LIST OF FIGURES.

LIST OF TABLES.

ABBREVIATIONS.

ACA	L-Azetidine-2-carboxylic acid
ANF	Atrial natriuretic factor
ATP	Adenosinetriphosphate
APS	Ammonium persulphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosurea
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanine monophosphate
DBP	Dibutyl phalate
DDSA	Dodecenyl succinic anhyride
DG	Diacylglycerol
DNA	Deoxyribonucleic acid
DMP-30	tris-Dimethylaminoethyl phenol
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis-(ß-aminoethylether) N,N,N',N'-tetraacetic acid
FUra	5-Fluorouracil
g	Acceleration due to gravity
HEPES	N-2-hydroxyethyl piperazine N'-2-ethanesulfonic acid
HN2	Mechlorethamine
hsp	Heat shock proteins
IP_3	Inositoltrisphosphate

i.p.	Intraperitoneal
Me-HN1	ß-dimethylaminoethylchloride
PBS	Phosphate buffered saline
PI	Phospatidylinositol
PIP	Phospatidylinositolphosphate
PIP_2	Phospatidylinositolbisposphate
PMSF	Phenylmethylsulfonylfluride
PPO	2,5-diphenyloxazole
Quin-2	quin-2 acetoxmethyl ester
r.p.m.	Revolutions per minute
RNA	Ribonucleic acid
RNase	Ribonuclease A
RPMI	Rosewell Park Memorial Institute
SDS	sodium dodecyl sulphate
sp. act.	specific activity
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol-13-acetate

1. INTRODUCTION.

1.1 General introduction.

The first attempt to treat cancer by chemotherapy was
in 1865 when physicians administered arsenic to patients
with leukaemia (Pratt and Ruddon 1979). In the last 40
years there has been an intensive programme of research to
discover new and effective therapies for malignant diseases.
During this time an unprecedented number of drugs have been
introduced into the clinic but, despite some successes, for
many types of cancer there is at present no effective
treatment. The discovery of new anti-cancer drugs has often
been characterised by serendipity. Initially, nitrogen
mustard (HN2) was developed as a chemical warfare agent
prior to the onset of World War II. However, the
identification of the cytotoxic effects of this drug lead to
its introduction into the clinic as a treatment for
malignant diseases. HN2 is a prototypical alkylating agent,
a class of drugs used extensively in cancer chemotherapy.
New compounds have been synthesised from the basic structure
of HN2 which exhibit an increased therapeutic index.

The search for a drug specifically cytotoxic to
malignant cells is the elusive philosopher's stone of cancer
chemotherapists. HN2, like many other anticancer drugs, has
a wide range of toxic side effects which include leucopenia
and thrombocytopenia. This is due to its lack of
specificity in that all proliferating cells, not just
malignant, are sensitive to the action of HN2. In theory,

the identification of a metabolic difference between malignant and normal cell populations could lead to the design of a drug which is specifically cytotoxic to neoplastic cells. Thus, a greater knowledge of tumour cell biology and the mechanism of action of anti-cancer drugs is necessary for the discovery of new and more effective therapies. Although much is understood about the primary lesions of drugs such as HN2, little is known about how they bring about cell death.

Berger (1986) emphasised that cell death is one area of tumour biology which is poorly understood and a greater appreciation of it would be of advantage to cancer chemotherapists. Two modes of cell death have so far been identified - necrosis and apoptosis. Necrosis is a state where excessive cellular damage leads to a loss of ion homeostasis and cellular integrity. In contrast, apoptosis is a "programmed" cell death (i.e. an active response of the cell to damage) and was first defined by Kerr et al (1972). The different types of cell death will be described in detail later. A greater understanding of the mechanisms by which cells die would allow advantage to be taken in the design of new anti-cancer strategies to enhance the cytotoxicity of drugs and, perhaps, protect normal cell populations.

HN2 is an archetypical drug that has largely been supplanted by more effective analogs. However, it is still used in combination chemotherapy (Mustargen; Oncovin;

Procarbazine; Predinosone; MOPP) for the successful treatment of Hodgkin's disease (Pratt and Ruddon 1979). HN2 is of use in the research laboratory as a probe to investigate the mechanisms of cell death.

Many primary lesions have been proposed as the cytotoxic targets of HN2 with much research focussing on its reaction with DNA. In contrast, only a few workers have investigated the cellular processes that lead to cell death after the administration of HN2. One of these events is a change in cell volume and little is known of its possible role and importance in HN2-induced cell death. It is at the plasma mebrane that many of the processes which regulate cell volume are found. One such process is the $Na^+-K^+-Cl^-$ cotransporter which is thought to be intimately involved in the regulation of cell volume (Geck and Heinz 1986).

Wilcock et al (1988) reported that in the L1210 murine leukaemic cell line the $Na^+-K^+-Cl^-$ cotransporter was selectively inhibited 4 hours after exposure to 10uM HN2. This inactivation was observed after the cell had become committed to die but before cell death had occurred. The nature of the inhibition of $Na^+-K^+-Cl^-$ cotransport by HN2 and its role and importance in HN2-induced cell death is the subject of this thesis.

1.2.1 The chemistry of alkylation by nitrogen mustard.

HN2 is an extremely reactive molecule and many biological compounds act as substrates for alkylation. Alkylation occurs through the covalent binding of the alkyl groups of HN2 to nucleophilic centres, by a process of nucleophilic substitution. Such nucleophilic groups as amino, carboxyl, sulfhydryl, or imidazole moieties, which in biological systems are present in nucleic acids or proteins, are potentially open to attack by HN2. The first step of the reaction, prior to alkylation, is the leaving of a chloride ion and the formation of a cyclic aziridinium ion which is positively charged and highly unstable. This proceeds by a unimolecular, first order (SN_1) reaction. The positive charge of the aziridinium ring is open to nucleophilic attack. This results in the opening of the aziridinium ring and the formation of a covalent bond with the nucleophile. This is a bimolecular second order (SN_2) reaction. The second halogeno-alkyl chain of HN2 then undergoes a similiar series of reactions and a second nucleophilic substitution occurs. This second series of reactions could result in the alkylation of a nucleophilic moiety within the same molecule, i.e. between DNA bases on opposite strands of the same molecule. Alternatively, the second side chain of HN2 may react with water resulting in a monoalkylated molecule. This ability to cross-link molecules is characteristic of HN2 and bifunctional alkylating agents.

1.2.2 The reaction of nitrogen mustard with DNA.

Auerbach and Robinson (1946) noted that HN2 was mutagenic and proposed that the nucleus was a major target of alkylation. A number of investigators observed that bifunctional alkylating agents exhibited a greater cytotoxicity to tumour cells than the corresponding monofunctional analogues (Goldacre et al 1949). Bifunctional alkylating agents, such as HN2, are able to form cross-links between bases on the same (intra-strand) or different (inter-strand) chains of the DNA double helix. Geiduscheck (1961) demonstrated that the denaturation of DNA could be reversed if it was treated with HN2, unlike native DNA. Therefore, he proposed that inter-strand cross-links between the DNA chains were being formed. Kohn et al (1966) showed that bacterial DNA treated with HN2 was more resistant to denaturation by sodium hydroxide and formamide than untreated bacterial DNA. Even at low concentrations of HN2 (5×10^{-7}M), which would correspond to 1 in 50,000 bases being alkylated, the HN2 treated DNA was still able to return to its bihelical structure after denaturation. Thus the concept that the formation of interstrand DNA cross-links as the lesion responsible for cell death was developed, although the consequences of this damage to DNA were never defined.

Price (1969), using monofunctional analogues of HN2, studied the alkylation of both denatured and native DNA by

isolating the end-products, using chromatography and ion-exchange columns. When using native DNA the only identifiable end-product was a N-7 alkylated guanine whilst with denatured DNA both N-7 alkylated guanine and N-1 alkylated adenine were isolated. This was due to the helical structure of DNA which left exposed the N-7 atom of the guanine molecule whilst the N-1 atom of adenine molecule remained hidden. Brookes and Lawley (1961) compared the products of alyklated nucleic acids and those of alkylated guanosine or guanylic acid. They showed that the alkylation site was the N-7 of guanine and that these products were either 7-alkylguanines or di(guanin-7-yl) derivatives. Using the Watson-Crick model of the DNA structure, they proposed that HN2 could theoretically form inter-strand cross-links between adjacent guanine bases.

The synthesis of DNA, RNA and proteins may be inhibited by the formation of DNA cross-links by HN2. However, it was noted that alkylating agents caused a greater inhibition of DNA synthesis than that of either RNA or protein synthesis (Brewer 1961; Crathorn and Roberts 1966; Wheeler 1967; Warwick 1963). It was proposed that this inhibition, measured by a decrease in the incorporation of radio-labelled thymidine, was due to the inactivation of the DNA template rather than of the polymerase enzyme (Wheeler, 1962). The cytotoxic effects of HN2 have been attributed to the blocking of DNA synthesis (Goldenberg 1966; Crathorn and Roberts 1966). However, it has been reported that the

DNA template for RNA synthesis is more sensitive to alkylation than that for DNA synthesis (Chmielewicz et al 1967; Ruddon and Johnson 1968). Ruddon and Johnson (1968) also reported that DNA in its nucleoprotein complex in vivo was much less sensitive to the effects of HN2 than purified DNA in vitro. Thus, it was suggested that, as cellular DNA may not be the most sensitive target of alkylation, other targets may be important in the cytotoxicity of HN2.

Rates of radiolabelled precursor incorporation into DNA has often been used to measure the effect of alkylating agents on DNA synthesis, as mentioned above (Roberts et al, 1971; Carthorn and Roberts, 1966; Brewer et al, 1961). However, these results must be treated with some caution. Dean & Fox (1984) reported that [^{3}H]-thymidine uptake did not reflect the actual rate of DNA synthesis in HN2 treated cells. This may be due to an increase in the amount of intracellular thymidine triphosphate and a consequent reduction in the incorporation of radioactive thymidine into DNA (Alexander, 1969). Also Grunicke et al (1975) showed that the alkylating agent Trenimon (2,3,5-Triethylene-iminobenzeoquinone) reduced the uptake of [^{3}H]-thymidine by affecting its transport mechanism into the cell, yet it did not alter the rate of DNA synthesis.

HN2 is more cytotoxic to rapidly proliferating cells than to quiescent cells (van Putten and Lelieveld, 1971). Mauro & Madoc-Jones (1970) found that mammalian cells were more sensitive to HN2 when in either the M phase of the cell

cycle or at the transition from G_1 to S. Roberts et al (1971) demonstrated that when HeLa cells in either S or late G_1 were exposed to low doses of sulfur mustard, a greater cytotoxicity was observed than if the cells were in early G_1. It was proposed that this was due to the cells in early G_1 being able to repair the DNA damage sufficiently before entry into S. However, Levis (1965) noted that human amnion cells (AUP cell line), treated with HN2, were still able to undergo several rounds of DNA replication. A number of workers, using cytochemical techniques, have noted that cells treated with HN2 become blocked in G_2 (Caspersson 1963). It has been proposed that the major biological action of HN2 is in this phase and appears to be independent of DNA replication (Decosse and Gelfont 1970). These opposing views may be reconciled by the fact that different cell lines were employed and that the different concentrations of HN2 used may have induced various modes of cell death.

The transforming activity of bacterial DNA was maintained despite the DNA containing at least two cross-links. This indicated that the formation of cross-links did not effect the biological activity of DNA (Kohn and Green, 1966). Examination of cell lines exhibiting differential sensitivities to alkylating agents have shown no difference in the inhibition of DNA synthesis, DNA cross-links and their rates of removal or initial and residual strand breaks (Yin et al 1973; Hesselwood 1978).

However, other investigators have found differences as judged by these criteria, but they were of such a magnitude as to inadequately account for the differences in sensitivity (Yin et al 1973; Stranberg et al 1982; Micetich et al 1983: Zwelling et al 1981).

A relationship between HN2 cytotoxicity and the degree of cross-linking of DNA, assayed by alkaline elution, was, however, demonstrated in L1210 cells (Ewig and Kohn, 1977; Ross et al, 1978). Ewig and Kohn (1977) compared cell survival and cross-linking kinetics of both HN2 and melphalan in murine L1210 leukaemic cells. They showed that DNA HN2-induced cross-links were repaired within 4 hours. However, the cross-links caused by melphalan were present in the L1210 cells for a longer period and this corresponded to an increased cytotoxicity observed with melphalan. However, it could be argued that these lesions remained unrepaired in cells committed to cell death. Roberts et al (1971) proposed that the lethal lesion of HN2 was the formation of DNA cross-links which lead to the inactivation of the DNA template. This could prevent the separation of the strands of the DNA double helix which is necessary for semi-conservative replication. This would result in the inhibition of DNA synthesis, although this is disputed as mentioned above. It was suggested that the cells that survive treatment with HN2 are those that repair the DNA cross-links before DNA synthesis is attempted (Roberts et al, 1971).

A number of studies of the effect of HN2 on the cell cycle would seem to indicate that the cross-linking of DNA is not the only lethal lesion necessary for cell death. Analysis of cells treated with melphalan using flow cytometry (Brox et al 1980) demonstrated that cell division was blocked in the G_2 phase of the cell cycle. Cells exposed for a short time to melphalan were able to overcome the G_2 block and continue the cell cycle. However, cells that had been exposed for longer time periods became irreversibly blocked in the S phase of the cell cycle before cell death. Dean and Fox (1984) showed that cells treated with HN2 exhibited an initial S phase delay and then accumulated in G_2. This would seem at odds with the concept that the essential cell lesion caused by HN2 is the cross-linking of DNA as the cells are still able to achieve a premitotic complement of DNA.

In an elegant series of experiments the nucleii of amoebae treated with HN2 were transferred to untreated animals and vice versa (Ord, 1956; Ord and Danielli, 1956). Those amoebae with treated nucleii and untreated cytoplasms were more sensitive to the cytotoxic effects of HN2. However, the amoebae with treated cytoplasms and untreated nucleii were more sensitive to the growth inhibitory effects of HN2. The authors concluded that both the cytoplasm and the nucleus may be target sites of HN2 activity.

As outlined above, the cross-linking of DNA and the subsequent inhibition of DNA synthesis has been considered

the major lesion of HN2 induced cytotoxicity. However, a number of experiments have undermined this theory. The fundamental criticism is that, despite treatment with HN2, cells are able to continue DNA synthesis so that a complete premitotic complement of DNA is attained. Alexander (1969) proposed that it was the lack or inaccuracy of DNA repair which was the real cytotoxic lesion of HN2. However, HN2 is an extremely reactive compound and inhibits many other cellular fuctions which may be important in the cytotoxicity of HN2. Thus, the target sites of HN2 induced cell death are still an area of some controversy.

1.3.1 Ion transmembrane transport systems.

The effects of alkylating agents on specific membrane processes will be reviewed in a later section (introduction 1.4.). Here, an outline of various ion transport systems will be described. The plasma membrane is a semi-permeable barrier. This allows the movement of water, by osmosis, and of sodium and potassium ions down their concentration gradients. However, the plasma membrane is impermeable to amino acids and glucose. The major role of the plasma membrane is to regulate the entry and exit of substances into and out of the cell so as to maintain internal homeostasis. An important facet of this homeostasis is the control of internal potassium concentration of the cell, which is maintained at between 120 and 140mM. The external concentration of potassium is only 5-10mM. Therefore,

potassium will move down its concentration gradient and out of the cell. In contrast, the external sodium concentration is higher than the internal concentration (140mM and 20mM respectively) resulting in a net movement of sodium down its concentration gradient into the cell. Thus, sodium must be pumped out of the cell and potassium back in.

Elevations in internal potassium levels are one of the earliest observed changes in mitogenesis and may have a possible role in the initiation and regulation of cell division (Leffert 1980). Necrotic cell death is characterised by the loss of ionic homeostasis. Ledbetter and Lubin (1974) reported that pertubation of the internal potassium homeostasis resulted in a loss of cellular metabolism. The ion transport systems involved in this homeostatic function, as well as cell proliferation and death, will be outlined.

A number of ionic transport systems exist in order to maintain these ionic gradients, of which the most important is the Na^+/K^+ATPase pump. The Na^+/K^+ATPase pump couples the free energy contained within an ATP molecule to the exchange of three sodium ions from within the cell for two potassium ions outside (Skou 1965). The activity of the Na^+/K^+ATPase pump is pharmacologically defined by the inhibition of potassium flux by the cardiac glycoside ouabain. Ouabain is able to inhibit the activity of the Na^+/K^+ATPase pump by competitive inhibition for the potassium binding site of the protein (Rossier et al 1987). The enzyme is thought to be

active as an alpha beta heterodimer. It has been proposed that the catalytic functions of the $Na^+/K^+ATPase$ pump are associated with the alpha-subunit (Rossier et al 1987). However, the activity of the $Na^+/K^+ATPase$ pump does not account for the total carrier-mediated movement of sodium and potassium across the plasma membrane.

The $Na^+-K^+-Cl^-$ cotransporter is also important in the maintenance of the internal ionic homeostasis and in the regulation of cellular volume. The $Na^+-K^+-Cl^-$ cotransporter will be reviewed further in the following sections 1.3.2 and 1.3.1.

The Na^+/H^+ antiport is another ion transmembrane transport system that is thought to be involved in maintaining the internal homeostasis of the cell. A proton is extruded and replaced by a sodium ion resulting in the alkalinisation of the cytoplasm without affecting the membrane potential. The Na^+/H^+ antiport is pharmacologically defined by its sensitivity to amiloride, (3,5,diamino-6-chloro-N-(diaminomethylene)-pyrazine-carboxamide), which competes for the binding site of sodium ions. It has been demonstrated that 1mM amiloride is able to inhibit Na^+/H^+ antiport activity and block DNA synthesis. However, at this concentration other cellular functions, such as the $Na^+/K^+ATPase$ pump, are also inhibited (Grinstein et al 1989). Growth factors were shown to stimulate the Na^+/H^+ antiport and raise the intracellular pH (Moolenar et al 1983). However, this work was performed in HCO_3^--free

media. Ganz et al (1989) demonstrated that arginine vasopressin (AVP), in the presence of HCO_{3-}, stimulated the Na^+-independent Cl^-/HCO_3^- exchanger resulting in a decrease in internal pH.

In addition to these mechanisms of sodium homeostasis, yet another, the Na^+/Ca^{2+} exchange, also plays a role. It has been identified in all cell types except in human erythrocytes (Schatzmann and Vincenzi 1969). The Na^+/Ca^{2+} exchange has a stoichiometry of $3Na^+$: $1Ca^{2+}$ and activity is dependent upon extracellular sodium (Blaustein and Nelson 1982).

A Ca^{2+}-sensitive channel for potassium efflux is an additional ion transport system, the activity of which is able to regulate the internal potassium homeostasis of the cell. It was first demonstrated by Gardos (1958). It has a role in cell volume and potassium homeostasis (Hoffman 1985; Grinstein et al 1984). Quinine is most frequently used to define this channel (Lew and Ferreria 1978).

This chapter has given a brief overview of some of the systems, found in the plasma membrane, that are involved in ion transport and volume regulation. However, the emphasis of this thesis is the activity of the Na^+-K^+-Cl^- cotransporter and its selective inhibition by HN2. Therfore, the next two sections will describe the Na^+-K^+-Cl^- cotransporter and the regulation of its activity in detail.

1.3.2 The Na^+-K^+-Cl- cotransporter.

The Na^+-K^+-Cl^- cotransporter is involved in the transport of sodium, potassium and chlorine ions across the plasma membrane. The presence of the Na^+-K^+-Cl^- cotransporter was not clearly defined in a mammalian system until 1980, when it was elucidated in murine Ehrlich ascites cells (Geck et al 1980). Since then it has been defined in a number of mammalian tissues such as the human colon adenocarcinoma HT-29 cell line (Kim et al 1988), the murine leukaemic L1210 cell line (Wilcock and Hickman 1988), human red blood cells (Duhm and Goebel 1984) and rat vascular smooth muscle cell (Owen 1984) as well as many other tissues (reviewed by Chipperfield 1986; Geck and Heinz 1986).

The Na^+-K^+-Cl^- cotransporter is pharmacologically defined by the inhibitory action of the 5' sulfamoyl-benzoic acid derivatives, compounds also known as loop diuretics because of their action on the ascending loop of Henle. These include, in an order of potency, furosemide, piretanide, bumetanide and benzmetanide (O'Grady et al 1987). The binding of [^{3}H]bumetanide requires the presence of both sodium and potassium ions in the external media (Forbush and Palfrey 1983). Increments in the external concentration of chloride reduced the level of inhibition of bumetanide (Haas and McManus 1983), indicating that bumetanide inhibited Na^+-K^+-Cl^- cotransport at a chloride binding site.

The Na^+-K^+-Cl^- cotransporter is also defined by its mode of operation. The stoichiometry of the Na^+-K^+-Cl^- cotransporter was taken to be 1:1:2 Na^+/K^+/Cl^- respectively, so that electroneutrality is maintained. It is bi-directional, having both an influx and efflux component in Ehrlich ascites (Levinson 1987) and 3T3 cells (Sussmann et al 1985). However, this was not discerned in either the murine leukaemic L1210 cell line (Wilcock and Hickman 1988) or human red blood cells (Kracke et al 1988), where in both cases the direction of the flux was inward. None of the three ionic species transported by the Na^+-K^+-Cl^- cotransporter can be fully replaced by related species. Potassium can only be fully replaced by rubidium (Geck and Heinz 1986). Sodium can only be partially replaced by lithium (i.e. there is a loss in cotransport activity) but not by choline or tetramethylammonium (Owen and Prastein 1985; Wilcock and Hickman 1988; Tas et al 1987). Chloride can only be partially replaced by bromide, not by gluconate, iodide or nitrate (Owen 1984; Tas et al 1987; Owen and Prastein 1985). However, in the mouse leukaemic L1210 cell line, bromide stimulated the Na^+-K^+-Cl^- cotransport in the absence of external chloride so that influx was greater than in the normal Kreb's-Ringer solution (Wilcock and Hickman 1988).

Photolabelling with bumetanide of porcine kidney outer medulla identified a protein of 34kDa molecular weight which was associated with cytoskeletal elements of the membrane

(Jorgensen et al 1984). Strangely, though bumetanide binding requires the presence of chloride ions, these studies were carried out in chloride-free media. A protein of 150kDa molecular weight, in the cortex of dog kidney, has also been identified by photolabeling studies. Binding of radiolabeled diuretics to the 150kDa protein required the presence of sodium, potassium and chloride ions, which is characteristic of the Na^+-K^+-Cl^- cotransport protein (Haas and Forbush 1987). In Ehrlich ascites tumour cells two proteins of ~76kDa and 38-39kDa have been isolated by using a bumetanide-sepharose affinity column (Feit et al 1988). The difference in the molecular weight of the Na^+-K^+-Cl^- cotransport proteins isolated from the two studies could be due to either the different systems employed (dog kidney and Ehrlich ascitestumour cells) or to different methodology.

1.3.3 The regulation of the $Na^+K^+Cl^-$ cotransporter.

Changes in cell volume (Baserga 1984) and ionic flux (Leffert 1980) have been observed during mitogenesis. In addition Wyllie (1981) has proposed that one of the criteria to distinguish necrotic from apoptotic cell death is alterations in cell volume. It is possible that the Na^+-K^+-Cl^- cotransport is important in volume regulation (Geck et al 1980) and ionic flux. In this section, the regulation of the Na^+-K^+-Cl^- cotransporter is reviewed in the context of both the control of mitogenesis and cell

death, and its inhibition by HN2 will be reviewed in section 1.4.2.

The role of the $Na^+-K^+-Cl^-$ cotransporter in volume regulation of mammalian cells, especially Ehrlich ascites, has been studied extensively. Treatment of cells with concentrations of diuretics which completely inhibited the $Na^+-K^+-Cl^-$ cotransporter resulted in no change in the volume of C6 glioma cells (Chassande et al 1988) but a decrease was observed in the volume of murine L1210 cells (Wilcock and Hickman 1988) and Ehrlich ascites (Geck et al 1980). An increase in osmolarity, by addition of mannitol, resulted in rapid cell shrinkage but no change in the internal ion concentration of Ehrlich ascites cells: under these conditions there was a twenty-fold increase in $Na^+-K^+-Cl^-$ cotransport activity (Geck et al 1981). This increased activity is known as volume regulatory increase (VRI). This was followed by a rise in internal ion concentration, concurrent with an increase in cell volume in a medium with a potassium concentration of 18mM. Treatment with 2mM furosemide prevented the rise in both internal ion concentration and cell volume (Geck and Pfeiffer 1985). Cells, preloaded with increasing concentrations of the nonmetabolizable amino acid alpha-aminoisobutyrate, under isotonic conditions exhibited a decreased $Na^+-K^+-Cl^-$ cotransport activity. In contrast, this parameter was unaffected in cells preloaded under hypertonic conditions as, it was proposed, the cells had not yet reached their

"reference volume". Thus, volume regulation involves a feedback control system which becomes activated once cell volume drops below a set value or "reference volume" (Geck and Pfeiffer 1985). However, the nature of the mechanism(s) by which cells sense their "set" volume is unknown.

The Na^+-K^+-Cl^- cotransporter, in contrast to the Na^+/K^+ATPase pump, is not associated with an increase in ATP hydrolysis (Geck et al 1980). However, ATP is necessary for the continued functioning of the Na^+-K^+-Cl^- cotransporter. Metabolic depletion blocked the cotransporter (Saier and Boyden 1984; Geck et al 1980) and only ATP could restore its activity (Palfrey and Rao 1983). There is speculation that ATP is needed to phosphorylate and thereby activate the protein (Saier and Boyden 1984; Geck and Heinz 1986).

Changes in cAMP levels in the cell are observed in cell death (Cho Chung 1980) and in the mitogenesis of some differentiated mammalian cells (Dumont et al 1989). In avian erythrocytes, stimulation of cAMP levels with catecholamines, 8-bromo-cAMP and adenosine analogues led to an increase in Na^+-K^+-Cl^- cotransport activity (Kregnow 1981; Palfrey and Greengard 1981) as did hypertonicity, NaF and deoxygenation, which were cAMP independent (Palfrey and Greengard 1981). This indicated that cAMP is not the only regulatory mechanism of the Na^+-K^+-Cl^- cotransporter. In mammalian tumour cells cAMP does not appear to be involved

in regulation. Treatment of Ehrlich ascites cells with adrenaline or isoprenaline did not alter potassium cotransport or cell volume (Geck and Pfeiffer 1985). However, isoproterenol inhibited $Na^+K^+Cl^-$ cotransport in endothelial cells (Brock et al 1986) and vascular smooth muscle cells (Smith and Smith 1987). Diuretic-sensitive potassium influx in mouse leukaemic L1210 cells was unaffected by incubation with 100uM dibutyryl cAMP (Wilcock and Hickman 1988). cAMP inhibited the $Na^+-K^+-Cl^-$ cotransporter in human fibroblasts (Owen and Prastein 1985) and rat vascular smooth muscle cells (Owen 1984; O'Donnel and Owen 1986a) and human red blood cells (Garay 1982). Data on the regulation of the $Na^+-K^+-Cl^-$ cotransport protein is often contradictory and Palfrey and Rao (1983) proposed that this heterogeneity may be due to an exhibition of tissue specificity.

In rat vascular smooth muscle cells, atrial natriuretic factor (ANF) stimulated both cGMP levels and the activity of the $Na^+-K^+-Cl^-$ cotransport in a concomittant manner (O'Donnel and Owen 1986a). The role of cGMP in the stimulation of the $Na^+-K^+-Cl^-$ cotransport protein was further confirmed by O'Donnel and Owen (1986b). $Na^+-K^+-Cl^-$ cotransport activity was depressed by incubation with an inhibitor of cGMP formation, whilst with 8-bromo-cGMP it was increased (O'Donnel and Owen 1986b). ANF did not stimulate either the Na^+/H^+ antiport or the Na^+/K^+ATPase pump. The ranking of various ANFs for their biological activity

correlated well with their ranked order for stimulation of the Na^+-K^+-Cl cotransport (O'Donnel et al 1987).

In mitogenesis there are changes in ionic transport and cell volume. The role of the $Na^+-K^+-Cl^-$ cotransporter in this event has been investigated by a number of workers. Stimulation of NIH 3T3 mouse fibroblasts with serum caused a five fold increase in furosemide-sensitive rubidium influx within two minutes. This was thought to be responsible for the increased intracellular potassium concentration in stimulated cells (Panet 1985). Treatment of cells in vitro with a number of hormones resulted in the stimulation of $Na^+-K^+-Cl^-$ cotransport. Foetal calf serum, insulin and alpha-thrombin stimulated furosemide-sensitive potassium uptake strongly whilst epidermal growth factor (EGF), 12-0-tetradecanoyl phorbol-13-acetate (TPA) and arginine-vasopressin (AVP) were found to be less effective (Amsler et al 1985; Owen 1984; Paris and Pouyssegur 1986). Bumetanide did not interfere with the hormonal stimulation of DNA synthesis. However, the initiation of DNA synthesis was not associated with an elevation of intracellular potassium concentrations. Therefore, although $Na^+-K^+-Cl^-$ cotransport activity was modulated by mitogenic hormones, it was concluded by these authors that it did not play a major role in the regulation of cell division (Paris and Pouyssegur 1986; Amsler et al 1985), although Panet (1985) disputes this. The co-incubation of EGF and TPA resulted in the additive stimulation of $Na^+-K^+-Cl^-$

cotransport activity. As EGF and TPA both act via different secondary messengers, it was proposed that $Na^+-K^+-Cl^-$ cotransport can be stimulated by a variety of phosphorylation reactions (Paris and Pouyssegur 1986).

The phophoinositide pathway is a bifurcating secondary messenger system which is thought to be involved in the control of cell division (Berridge and Irvine 1989). The stimulation of $Na^+-K^+-Cl^-$ cotransport by the phosphoinositide pathway has been investigated by a number of workers in order to ascertain the relationship between these two processes and cell proliferation. Phosphatidylinositol (PI), resident in the plasma membrane, can be phosphorylated to phosphatidylinositolphosphate (PIP) and then to phosphatidylinositolbisphosphate (PIP_2; reviewed by Berridge and Irvine 1989). Stimulation of some receptors by agonists activates phospholipase C through the intermediate action of a GTP-binding protein (Birnbaumer et al 1987). Phospholipase C catalyses the breakdown of PIP_2 to diacylglycerol (DG) and inositoltrisphosphate (IP_3). The protein kinase C enzyme is activated by DG and IP_3 brings about a rise in internal calcium levels.

Inositol phosphate formation can be induced by alpha-thrombin (Paris and Pouyssegur 1986), NaF with $AlCl_3$ and by vandate in hamster fibroblasts (Paris and Pouyssegur 1987). The stimulation of inositol phosphate can be inhibited by preincubation of the cells with pertussis toxin. This indicates that a GTP-binding protein couples

with the alpha-thrombin receptor and that $AlCl_3$ with NaF and vandate directly act on the GTP-binding protein. Alpha-thrombin, or $AlCl_3$ with NaF, stimulated bumetanide-sensitive [86]rubidium[+] uptake in hamster fibroblasts (Paris et al 1987); this indicates that there may be an activation of $Na^+-K^+-Cl^-$ cotransport via the stimulation of the phosphoinositide pathway.

TPA can stimulate the protein kinase C by its ability to mimic diacylglycerol (Nishizuka 1986). Though Owen (1984) claimed that TPA can stimulate the $Na^+-K^+-Cl^-$ cotransport in vascular smooth muscle cells, she was unable to reproduce this phenomenon, and in a later study observed an inhibition (Owen 1985). This was in agreement with a number of investigators who noted an inhibition of $Na^+-K^+-Cl^-$ cotransport by TPA (Sussman et al 1985; O'Brien and Krzeminski 1983). Treatment of 3T3 cells with a mitogenic concentration of TPA caused not only an inhibition of furosemide-sensitive potassium uptake but also an increase in intracellular potassium concentration and a decrease in cell volume. A 3T3 cell line with a reduced $Na^+-K^+-Cl^-$ cotransport activity, isolated by its ability to grow in a low potassium medium, expressed a smaller decrease in cell volume in response to TPA (Sussmann and O'Brien 1985). A study comparing parental and mutant cell lines demonstrated a lower mitogenic response to TPA than in the latter (O'Brien and Prettyman 1987). Incubation of 3T3 cells with the cell permeable diacylglycerol

sn-1,2-dioctoanoylglycerol resulted in the inhibition of Na^+-K^+Cl^- cotransport via a PKC-dependent mechanism (O'Brien et al 1988). This would suggest that the Na^+-K^+-Cl^- cotransport is regulated in some manner by the phosphoinositide signalling pathway through the action of PKC. Thus, the Na^+-K^+-Cl^- cotransport activity may be modulated in mitogenesis by growth factors which act via this pathway.

Increases in internal calcium concentration are observed in both mitogenesis and cell death. The rise in calcium is thought to be pivotal in the control of both these processes (Trump and Berezesky 1987). Therefore, the increased activity of the Na^+-K^+-Cl^- cotransporter observed in mitogenesis (Panet 1985) may be a consequence of changes in the internal calcium concentration of the cell. However, the Na^+-K^+-Cl^- cotransporter was unaffected by an increases in the internal calcium concentration of endothelial cells (Frelin et al 1986; Owen 1984). A reduction in the cytosolic concentration of calcium in Ehrlich ascites tumour cells, by the use of the calcium chellator EGTA, did not alter Na^+-K^+-Cl^- cotransport activity (Geck and Pfeiffer 1985). The stimulation of bumetanide-sensitive potassium flux by bradykinin in endothelial cells was inhibited by the addition of EGTA or $LaCl_3$ which blocked calcium rises (Brock et al 1986). In vascular smooth muscle cells, the stimulation of Na^+-K^+-Cl^- cotransport by angiotensin II was absolved by blocking calcium influx (Owen and Ridge 1989).

$Na^+-K^+-Cl^-$ cotransport activity was stimulated by the addition of calcium ionophore in endothelial cells (Brock et al 1986) and vascular smooth muscle cells (Smith and Smith 1987). $Na^+-K^+-Cl^-$ cotransport activity can also be stimulated by an increase in the internal pH of hamster fibroblasts (Paris and Pouyssegur 1986) although the cells were incubated in a HCO_3^--free media. Thus, changes in $Na^+-K^+-Cl^-$ cotransport activity during mitogenesis or cell death may be due to the internal pH changes characteristic of these processes.

1.4.1. The reaction of nitrogen mustard with the plasma membrane.

In contrast to the classical hypothesis that DNA is the main target of nitrogen mustard, an alternative school of thought has advocated that the plasma membrane may play an essential role in cell death. As early as 1947 Peters proposed that sulphur mustard caused cell death by altering membrane permeability.

The human erythroycte is a good model to investigate the effcts of alkylating agents on the plasma membrane as it has a well defined membrane and is enucleated. Metabolically induced shape changes of human erythrocytes were inhibited by incubation with 2mM tris(2-chloroethyl)amine (Wildenhauer et al 1980). An investigation of red blood cell membrane proteins, by sodium dodecyl sulphate polacrylamide gel electrophoresis, showed

that the major cytoskeletal protein spectrin became cross-linked, either with itself or other proteins, at concentrations of tris (2-chloroethyl)amine that maintained cell shape. A more detailed study, using radio-labelled tris (2-chloroethyl)amine, indicated that glycophorin (PAS 1) and the protein in band 7 were also labelled with the compound (Wildenhauer and Weger 1979).

Pretreatment of nucleated chicken erthrocytes, their ghosts, rabbit bone marrow and lung cells with either nitrogen or sulphur mustard protected those cells from swelling when placed in a nucleoprotein solvent (Levy 1965). This phenomenon was observed at concentrations of either type of mustard (10^{-6}M) which did not significantly alter nucleoproteins. Levy (1965) proposed that as cell swelling was dependent on membrane fluidity, the loss of the ability of cells to swell indicated that nitrogen or sulphur mustard treatments had significantly altered this property.

The maintenance of an optimum membrane fluidity is essential for the continued functioning of the cell (Pang 1979) and its biological activity (Melchion and Stein 1976). The studies mentioned in the previous paragraph indicate that the cross-linking of membrane molecules by alkylating agents leads to a loss in membrane fluidity. Using a variety of techniques, a number of anti-tumour agents have been shown to alter the membrane permeability of the cell (reviewed by Tritton and Hickman 1984).

Grunicke investigated the effects of nitrogen mustard

on membrane fluidity (Grunicke et al 1982 and 1985a). In the earlier study the fluorescent polarisation of diphenylhexatriene in Ehrlich ascites cells was monitored. Due to its lipid solubility, diphenylhexatriene can partition into the membrane and can then be excited with polarised light. If the probe is able to rotate before emission then a lower level of polarisation will be observed. The amount of rotation is determined by the fluidity of the local enviroment so that a high degree of polarisation is associated with low membrane fluidity and vice versa. Grunicke et al (1982) noted a decrease in membrane fluidity after treatment with nitrogen mustard which was synchronous with the loss of cell proliferation and the activity of the Na^+/K^+ATPase pump. An Ehrlich ascites cell line that was resistant to nitrogen mustard did not show any change in membrane fluidity, as determined by this method, when exposed to nitrogen mustard.

Grunicke et al (1985a) further investigated the effect of HN2 on membrane fluidity as the interpretation of data from diphenylhexatriene flourescence polarisation had been criticised (Bouchy et al 1981). The fluoresecent probe, diiodofluorescein iodoacetamide, can become associated with integral membrane proteins. As with the diphenylhexatriene, diiodofluorescein is excited with polarised light and if the probe is able to rotate before emission, then a lower level of polarisation will be observed. Changes in membrane fluidity would alter the rotational diffusion of the

membrane proteins and this can be measured by analysis of the flourescence emitted by the probe. Ehrlich ascites tumour cells, treated with 10^{-4}M concentrations of nitrogen mustard for 30 minutes, exhibited increased membrane fluidity.

The lateral diffusion of lipids in the membrane of Ehrlich ascites cells after nitrogen mustard treatment was investigated (Grunicke et al 1982). The method used was based on the quenching reaction between the amphiphilic cation cetylpyridinium and the fluorescent probe pyrene. The quenching molecule cetylpyridinium is able to move in the plane of the bilayer, the speed of which is dependent on membrane fluidity. Vesicles of Ehrlich ascites cells, treated for one hour with 10^{-4}M nitrogen mustard, showed no difference in membrane fluidity compared to controls.

The binding of lectins, such as concanavalin A, can also be used to investigate alterations in the properties of the plasma membrane. An increase in the binding of [3H] concanavalin A to human fibroblasts was observed after exposure to ionising radiation (Koetles et al 1979). This increase was observed between 5 minutes and three hours after treatment, indicating a rapid change in membrane fluidity. However, Ankel et al (1986) showed no change in this parameter for the L5178Y mouse leukaemic cell line when insulted with 10^{-3}M concentrations of nitrogen mustard.

Grunicke et al (1985a) proposed that nitrogen mustard did not cause any change membrane fluidity, as determined by

the quenching of the probe pyrene. The observed reduced rotational diffusion of membrane constituents labelled with diiodofluorescein iodoacetamide was as a result of protein-protein cross-links produced by the bifunctional alkylating agent. Similiarly, the decrease in fluidity of the membrane observed with diphenylhexatriene was explained as the association of the probe with immobilized membrane proteins. Ankel et al (1986), using ESR spin-label probes, confirmed that the membrane fluidity of Ehrlich ascites cells, treated with the alkylating agent trenimon, was unaffected.

1.4.2 The effect of nitrogen mustard on membrane transport processes.

Alkylating agents have been shown to interfere with a variety of transport processes essential to the continued functioning of the cell. Grunicke et al (1975) demonstrated that Ehrlich ascites cells treated in vivo with 0.6uM trenimon were still able to incorporate thymidine into DNA, but the rate of incorporation of radiolabelled thymidine was found to be reduced due to a decreased transport of the nucleoside into the cell, as discussed in section 1.2.2.

A variety of mustards have been shown to cause a decrease in the incorporation of radiolabelled amino acids into proteins (Wheeler 1962). However, whether this phenomenon was as a consequence of reduced protein synthesis or interference in the transport of amino acids, was not

discussed. Ihlenfeldt et al (1981) showed that in vivo the uptake of the non-metabolizable amino acids 2-amino-isobutyric acid and cycloleucine into Ehrlich ascites cells was depressed at concentrations of 10^{-6}moles/kg trenimon. This inhibition occurred 4 hours after drug administration. Baxter et al (1982) reported that in PC6A plasmacytoma cells the uptake of alpha-aminoisobuytric acid, but not cycloleucine, was inhibited by a 4 hour incubation with 10uM HN2. However, Ankel et al (1986) reported a lack of correlation between concentrations of alkylating agents needed to inhibit cell growth and alter uptake of amino acids. In the L5178Y murine leukaemic cell line a 1 hour incubation with 10^{-6}M Trenimon, which completely inhibited cell division, did not alter the uptake of aminoisobutyric acid. In contrast, concentrations of less than 10^{-4}M HN2 and HN3 (tris(2-chloroethyl)-amine) stimulated uptake of the amino acid. Therefore, Ankel et al (1986) suggested that the critical target for alkylating agents in cell membranes had not been identified.

It is essential for cell integrity that potassium concentrations are maintained within set limits otherwise cellular functions such as macromolecular synthesis are terminated (Ledbetter and Lubin 1977). The loss of ability to maintain potassium homeostasis may result in cell swelling and rapid cell death, as observed in necrosis. Alternatively, the reduction of potassium uptake may be

responsible for the decrease in cell volume which is characteristic of apoptotic cell death (reviewed in section 1.6.2).

In experimental systems ^{86}rubidium$^+$ is used to investigate potassium fluxes, as it is a complete congener (Meares et al, 1978) and has a longer half-life than radioisotopes of potassium ($t_{1/2}$ ^{42}K is 12 hours).

Ehrlich ascites cells were treated in vivo with 10^{-6}moles/kg trenimon. After 4 hours ^{86}rubidium$^+$ uptake by the Na$^+$/K$^+$ ATPase pump was inhibited (Grunicke et al 1979). The loss of Na$^+$/K$^+$ ATPase pump activity in Ehrlich ascites cells was mirrored by the loss of proliferative capacity after treatment with trenimon, nitrogen mustard and phosphoramide mustard but chlorambucil did not alter ^{86}rubidium$^+$ uptake (Grunicke et al 1983). Similarly, ^{86}rubidium$^+$ uptake in PC6A plasmacytoma cells was inhibited by 40% 4 hours after treatment with 10^{-5}M HN2 (Baxter et al 1982). The inhibition of ^{86}rubidium$^+$ uptake was attributed to a reduced activity of the Na$^+$/K$^+$ ATPase pump. In contrast, the monofunctional analogue of nitrogen mustard did not inhibit ^{86}rubidium$^+$ uptake. The bifunctionality of HN2, thus appeared to be important in the inactivation of the Na$^+$/K$^+$ ATPase pump, possibly, it was suggested, due to proximal sulfhydryl groups of the protein becoming cross-linked by HN2 (Baxter et al, 1982).

The Na$^+$-K$^+$-Cl$^-$ cotransporter is also important in the transmembrane transport of potassium ions and the

maintenance of both ionic and volume homeostasis of the cell (section 1.3.1). Further investigations of the inhibitory effect of HN2 on ^{86}rubidium$^+$ uptake by the Na$^+$-K$^+$-Cl$^-$-cotransporter in the Ehrlich ascites tumour cell line was investigated by Doppler et al (1985). It was demonstrated that the inhibition of ^{86}rubidium$^+$ uptake by the Na$^+$-K$^+$-Cl$^-$ cotransporter after 4 hours exposure to HN2 was coincident with the loss in proliferative capability of the cells. At 2uM concentrations of HN2, a 50% inhibition of the Na$^+$-K$^+$-Cl$^-$ cotransport system was noted but there was no loss of activity of the Na$^+$/K$^+$ATPase pump. Loss of Na$^+$-K$^+$-Cl$^-$ cotransport activity and the degree of DNA cross-linking was investigated over 32 hours after 2uM HN2 treatment (Grunicke et al 1985a). During the first 12 hours the recovery in activity of the Na$^+$-K$^+$-Cl$^-$ cotransporter was comparable to the removal of DNA cross-links. However, after 12 hours there was a gradual loss in ^{86}rubidium$^+$ uptake which Grunicke et al (1985) suggested was due to an increase in the number of dead cells.

2uM HN2 caused a 75% inhibition of cell multiplication and a 50% inhibition of the Na$^+$-K$^+$-Cl$^-$ cotransporter (Grunicke et al 1985a). This suggested the possibility that there may be a relationship between the anti-tumour activity of HN2 and the impairment of the activity of the Na$^+$-K$^+$-Cl$^-$ cotransporter. However, concentrations of furosemide which completely inhibited the Na$^+$-K$^+$-Cl$^-$ cotransporter did not cause a significant level of cell death. Grunicke therefore

concluded that the cytotoxic action of HN2 could not be explained by the inhibition of the $Na^+-K^+-Cl^-$ cotransporter alone. However, it was proposed that this phenomenon could enhance the effect of other lesions. Thus, the cytotoxic action of HN2 could be muti-modal. However, 200uM furosemide did not increase the toxicity of 2uM HN2 though this would appear pointless as the $Na^+-K^+-Cl^-$ cotransporter was already severly inhibited. Furthermore, coincubation of cells withfurosemide was not investigated at lower concentrations of HN2 (Grunicke et al 1985a).

The selective inhibition of the $Na^+-K^+-Cl^-$ cotransporter was also observed in L1210 murine leukaemic cells treated in vitro with 10uM HN2 (Wilcock et al 1988). This phenomenon occurred gradually, reaching saturation after 3 hours incubation in a Krebs-Ringer buffer. The inhibition of $^{86}rubidium^+$ uptake was not observed with either adriamycin (Chahwala and Hickman 1985), a monofuctional analogue of HN2 or the more slowly reacting chlorambucil (Wilcock, 1987). Incubation of L1210 cells with HN2 for as little as 10 minutes was sufficient for the full cytotoxic effects of the drug to be exerted. However, after 3 hours incubation with 10uM HN2 there was no change in the membrane potential of cells, ATP levels, intracellular pH or in the ability of the cells to exclude trypan blue. Thus, the loss in the activity of the $Na^+-K^+-Cl^-$ cotransporter appeared to follow lethal damage but preceded cell death. However, a similiar inhibition

was not observed after 4 hours exposure to a lower but also toxic doses (1uM) of HN2.

The inhibition of the Na^+-K^+Cl^- cotransporter was also concomittant with a 30% decrease in cell volume, as measured by tritiated water, so that the internal potassium concentration was maintained at control levels (Wilcock et al 1988). Doppler et al (1985) noted that no change in volume was observed in Ehrlich cells after 4 hour incubation with 2uM HN2. This difference may be explained by the observation that under isoosmotic conditions Ehrlich ascites cell volume was unaffected by loop diuretics (Doppler et al 1985: Hendil and Hoffman 1974), and so may be similarly insensitive to HN2. The effects of HN2 on the volume of L1210 cells are compatible with the inhibition of the cotransporter.

Na^+-K^+Cl^- cotransport, in an Ehrlich ascites cell line resistant to HN2, was unaffected by HN2 (Doppler et al 1985). In an L1210 cell line resistant to HN2, no diuretic-sensitive potassium transport could be defined. However, further work to investigate whether this phenomenon was either due to a mutation or a complete inactivation of the $Na^+K^+Cl^-$ cotransport protein was not undertaken (Wilcock 1987). Therfore, the importance of the inhibition of the Na^+-K^+-Cl^- cotransport for HN2 toxicity is an area that requires further investigation. This will be discussed further in the aims and objectives section.

1.5 Target sites of nitrogen mustard and cell death.

Many lesions, other than to DNA, have been noted after HN2 treatment and these have been extensively reviewed (Wheeler, 1962; Warwick, 1963). In the above sections the possible target sites for HN2 which may produce a cytotoxic lesion have been discussed. The cross-linking of DNA by HN2 is generally considered to be the only lesion which will induce cell death (section 1.2.2). However, other investigators have proposed that the membrane, and especially the transport proteins therein, could also be considered as a target site for HN2. In an extensive review of membrane lesions induced by anti-cancer drugs, Tritton and Hickman (1984) proposed that their cytotoxic action could be exerted in a multimodal fashion. For example, the result of exposing cells to hypotonic shock is a degradation of their DNA (Williams and Little 1974). Therefore, the disruption of internal ionic homeostasis and levels of DNA damage may be closely interrelated. HN2 could, hypothetically, also act in a multimodal way as levels of DNA damage could be increased by other lesions, including those ocurring at the plasma membrane.

In contrast to these ideas, Sakomoto and Elkind (1969) studied the combined effects of irradiation and HN2 treatment on the survival of Chinese hamster cells. They discovered that HN2 treatment did not affect the survival of cells previously exposed to irradiation or vice versa. This, they argued, indicated that the cytotoxic lesions

occurred either independently at the same or at different sites. The authors also noted that there was no sublethal injury to cells treated with HN2 and that a single lesion was sufficient to cause cell death.

Some of the possible primary lesions of HN2 has been reviewed in previous sections. There has been much discussion and research into the target sites of HN2 action. However, the question of how and why cells die has often been left unanswered. The different types and processes of cell death will now be reviewed in section 1.6.

1.6.1 Cell death.

The inhibition of $Na^+-K^+-Cl^-$ cotransport, by a 4 hour treatment with 10uM HN2, was observed to occur after lethal damage, but preceded actual cell death (Wilcock et al, 1988). It is possible that the selective inhibition of the $Na^+-K^+-Cl^-$ cotransporter is an early event in cell death and represents a cellular response to damage rather than a direct lesion. Therefore, an appreciation of the different types cell death and the events observed in this process. Such an understanding might help us to define the importance and role of this selective inhibition of ion transport in the process leading to cell death induced by HN2.

Historically, cell death has been regarded as a degenerative process induced by excessive cell injury. Concepts of imbalanced growth (Rueckert and Mueller 1969) and competing metabolisms (van Ankeren and Wheeler 1985)

have been promoted as the processes by which cell death occurs. It has been proposed that the cytotoxicity of HN2 is due to the lack or inaccurate repair of DNA damage (Alexander 1969). However, none of these theories attempt to explain the precise mechanisms by which cell death occurs.

More recently there has been a greater appreciation of the possible role of cell death in the developmant of new anti-cancer strategies (Berger 1986). Cells, when insulted by a variety of agents, can respond in a number of ways. Anti-cancer chemotherapeutic drugs may induce cell death directly by the phenomenon of either necrosis or apoptosis (Wyllie 1981). Alternatively, chemotherapy may induce differentiation so that new phenotypes are expressed in which a reduced proliferative capability is exhibited. Mitotic cell death is a possible consequence of administration of cytotoxic drugs, in which cells have lost their ability to divide due to excessive nuclear damage. Both of these phenomenon can be observed before the onset of cell death mediated by either one of the two processes of necrosis or apoptosis.

1.6.2 **Necrosis and apoptosis.**

A number of criteria distinguish necrosis from apoptosis. Necrosis is most often associated with an acute and gross departure from physiological conditions, such as hypoxia, changes in enviromental temperature and

complement-mediated cytolysis (Wyllie 1981). Growing solid tumours frequently include zones of necrosis which are almost certainly a result of hypoxia and deprivation of other substances (Tomlinson and Gray 1956). Apoptosis, in contrast, appears to be a physiologically induced type of cell death and can occur in normal tissue turnover. It was first defined by Kerr et al (1972). One of the earliest observations of apoptosis was in embryogenesis where loss of appendages was observed (apoptosis is Greek for "the falling of leaves"). It occurs spontaneously in growing tumours and its rates are increased in tumours in regression (Kerr et al 1972).

It has been proposed that volume changes are one of the best morphological characteristics for distinguishing the two forms of cell death (Wyllie 1981). In necrotic cells a mild degree of swelling of the cytoplasm and cellular organelles, especially the mitochondria is observed. The mitochondria will continue to increase in size until they rupture. This is then followed by extensive swelling of the cell, dissolution of cytoplasmic organelles and finally the rupturing of the cell membrane. In contrast, apoptosis is characterised by a decrease in cell size and the organelles remain structurally intact. The mechanism by which cell volume decreases in apoptosis is unknown. Apoptotic cells in vivo eventually become phagocytosed by neighbouring cells. In contrast, cells grown in vitro which are apoptotic will undergo a secondary necrosis in which the

cells swell and eventually the membranes rupture. Thus, apoptotic cells can only be clearly distinguished from necrotic cells in the early stages of cell death.

Viability is often assessed by the ability of the cell to exclude vital dyes such as trypan blue and eosin. Thus, an increase in permeability is directly correlated with loss of viability. Apoptotic cells are still able to exclude vital dyes until secondary necrosis occurs. A number of investigators (Bhuyan et al 1976; Roper and Drewinko 1976) have noted the insensitivity of this method to assess the lethal effects of cytotoxic cancer-chemotherpeutic drugs.

Lymphoma cells treated for 18 hours with 0.1ug/ml HN2 showed a doubling in cell volume as measured by a Coulter counter (Alexander 1969). Cell volume was not determined at earlier time points, thus this increase in cell size could have been due to secondary necrosis. When Ehrlich ascites cells were treated with 2uM HN2 no increase in cell volume, as measured by a Coulter Counter, was observed until 12 hours after treatment and no loss in cell viability, as measured by trypan blue exclusion, was noted until 24 hours (Doppler et al 1985). However, when L1210 murine leukaemic cells were exposed to 10uM HN2 for 4 hours, cell volume, as measured by tritiated water, decreased by 30% (Wilcock et al 1988).

The mitochondria of necrotic cells swell. Eventually the cristae rupture and this is usually concomittant with the appearance of electron dense areas in the matrix of

mitochondria. However, in apoptotic cells the mitochondria remain structurally intact and functional though they become compacted together. This reflects the nature of each type of cell death. In necrosis the typical morphological changes are a consequence of a loss in homeostatic ion control. This loss of ion permeability may result directly from membrane damage (i.e. by complement) or indirectly by a depletion of cellular energy (Sato and Yonei 1987). Ion leakage across the plasma membrane may occur due to a reduction in ATP production as a consequence of mitochondrial damage. The correct internal ion concentration will not be maintained by the necrotic cell, macromolecular sythesis will thus be terminated and the membrane integrity of the cell will not be maintained. In contrast, the mitochondria, in apoptotic cells, are still fuctional so that ATP levels do not fall (Wyllie et al 1980). An appropriate ionic homeostasis is maintained, macromolecular synthesis is not disrupted and the membrane integrity of the cell will be maintianed until the onset of secondary necrosis.

When myeloma cells were treated in vivo with melphalan changes in nuclear structure occured before those in mitochondria (Bocciarelli and Violante 1960). Four hours after exposure of the murine leukaemic L1210 cell line to 10uM HN2, levels of ATP were not significantly different from control levels (Wilcock et al 1988). Although the $Na^+-K^+-Cl^-$ cotransporter was inhibited under these same

conditions, the mitochondria were presumably undamaged as there was no reduction in ATP levels. These observations would argue against the idea that this concentration of HN2 in L1210 cells induced necrosis.

1.6.3 Nuclear changes in necrosis and apoptosis.

Wyllie (1981) proposed that necrosis and apoptosis could be distinguished by the early changes in the nucleus. An early stage in necrosis is the marginal clumping of loosely textured nuclear chromatin. The chromatin is often uniformly compacted (pyknosis), but with the swelling of the nucleus and the rupture of its membrane, the marginated masses may become evident as small discrete masses (karyorrhexis). The nucleolus becomes compacted and remains so until cytoplasmic degradation is advanced. All basophilia (i.e. the ability of basic stains such as methylene blue to stain the nucleus) is eventually lost leaving a nuclear "ghost" (karyolysis).

In the early stages of apoptosis most of the chromatin becomes aggregated in large compact granular masses on the periphery of the nucleus. The nuclear membrane can become convoluted and at later stages may become grossly indented. The nucleus, in the later stages of apoptosis, breaks up into a number of discrete fragments in which the chromatin occupies the entire surface or is arranged in crescentic caps. The nuclear fragments can slough offf from the cell surface to form membrane-bound 'apoptotic bodies' containing

cytoplasmic organellles. In contrast to necrotic cells, the basophilia of apoptotic cells is not lost. As mentioned above, cells, grown in suspension, that become apoptotic do not become phagocytosed but instead undergo secondary necrosis (Wyllie et al 1981). Apoptotic cells that undergo secondary necrosis can be distinguished by the presence of nuclear fragments in which segregated masses of dense chromatin are still evident.

Investigations of chromosome damage in rat Walker carcinoma 256 cells after 1mg/kg i.p. administration of HN2 revealed that chromosome breaks and bridges occurred at early time points (Koller 1957). Seventy-two hours after treatment, chromosomes became fragmented and the nuclei were pyknotic. Cobb (1960) noted intranuclear vacoules and multiple micronuclei in HeLa cells treated with 0.5mM HN2 and that they were still basophilic 6 days after treatment. An increase in nuclear density followed by fragmentation of the nucleus and the disappearance of nucleoli was observed in myeloma cells treated with melphalan (Bocciarelli and Violante 1960). Thus, the report by a number of workers of the presence of micronucleii and basophilia in HN2 treated cells would indicate apoptotic cell death may have occurred under the conditions described.

1.6.4 <u>Calcium levels in apoptosis and necrosis.</u>

Calcium is considered to play a major role in cell death. Free calcium concentrations within the cell are maintained within very strict limits of between 10 and 200nM. This is despite a high external calcium concentration of 1mM. Calcium leaks down its concentration gradient into the cell and is buffered by three systems. Transport proteins in the plasma membrane can extrude excess calcium or alternatively it can be stored intracellularly either in the mitochondria or the endoplasmic reticulum (Orreniue <u>et al</u> 1989). All these systems have a prerequisite for energy, thus the loss of ATP in necrotic cells will lead to a rise in intracellular calcium.

Increases in intracellular calcium levels were observed in hepatocytes treated with a variety of membrane-active toxins (Schanne <u>et al</u> 1979). The rise in internal calcium preceded the loss in cell viability as determinmed by exclusion of trypan blue and incubation in a Ca^{2+}-free media prevented cell death (Schanne <u>et al</u> 1979). Similiarly, in apoptotic cells internal calcium rises are observed which appear to be pivotal in the regulation of this pathological process (Orrenius <u>et al</u> 1989). When immature thymocytes were treated with the glucocorticoid hormone methylprednisolone calcium rises were observed in a dose-related manner (McConkey <u>et al</u> 1989).

In apoptotic cells the DNA is cleaved at internucleosomal sites by an endonuclease enzyme (Wyllie

1980; Wyllie 1985; section 1.6.5). McConkey et al (1988) reported that the activation of the endonuclease enzyme was dependent on rises in cytosolic free Ca^{2+}. The fragmentation of DNA could be prevented by the incubation of cells in a Ca^{2+}-free media or by buffering the rises in Ca^{2+} with quin-2AM (McConkey et al 1988;1989). The cytosolic rise in Ca^{2+} by toxic concentrations of methylprednisolone could be prevented by inhibiting protein synthesis (McConkey et al 1989). Thus, it was proposed that the rise in internal free calcium was due to the synthesis of a heat-labile factor which may act as a Ca^{2+} pore (McConkey et al 1989).

Blebbing of the cell membrane, in which the surface becomes convoluted and protrusions appear, is often observed in cell death . This phenomenon is considered to be an early cytotoxic event and is a consequence of the disruption of the organization of the cytoskeleton. A sustained rise in cytosolic calcium induces skeletal alterations by two independent mechanisms which lead to plasma membrane blebbing (Orrenius et al 1989). Firstly, an increased calcium concentration may cause the dissociation of actin filaments from alpha-actinin, a protein which connects the microfilaments to actin-binding proteins in the plasma membrane. Secondly, calcium-dependent proteases such as calpains are activated. Targets of these enzymes include cytoskeletal elements such as actin-binding proteins and integral membrane proteins. Incubation of liver cells with

inhibitors of calcium activated proteases can delay or even prevent cytotoxic effects (Nicotera et al 1986).

Necrosis, induced by free radical formation, caused rapid increases (less than one hour) of internal free calcium levels. These rises were concommitant with the appearance of cellular blebbing (Jewell et al 1982). Bleb formation in hepatocytes, treated with metabolic inhibitors such as KCN and iodoacetate, was not dependent on a rise in internal calcium, which only increased in parallel with dye leakage (Nieminen et al 1988). Thus, in apoptosis subtle calcium rises may be important in regulating the onset of cell death but in necrosis, induced by metabolic inhibitors, gross rises in calcium may be a consequence of the loss of ionic homeostasis.

1.6.5 Protein synthesis and cell death.

Another distinguishing characteristic of apoptosis is its dependence on protein synthesis. The action of inhibitors of protein synthesis can suppress apoptosis in glucocorticoid-treated thymocytes (Wyllie 1980). In vivo, cycloheximide was able to protect bone marrow and gut epithelial cells from the toxic effects of HN2 (Liebermann et al 1970; Ben-Ishay and Farber 1975). Both groups of investigators observed that cycloheximide could be administered up to 40 minutes after the HN2 treatment and cells would still be protected. Liebermann et al (1970) proposed that after an insult there needed to be an active

metabolic response, i.e. protein synthesis, before cell death would occur.

Condensation of the chromatin, typical of apoptosis, is one of the earliest observations to be made after lymphoid cells are treated with toxic doses of methylprednisolone. This morphological change is thought to be closely associated with the activation of intracellular endonuclease enzymes (Wyllie 1985). Agarose gel electrophoresis showed that the DNA appeared "laddered" and that the fragments were multiples of units of approximately 180bp. Thus, in apoptosis, the activation of the endonuclease enzyme will cut DNA at internucleosomal sites. The treatment of HL-60 cells with a variety of cytotoxic agents induced DNA fragmentation typical of endonucleoytic cleavage (Kaufmann 1989). In necrosis, endonuclease and lysosomal proteins are activated leading to a general dissolution of DNA so that karyorrhexis is observed.

Endonuclease activation was also also observed when thymocytes were exposed to TCDD (2,3,7,8,-tetrachloro-dibenzo-p-dioxin). This activity was dependent on an internal calcium rise, as buffering with Quin-2AM prevented endonuclease activation (M^{C}Conkey et al 1988; section 1.6.4). The occurrence of endonuclease fragmentation of DNA in apoptosis can be inhibited by inhibitors of protein synthesis (Wyllie et al 1984). However, the importance and role of endonucleolytic cleavages in cell death has been recently questioned by

Kaufmann (1989). Inhibitors of protein and RNA synthesis did not prevent the fragmentation of the DNA of HL-60 cells treated with toxic doses of etoposide and Kaufmann was unable to identify the endonuclease enzyme from treated isolated nuclei. Thus, Kaufmann proposed that the enzyme may be released from cytoplasmic lysosomes. Kaufmann also noted that other nuclear proteins were degraded concomitantly with DNA fragmentation, and so he questioned the selectivity of this process.

1.6.6 Cell death and the expression of immediate-early genes.

A number of investigators have reported that specific gene expression was enhanced in cells in response to injury and stress. In particular levels of heat shock proteins (hsp) and certain oncogenes (c-fos and c-myc) have been reported to alter in response to cell damage (Hollander and Fornace, 1989; Futscher and Erickson, 1990; Buttyan et al, 1988). It is considered possible that changes in the expression of these genes may be involved in drug-induced cell death.

c-fos is the normal homologue of the retroviral v-fos oncogene. The fos gene encodes a nuclear protein which binds to the AP-1 binding site (Rauscher et al 1988) and can enhance transcription from multiple promoters such as human metallothionein and mouse $alpha_1$ (III) collagen (Setoyama et al 1986). Increased levels of fos RNA are expressed in some

cell types during differentiation and in cells stimulated with growth factors and mitogens (reviewed by Verma, 1986). Nishikura and Murray (1987) reported that antisense fos RNA prevented the proliferation of quiescent 3T3 cells stimulated by platelet-derived growth factor. Heat shock and inducers of heat shock protein have been demonstrated to increase levels of fos RNA in HeLa cells (Andrews et al 1987). This implies that the fos protein may activate the genes that are involved in the cell's general response to injury. fos RNA was increased in Chinese hamster ovary (CHO) cells 4 hours after treatment with 40uM HN2 but a lower dose of HN2 (8uM), which was also toxic, did not enhance the expression of fos RNA (Holander and Fornace 1989). Heat shock of cells induced the transcription of fos RNA but no lethality was observed. Thus the role of fos in the response of the cell to injury and death is still to be elucidated (Hollander and Fornace 1989).

Heat shock proteins (hsp's) are expressed in response to hyperthermia as well as heavy metals, amino acid analogues and oxidising agents. The precise intracellular mechanisms that induce hsp production are unknown although the presence of damaged proteins appears to be a major contender (reviewed by Lindquist, 1986). Hsp's are found in all cells, are highly conserved, and can be catergorized into three major hsp families. Hsp proteins of a 90kd molecular weight are produced by heat shock and are soluble, cytoplasmic proteins. The 90 kd hsp are abundant cellular

proteins of which only a small proportion is associated with other cellular components. The two main proteins of the hsp 70 family are a glucose-regulated protein of 78kd and a 72 kd protein. Induced hsp 70 protein is found mostly in the nucleus of heat-shocked cells (Velazquez and Lindquist 1984). Serum stimulation of quiescent cells will induce transcription of hsp 70 (Wu and Morimoto 1985) and it is preferentially expressed in the S phase of the cell cycle (Milarski and Morimoto 1986). The final class of hsp is a heterogenous group of small molecular weight proteins which include ubiquitin. The role of hsp is yet to be elucidated but it appears to be involved in the cell's response to stress and injury (Lindquist 1986).

Recently, Futscher and Erickson (1990) reported changes in c-myc and c-fos expression in the human tumour Colo320Hsr cells after exposure to concentrations of HN2 which produced a 1 or 3 log cell kill. mRNA levels of c-myc increased 2- to 3-fold immediately after drug exposure but was depressed by approximately 5- fold after 12 hours. By 24 hours levels of c-myc mRNA were approaching those of control cells. mRNA c-fos levels increased 3- to 4-fold after drug administration and slowly fell back to control levels by 24 hours. In contrast, levels of ß-actin or N-ras were unaffected. Thus, Futscher and Erickson (1990) proposed that alkylating agents altered the expression of specific transforming genes and that these changes were indicative of cell death.

In prostatic cell death induced by castration in male rats a "reactive cascade" of gene activity was observed (Buttyan et al 1988). A transient increase in the transcription of c-fos was observed between 24 and 36 hours after castration. This was followed by an increase in expression of the c-myc protein after 72 hours and hsp 70 after 96 hours. This cascade of gene activity was concomitant with maximum cell death in the prostrate gland. Paradoxically, the cascade of gene activity mimics that observed in proliferating cells. Buttyan et al (1988) proposed that increases in internal calcium from either cell division or injury stimulates levels of fos which in turn enhances the transcription of myc and hsp 70.

In two human colon adenocarcinoma cell lines, treatment with equitoxic concentrations of BCNU induced HSP 70 RNA in Mer$^+$ HT-29 cells but not in Mer$^-$ BE cells. Other DNA-damaging agents such as chlorambucil did not induce HSP 70 synthesis in the HT-29 cell line (Schaefer et al 1988). It was concluded that HSP70 induction was neither as a consequence of DNA-damage nor of cell death in this study. However, CHO cells treated with 40uM HN2 showed increased levels of two heat shock inducible RNA transcripts which were ubiquitin and HS18 though none of these transcripts were increased by a lower but still toxic concentration of HN2 (8uM) (Fornace et al 1989). The possible role of hsp's induced by the amino acid analog azetidine carboxylic acid in HN2 cytotoxicity is investigated in this thesis.

1.7. Aims and objectives.

The properties of the Na^+-K^+-Cl^- cotransporter in the L1210 murine leukaemic cell line were identified by Wilcock and Hickman (1988). Incubation of L1210 cells with 10uM HN2 for 4 hours resulted in the selective inhibition of the Na^+-K^+-Cl^- cotransporter (Wilcock et al 1988). This selective inhibition of transport has also been observed in the Ehrlich cell line (Doppler et al 1985; Grunicke et al 1985). The aim of this project was to investigate the nature of the selective inhibition of the Na^+-K^+-Cl^- cotransporter after HN2 treatment and to attempt to understand its role and importance in HN2-induced cell death in L1210 cells.

1. I wished to investigate whether the inhibition of Na^+-K^+-Cl^- cotransport was due to a direct interaction between the cotransporter and HN2. Wilcock et al (1988) noted that the inhibition of the Na^+-K^+-Cl^- cotransporter was only observed with HN2, and with neither the monofunctional analog of HN2 nor chlorambucil, and may be a consequence of a direct alkylation of the cotransporter. The effects of HN2 on ^{86}rubidium$^+$ uptake were investigated in human erythrocytes in which the potassium transport systems are similiar to those found in the L1210 cell line. Human erythrocytes are enucleate cells and so unable to express any new proteins. Any inhibition of ^{86}rubidium$^+$ uptake observed in these cells would be the consequence of a

direct inactivation of potassium transport systems by the formation of HN2 cross-links, rather than a cellular response to damage expressed, perhaps, through the synthesis of new protein(s).

2.   Elkind and Sakomoto (1969) proposed that the cytotoxicity of HN2 was the consequence of a single lesion. However, other investigators suggested that HN2 cytotoxicity may be the combined result of several interactions (Tritton and Hickman 1984). The effect of the inhibition of $Na^+-K^+-Cl^-$ cotransport by loop diuretics on the toxicity of low levels of HN2 in murine leukaemic L1210 cells was investigated. van Ankeren and Wheeler (1985) observed that cytotoxicity of irradiated cells could be enhanced by incubation in a hypertonic media. I wished to investigate this protocol using the murine leukaemic L1210 cells treated with HN2 in order to ascertain if a change in the activity of the $Na^+-K^+-Cl^-$ cotransporter may be responsible for enhanced cytotoxicity.

3.   Wilcock et al (1988) reported that potassium transport in the murine leukaemic L1210 cell line was inhibited 4 hours after exposure to 10uM HN2. However, it was unaffected by either 1 or 0.5uM HN2 though both of these concentrations of HN2 were cytotoxic but not equitoxic to L1210 cells. I wished to determine whether there was any inhibition in potassium transport at later times after exposure to HN2 at

these concentrations. Changes in cell volume are one
criterion by which apoptosis and necrosis can be
distinguished (introduction 1.6.2). Wilcock et al (1988)
reported a decrease in the volume of L1210 cells incubated
with 10uM HN2. The patterns of cell death induced by the
three concentrations of HN2 (10,1 and 0.5uM) were
investigated so that any differences could be correlated
with changes in potassium transport.

4.   A number of investigators have reported that cycloheximide
protected cells from the toxic effects of HN2 (Ben-Ishay and
Farber 1975; Lieberman et al 1970; Weissberg et al 1978). I
wished to determine if an inhibitor of protein synthesis
(i.e. cycloheximide) could protect murine leukaemic L1210
cells from the toxic effects of HN2 and if this correlated
with any loss of inhibition in potassium transport.

5.   It was reported that l-histidinol, an analog of the
essential amino acid histidine, enhanced the cytotoxicity of
BCNU against the murine lymphoctyic leukaemic cell line P388
(Warrington and Fang 1989). No convincing hypothesis has
been put forward to explain this phenomenon. However, amino
acid analogs have been shown to enhance the synthesis of
heat shock proteins (hsp's) in a variety of systems (Burdon,
1986). This protocol was repeated in vitro using the murine
leukaemic L1210 cell line and HN2 in order to investigate
whether the synthesis of hsp's was correlated with any

enhancement of cytotoxicity of HN2.

In summary, the work described here attempts to determine whether the specific inhibition of the Na^+-K^+-Cl^- cotransporter, previously reported by this laboratory, was symptomatic of a particular process of cell death rather than a direct lesion inflicted at the level of the cell membrane.

2.MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Purchased from Sigma Chemical Company Limited, Poole, Dorset, England.

Ammonium persulphate, adenosine triphosphate (ATP), ATP assay buffer, L-azetidine carboxylic acid, bromophenol blue, coomassie blue R250 stain, cycloheximide, ß-dimethylaminoethylchloride (MeHN1), dimethytsulfoxide, dithiothreitol (DTT), ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), furosemide, glucose, glycine, N-2-hydroxyethyl piperazine N′-2-ethanesulfonic acid (HEPES), luciferase-luciferin reagent, magnesium chloride, mechlorethamine (nitrogen mustard), potassium chloride, potassium hydroxide, osmium tetroxide, ouabain, phenylmethylsulfonylfluride, propidium iodide, 2-((2-bis(carboxymethyl)-amino-5-methylphenoxy)-methyl)-6-methoxy-8-bis(carboxymethyl)aminoquinolone (Quin-2), ribonuclease A, sucrose, N,N,N′,N′-tetra-methylethylenediamine (TEMED), triethanolamine, triton X-100, trizma base, trypan blue.

2.1.2. Purchased from BDH Chemicals Limited, Poole, Dorset, England.

N, N′-methylenebisacrylamide, calcium chloride, 2,5-diphenyloxazole, ethylenediaminetetraacetic acid (EDTA), potassium dihydrogen orthophosphate, phenol red, sodium dodecyl sulphate, sodium hydrogen carbonate,

trichloroacetic acid.

2.1.4. Purchased from FSA Laboratory Supplies, Loughborough, Leicestershire, England.

Acrylamide, ethanol, 90% formic acid, glacial acetic acid, magnesium sulphate, methanol, 60% perchloric acid, Optiphase scintillation fluid, sodium hydroxide pellets, sodium chloride.

2.1.5. Purchased from Gibco Limited, Paisley, Glasgow, Scotland.

Calcium-magnesium free PBS, horse serum, Rosewell Park Memorial Institute medium 1640 (RPMI 1640) with 25mM HEPES and L-glutamine, methionine-free RPMI 1640 with 25mM HEPES and L-glutamine, X10 concentrate RPMI 1640 media without L-glutamine and sodium hydrogen carbonate.

2.1.6. Purchased from Hopkins and Williams Limited, Chadwell Heath, Essex, England.

Dow Corning 550 silicone fluid.

2.1.7. Purchased from E.F. Fullman Inc., Latham, N.Y. 12110.

Araldite 506, dibutyl phalate (DBP), dodecenyl succinic anhydride (DDSA), tris-dimethylaminoethyl phenol (DMP-30).

2.1.8. Purchased from Eastman Kodak Company, Rochester. New York. 14650.

D-19 developer, fixative, X-OMAT Kodak film (13cm x 18cm).

2.1.9. Purchased from Amersham International PLC, Amersham Laboratories, Amersham, Buckinghamshire. HP7 9LL.

Inulin-[^{14}C] carboxylic acid (sp. act. 2-10mCi/mmol), L-[4,5-^{3}H] leucine (sp. act. 25-50Ci/mmol), L-[^{35}S] methionine (sp. act. 15mCi/ml), ^{86}rubidium chloride (sp. act. 1-8mCi/10uM).

2.1.10. Purchased from EM scope Laboratories LTD, Kingsnorth Industrial estate, Ashford, Kent.

25% E.M. grade glutaraldehyde, propylene oxide.

2.1.11. Tissue culture materials.

50ml and 250ml culture flasks, 1ml cell vials, 6 and 24 well tissue culture plates were obtained from Nunclon, Kamstrup, Roskilde, Denmark. 30ml sterile universals, 7ml bijou bottles and sterile pipettes were purchased from Sterilin LTD, Feltham, England.

2.1.12. Donated chemical.

Bumetanide was the kind gift of Leo Pharmaceutical Products, Denmark.

2.2. SOLUTIONS AND BUFFERS.

2.2.1. 5% Noble Agar solution.

Noble Agar	5g
dH_2O	to 100ml.

Solution was autoclaved for 20 minutes at $120^{\circ}C$.

2.2.2. Kreb's-Ringer buffer.

NaCl	118mM
KCl	5mM
$NaHCO_3$	25mM
KH_2PO_4	1.17mM
$MgSO_4$	1.18mM
Glucose	5.5mM
Phenol Red	10mg/L

The solution was gassed for twenty minutes under 5% CO_2, 1.27mM $CaCl_2$ was added and then gassed for a further twenty minutes.

HCl (1M)	to pH 7.4

2.2.3.1. Red blood cell PBS - Buffer A.

Calcium-magnesium free PBS from Gibco supplemented with :

Glucose	5mM
Tris(trizma base)	15mM
HCl (1M)	to pH 7.4

2.2.3.2. Red blood cell PBS - Buffer B.

Calcium-magnesium free PBS from Gibco supplemented with :

Glucose	5mM
Tris(trizma base)	15mM
$CaCl_2$	1mM
$MgCl_2$	1mM
HCl(1M)	to pH 7.4

2.2.4. Gel electrophoresis.

2.2.4.1. Resolving gel (7.5% w/v).

30% acrylamide	10ml
1% bisacrylamide	7.73ml
1M Tris (trizma base) pH8.9	14.9ml
20% SDS	0.2ml
dH_2O	7.1ml

2.2.4.2. Stacking gel (5% w/v).

30% acrylamide	3.34ml
1% bisacrylamide	2.6ml
1M Tris (trizma base) pH6.8	2.5ml
20% SDS	0.1ml
dH_2O	11.4ml

2.2.4.3. Electrode buffer.

Glycine	14.4g/L
SDS	1g/L
Tris(trizma base)	3g/L
NaOH (1M)	to pH 8.3

2.2.4.4. Coomaissie blue R250 stain stock solution.

Coomassie blue R250 stain	24g
Methanol	300mls
Glacial acetic acid	60mls
dH$_2$O	600mls

2.2.4.5. Gel Stain.

Coomassie blue R250 stock	0.25% v/v
Methanol	50% v/v
Glacial acetic acid	10% v/v
dH$_2$O	39.75% v/v

2.2.4.6. Gel Destain.

Methanol	43% v/v
Glacial acetic acid	7% v/v
dH$_2$O	50% v/v

2.2.4.7. Sample buffer.

20% SDS	10%v/v
1M Tris (trizma base) pH 7.0	5% v/v
60% Sucrose	5% v/v
0.9% Bromophenol blue	40% v/v
dH$_2$O	40% v/v
DTT	25mg/ml

2.2.5. Electron microscopy.

2.2.5.1. 0.2M PBS.

2.7% KH$_2$PO$_4$	56.2% v/v
0.8% NaOH	45.8% v/v

2.2.5.2. Fix 1. 2.5% gluteraldehyde in 0.1M buffer.

0.2M PBS	50% v/v
25% E.M. grade gluteraldehyde	10% v/v
dH$_2$O	40% v/v

2.2.5.3. Fix 2. 1% osmium tetroxide in 0.1M buffer.

2% osmium tetroxide	50% v/v
0.2M PBS	50% v/v

2.2.5.4. Embedding Resin.

Epon 812	24% v/v
Araldite 506	31.7% v/v
DDSA	40.4 v/v
DBP	1.4% v/v

The solution was mixed well for 20 minutes

DMP 30	2.4% v/v

The solution was again mixed well for 20 minutes.

2.2.6. Buffer for flourometric determination of internal calcium.

NaCl	140mM
KCl	5mM
$NaHCO_3$	2.8mM
$CaCl_2$	1.5mM
$MgCl_2$	1mM
$MgSO_4$	0.6mM
Glucose	5.6mM
HEPES	15mM
HCl (1M)	to pH 7.4

2.3   METHODS.

2.3.1 Maintenance of the L1210 murine leukaemia cell line in vitro.

The cell line employed was the murine leukaemic cell line L1210, obtained from Flow Laboratories (Glasgow). Cells were incubated at $37^{\circ}C$ in 10% horse serum in RPMI 1640 media (with 25mM HEPES), and gassed with 5% CO_2 in air.

Cultures were initiated at densities of between 0.3-4 $x10^5$ cells/ml. Under the conditions described above L1210 cells exhibited a doubling time of between 12 to 14 hours, reaching a plateau of growth at a density of $1-2x10^6$ cells/ml.

2.3.2.   Preparation of a furosemide resistant L1210 cell line.

This cell line was designated L1210FR. Cells were harvested from an exponentially growing culture and treated with 1mM furosemide. Furosemide was dissolved in DMSO at x200 the required concentration so that the final concentration of DMSO did not exceed 0.5-0.75% v/v. L1210 cells which grew after this treatment were harvested and washed twice in RPMI 1640 and treated with increasing concentrations of furosemide. By the twelfth passage the cells were able to grow in 5mM furosemide, a concentration which caused 100% inhibition of cell growth in control

L1210 cells (results 3.3.2). L1210FR cells were used for experimental work between passage numbers 18 and 22.

2.3.3. Storage of cells in liquid nitrogen.

Cells in exponential phase of growth were harvested by centrifugation at 350g (1,500 rpm) for 5 minutes. The cells were washed once in RPMI 1640 and resuspended to 90% of the volume of RPMI 1640 needed for a final cell density of $2x10^6$ cells/ml. DMSO was added at 10% of the final volume. Cells were allowed to stand for 10 minutes on ice and then 1ml aliquots were dispensed into sterile plastic vials. The vials were cooled overnight in a Union Carbide BF-5 Biological Freezer at a rate of 1^OC per minute. They were stored under liquid nitrogen until required.

2.3.4. Establishment of new cultures from frozen cells.

Vials containing cells were removed from liquid nitrogen and warmed in water at 37^OC. Once thawed, 1ml of cells from the contents of the vials were pipetted into 15ml of RPMI 1640 media with 20% horse serum. The final concentration of DMSO was between 0.5 and 0.75% v/v. Cells were incubated until reaching stationary phase, after 48 hours, whereupon they were subcultured with normal growth media (10% horse serum).

2.3.5. Estimation of cell number.

A sample of between 0.2 and 1.0ml was withdrawn from the cell culture and made up to 10ml with Isoton. Cells were counted using a ZM Coulter Counter. The settings on the Coulter Counter for L1210 cells are as below :

Amplification : 4

Current : 120

Lower threshold : 20

Upper threshold : 99

Gain : 10

2.3.6. Estimation of cell viability.

Cell viability was estimated using the vital dye trypan blue. A cell suspension of 200ul was mixed with 50ul of filtered 0.2% trypan blue in PBS. After mixing the cells were introduced into a haemocytometer counting chamber and examined under a microscope. Those cells excluding the dye were counted as viable and this was taken as a percentage of the total cell number.

2.3.7. Estimation of drug cytotoxicity to L1210 cells.
2.3.7.1. Estimation of drug cytotoxicity by continous incubation in suspension culture.

Cells were seeded at a density of 3×10^4 cells/ml so that within 72 hours the control cells would reach plateau phase (section 2.3.1.). The appropriate drug was added to the cells and then 2ml duplicate aliquots were dispensed

into each of the wells of a 24 well plastic tissue culture plate. The plates were incubated for 72 hours at $37^{\circ}C$ in an atmosphere of 5% CO_2 at full humidity. After 72 hours the cells were mixed and 200ul samples were removed for counting by a model ZM Coulter Counter. Once cell numbers were determined percentage inhibitions were calculated using the following equation :

$$I = 1 - [T_f - C_i / C_f - C_i] \times 100\%$$

where C_i = initial cell number per ml

C_f = final cell number per ml of control samples

T_f = final cell number per ml of treated samples

I = percentage inhibition of growth

2.3.7.2. Estimation of drug cytotoxicity by clonogenic assay.

This method is based on that described by Chu & Fischer (1968) as modified by Wilcock (1987). Cells were harvested from culture and spun at 350g (1,500 rpm) for 5 minutes. The cells were suspended to a density of 2×10^5 cells/ml in RPMI 1640. The drug was added, and the cells were incubated at $37^{\circ}C$ for the appropriate time. They were washed twice in RPMI 1640 and resuspended to a density of 2×10^5 cells/ml. Serial dilutions were then performed in order to obtain cell densities at x10 th, required number per cloning dish.

When required, bottles of 5% solutions of Noble Agar (section 2.2.1.) were heated to $100^{\circ}C$, then removed to a $44^{\circ}C$ water-bath when molten. When the agar had cooled to

touch, but was still molten, it was diluted 1 in 10 with RPMI 1640 and 10% horse serum. The cooled agar and cell culture medium were then mixed, a 0.5ml aliqout removed, and kept at 44°C until needed. 0.5ml of the cell suspensions was added and mixed. Triplicate 4ml samples were pipetted out into the wells of a 6 well tissue culture dish (Nunc).

The dishes were incubated at 37°C in an atmoshere of 5% CO_2 and full humidity for a week. The dishes were examined with a light microscope under x4 magnification and colonies of greater than 50 cells were counted.

2.3.8. Measurement of ^{3}H-leucine incorporation into L1210 cells.

Cells were harvested in exponential phase of growth and washed twice in a Krebs-Ringer solution at pH 7.4(section 2.2.2.). They were resuspended at a final cell density of 5×10^6/ml, allowed to preincubate for 15 minutes in 5% CO_2 in a shaking water bath at 37°C, during which time drugs were added. At the end of this period 5uCi/ml of ^{3}H-leucine was added. At the appropriate time points duplicate samples of 200ul were removed and the aliquots were layered onto Whatman GF/C glass microfibre filters (2.5cm in diameter) which had previously been soaked for 1 hour in a saline solution. The filters were washed three times with ice-cold PBS (Dulbecco), then 5ml of ice-cold 20% trichloroacetic acid. The filters were washed a further three times with PBS and dried with one wash of methanol before being placed

into glass scintillation vials and dried for 4 hours in a warm cabinet. Optiphase (10ml) was then added to each sample and placed in a 2000CA Tri-Carb liquid scintillation counter.

2.3.9. Measurement of ^{86}Rubidium^{+} influx into L1210 cells.

L1210 cells were harvested in the exponential phase of growth and washed twice in a Kreb's-Ringer bicarbonate buffer (section 2.2.2.) or RPMI 1640 at 350g (1,500 rpm) for 5 minutes. The cells were suspended in the media at a final density of 5×10^6/ml. They were incubated for a set time at 37°C under 5% CO_2 in a shaking water bath. Potassium transport inhibitors were added 5 minutes before the addition of ^{86}rubidium^{+} chloride at an activity of 5uCi/ml. Triplicate samples of 200ul were aliquoted from the samples at each time point and carefully layered into 0.4ml Microfuge tubes containing 100ul of 10 parts Dow-Corning 550 silicon oil to 3 parts corn oil (Mazola) which was suspended on top of 50ul of 90% formic acid. The Microfuge tubes were then spun at 900g for 30 seconds in a Beckmann B microfuge. The tubes were frozen in liquid nitrogen and cut at the acid-oil interface. The acid fraction, containing the cells, and the supernatant fraction were placed into LP3 tubes and counted in a 1282 Compugamma gamma-counter (LKB Wallac, Somerset Boulevard, Milton Keynes).

2.3.10. Red Blood cell experimental methodology.

2.3.10.1. Measurement of potassium influx into Human Red Blood Cells.

This method is based on that of O'Neill and Mikkelsen (1987). Blood samples, taken from healthy volunteers, were centrifuged at 350g (1,500 rpm) for 5 minutes and washed twice at room temperature with Buffer A (section 2.2.3.1.). The red blood cells were resuspended to a hematocrit of 50% and 50ul added with 150ul of incubation Buffer B (section 2.2.3.2.) to 1.5ml Microfuge tubes (Beckman). These samples were then preincubated for 15 minutes in a shaking water bath at 37°C during which time potassium transport inhibitors were added. At the end of the preincubation period 0.1uCi ^{86}rubidium^{+} chloride was added to each sample so that the final incubation volume was 250ul. At appropriate time points, 1ml of ice-cold PBS was added to each of the samples and centrifuged at 900g for 10 seconds in a Beckmann B Microfuge followed by a further two rinses with PBS. The pellet was then resuspended with 300ul 5% trichloroacetic acid, and centrifuged again. The supernatant was decanted into scintillation vials and 5ml of Optiphase added. The samples were then placed in a 2000CA Tri-Carb liquid scintillation analyser and the radioactivity measured.

Rubidium influx into the red blood cells was considered to represent that of potassium influx (Meares et al 1978). The flux inhibited by the action of 0.1mM ouabain was

considered to be due to the Na^+/K^+ ATPase pump. The rubidium influx due to the $Na^+-K^+Cl^-$ cotransporter was pharmacologically defined as that influx in the presence of 0.1mM ouabain which was further inhibited by 1mM furosemide or 0.1mM bumetanide.

2.3.10.2. Determination of haemolysis by spectrophotometric methods.

Red blood cells were prepared as described in the previous section (2.3.10.1.). At appropiate time points they were spun at 900g for 10 seconds in a Beckman B Microfuge. The supernatant was decanted off into plastic cuvettes. Absorbance was measured at 540nm in a CE595 double beam spectrophotometer. Haemoglobin absorbs light at 540nm and so this value represents the amount of haemolysis. The values of absorbance was compared for control and nitrogen mustard treated red blood cells.

2.3.11. Gel electrophoresis.
2.3.11.1. Resolving gel (7.5% w/v).

The resolving gel solution was prepared as described in section 2.2.4.1. TEMED (20ul) and 200ul of a freshly prepared 10% w/v APS solution were added to catalyse the polymerisation of acrylamide. This mixture was introduced between two glass plates (16cm x 20cm) in a gel casting unit. The surface of the gel was overlaid with 2ml of distilled water and allowed to polymerise.

2.3.11.2. <u>Stacking gel (5% w/v)</u>.

The stacking gel solution was prepared as described in section 2.3.4.2. A teflon comb with 10 teeth was introduced between the two glass plates such that the distance between the teeth and the resolving gel was approximately 3cm. TEMED (20ul) and 200ul of a freshly prepared 10% w/v APS solution were added to catalyse the polymerisation of acrylamide. This was introduced onto the resolving gel and left to polymerise before use.

2.3.11.3. <u>Running conditions</u>.

After the gels were cast and allowed sufficient time for complete polymerisation, the teflon combs were removed and the sample wells rinsed with distilled water. Protein samples were loaded into the sample wells using a Hamilton syringe. The gel was placed into a discontinuous buffer electrophoresis apparatus and the upper and lower buffer chambers were filled with the electrode buffer (section 2.2.4.3.). The gel was electrophorezed at either 50mA/gel constant current (during daytime) or 55v constant voltage (overnight).

2.3.11.4. Gel staining and destaining.

After electrophoresis, the polyacrylamide gel was carefully removed, rinsed with water and incubated with gel stain (section 2.2.4.5.) for 1-2 hours. The gel was briefly rinsed with water and gel destain was added (section 2.2.4.6.) with a length of wool to absorb the excess stain. The destaining was continued until the background of the gel was clear.

2.3.11.5. Gel drying.

Cellophane sheets were soaked in a 5mM glycine solution for 10 minutes. The wet gel was placed between two sheets of cellophane on a glass plate and all air bubbles were expelled. The sheets were sealed using gel dividers and bulldog clips. The plates were left overnight in a fume cupboard to dry.

2.3.12.1. Preparation of samples for fluorography.

2×10^6 cells were harvested from drug-treated samples. These were spun down at 350g (1,500 rpm) for 5 minutes and resuspended in methionine-free RPMI 1640 media and transferred to 1.5 ml Microfuge tubes and washed twice in the methionine-free RPMI 1640 media. The cells were resuspended to 100ul in methionine-free RPMI 1640 with 10% horse serum and 5uCi of ^{35}S-methionine. The samples were incubated in a shaking water bath at 37^0C for 2 hours, after which the cells were washed twice in phosphate-buffered

saline solution and resuspended in 320ul of sample buffer (section 2.2.4.7.). The samples were boiled for 5 minutes, spun at 10,000 rpm for 30 seconds and then loaded onto the gels and electrophorised as described previously (2.2.11.3.).

2.3.12.2. Preparation of fluorographs.

The gels were stained and destained as described above (2.3.11.4.). The gels then underwent a procedure to enhance the quality of the autoradiograph as described by Bonner and Laskey (1974). They were first given two twenty minute washes in DMSO and washed for 1 hour in a 20% PPO/DMSO solution. The gels were left in a flow of tepid water for 2 hours and dried as described above (2.3.11.5.). A Kodak medical film (13cm x 18cm) was exposed to the dried gel in the dark for 3 days at $-70^{O}C$. The fluorograph was developed by incubation for 5 minutes in a Kodak D-19 developer, followed by a 5 minute incubation in a fixative solution and then washed in water and hung to dry in a warm cabinet. The fluorographs were analysed with the aid of a LKB ·laser densitometer ultrascan system (LKB, Bromma, Sweden).

2.3.13.1. Preparation of scanning electron micrographs of L1210 cells.

Cells were harvested, spun down, and washed once in E.M. PBS (section 2.2.5.1.). The cells were resuspended in an ice-cold glutaraldehyde fixing solution (section 2.2.5.2.) and fixed for 1 hour at 4^0C, after which the cells were washed twice in E.M. PBS and twice in distilled water. The cells were dropped onto formvar coated grids and left to dry overnight.

2.3.13.2. Preparation of transmission electron micrographs of L1210 cells.

Cells were harvested, spun down and washed once in E.M. PBS (section 2.2.5.1.). The cells were resuspended in an ice-cold glutaraldehyde fixing solution (section 2.2.5.2.) and fixed for 1 hour at 4^OC. The cell pellet was rinsed twice in E.M. PBS and twice in distilled water. The cell pellet was incubated at room temperature in a 1% osmium tetroxide solution (section 2.2.5.3) for 1 hour after which the pellets were transferred to a 70% ethanol solution for 15 minutes. The cell pellets were washed for 15 minutes in 70% ethanol, then 90% ethanol, twice in a 100% ethanol and a final wash in dried ethanol. The samples were washed twice for 15 minutes in propylene oxide and then for 30 minutes in an equal mixture of propylene oxide and embedding resin (section 2.2.5.4.). The pellets were transferred into plastic bottle caps filled with the embedding resin and left

overnight in an incubator at 60°C.

2.3.14. Determination of cell size.

2.3.14.1. Determination of cell size by Coulter Counter.

A model ZM coulter counter was used to measure cell size and volume. The settings used were :

Current : 120

Full scale : 10

Attenuation : 4

It was assumed that there was a normal population of cell size distribution and that the cells were perfectlly spherical. The cell volume was measured by counting the total number of cells. The threshold volume was altered until half the original cell count was recorded and then read. This value was assumed to represent the average size of the cell population being investigated.

2.3.14.2. Determination of cell volume by equilibartion of radioisotopes.

Cells (5×10^6) were harvested and washed twice and resuspended in 1ml of RPMI 1640. The cells were incubated for 20 minutes in a shaking water bath at 37°C under an atmosphere of 5% CO_2 after which 1uCi/ml of ^{3}H-H_2O and 1uCi/ml of ^{14}C-inulin carboxylic acid were added. The cells were allowed to incubate for 10 minutes. Aliquots of 200ul were pippetted into 0.4ml microfuge tubes with an oil barrier (10 parts Dow Corning 550 silicon : 3 parts corn

oil) over a 90% formic acid layer. The tubes were spun at 900g in a Beckmann B Microfuge for 30 seconds and frozen in liquid nitrogen. The tubes were cut at the acid-oil boundary and the two fractions placed in pony vials with 4ml of scintillant and counted. The ^3H-H_2O estimated the total water in the system and the ^{14}C-inulin carboxylic acid the extracellular space. Cell volume was calculated by subtraction of these two values.

2.3.15. Determination of internal calcium levels using fluorimetric techniques.

This method is based on that desrcibed by Tsien et al (1982). Cells were harvested (1.5×10^7) and were spun down at 350g for 5 minutes and resuspended in 10mls of RPMI 1640. 5ul of a 40mM stock solution of the flourescent dye Quin-2 was added to the cells and incubated for 1 hour at 37^OC. The cells were washed in RPMI 1640 and resuspended in 2ml of neutrophil buffer (section 2.2.6.), transferred to a quartz glass cuvette and placed in a Perkin Elmer LS-5 luminescence spectrophotometer. Cells were excited with uv light at 332nm and emission was measured at 492nm. The cells were allowed to equilibrate for 8 minutes and then a 20ul of 10% Triton x100 was addded to disrupt the cells in order to obtain the maximum calcium signal. EGTA (200ul of a 250mM solution) was added to chellate all free calcium and so determine the minimum signal. The concentration of free internal calcium was calculated by the following equation:

$$[Ca^{2+}]_i = \text{minimum signal/maximum signal} \times Kd$$

The Kd value for Quin-2 is 120nM as stated by Tsien et al (1982).

2.3.16. Determination of ATP concentrations of the L1210 cells.

Cells were harvested (1×10^7) and washed twice in ice-cold 0.9% NaCl saline solution, transferred to 1.5ml Microfuge tubes and resupended in 0.5ml of the saline solution. 75ul of a 12% perchloric acid solution was added and a left on ice for 15 minutes. The cells were spun at 2,900g (13,000 rpm) for 2 minutes in a Heraeus Biofuge A, the supernatant was pipetted off and neutralised with solution of KOH (2M) and triethanolamine (0.5M). The samples were spun again at 2,900 (13,000 rpm) and the supernatant was transferred to fresh tubes and frozen at $-20^{\circ}C$ until analysis.

A 15 ul sample was mixed with 1ml of ice-cold ATP assay buffer and 15ul of the luciferase-luciferin reagent. The luminescence was measured immediately against a blank of 15ul luciferase-luciferin reagent and 1ml of the buffer in a 1250 luminometer (LKB Wallac, Somerset Boulevard, Milton Keynes) attached to a Data Trace potentiometric chart recorder (Gallenkamp, Belton Road West, Loughborough). ATP content was read from a plot of the relationship between luminescence and ATP content of standard solutions (0-2ug/ml).

2.3.17. Flow cytometry techniques.

Flow cytometry (FCM) is a technique that allows multiple parameters to be studied in individual cells. Cells in suspension are passed through a laser beam at a rate of several thousand per second. Light that is emitted from the flourochrome molecule or is scatterd by the cell is recorded and analysed.

2.3.17.1. Determination of cell size and viability by flow cytometry.

The membrane integrity of cells was assayed by propidium iodide (PI) staining. Cells which exhibited red fluorescence were counted as dead since the PI dye was able to enter the cell and bind to nucleic acids. Forward Light Scatter (Fls) may be used as a measure of cell size.

Cells in exponential phase of growth were harvested and washed twice in RPMI 1640. After twenty minutes of incubation with nitrogen mustard, 10% horse serum was added. The cells were gassed with 5% CO_2 and left for the appropriate time at $37^{\circ}C$. Cells were harvested (10^6) and washed once in RPMI 1640, transferred to a 1.5ml Microfuge tube and gassed with 5% CO_2. The samples were kept on ice until analysis when propidium iodide was added to a final concentration of 32uM. The samples were then processed on a FACS 440 flow cytometer using an argon laser operating at an excitation wavelength of 448nm. Data from 10^4 events were accumulated and processed on a Consort 40 computer.

Propidium iodide emission fluourescence was recorded in order to determine cell viability. Forward light scatter was recorded for cell size.

2.3.17.2. Cell cycle analysis by flow cytometry.

For cell cycle analysis cells were fixed and stained essentially using the method of Gray and Coffino (1979). Cells were harvested (10^6) from culture and washed twice in ice-cold PBS. The cells were fixed by the dropwise addition of ice-cold 70% ethanol with constant agitation to prevent clumping. The fixed cells were stored at 4^OC in the dark for up to two weeks prior to analysis.

The fixed cells were washed once in PBS and transferred to 1.5ml Microfuge tubes. 1ml of a RNase and propidium iodide solution (both at a final concentration of 50ug/ml) was added and incubated at 37^OC for twenty minutes. This procedure degraded the RNA so that only the binding between the fluorochrome to the DNA was recorded. The samples were stored on ice until they were processed on a FACS 440 flow cytometer using an argon laser operating at an excitation wavelength of 448nm. The data was recorded and analysed on a Consort 40 computer.

3.RESULTS AND DISCUSSION.

Pages 100

Missing page(s) from the bound copy

3.1.1. The toxicity of nitrogen mustard to L1210 murine leukaemic cells in vitro.

Introduction.

Two methods have been used to determine the toxicity of HN2 to L1210 murine leukaemic cells. The first is the measurement of inhibition of cell growth (methods 2.3.7.1.). L1210 cells were exposed to HN2 and incubated for 72 hours. The cell count of treated cells was expressed as a percentage of control growth. Alternatively, toxicity was assayed by determining the clonogenic potential of treated cells (methods 2.3.7.2.).

Results.

The toxicity of HN2, as assayed by continous incubation, is demonstrated in Figure 1A. The IC_{50} concentration of HN2 is approximately 170nM and 10uM, 1uM and 0.5uM HN2 all caused >90% inhibition of cell growth.

The surviving fraction, as determined by clonogenic assay, is displayed in Figure 1B. L1210 cells, in RPMI 1640 media, were exposed to various concentrations of HN2 for 1 hour. The mean control plating efficiency was 60±18%. Treatment of L1210 cells with 10uM HN2 for 1 hour resulted in a 4 log cell kill, a 3 log cell kill for those treated with 1uM and a 2 log cell kill in L1210 cells treated with 0.5uM HN2.

Discussion.

Wilcock (1987) reported that in L1210 cells the IC_{50} of HN2, determined by the inhibition of growth, was approximately 100nM which compares well with the value stated above. Wilcock also reported that a 1 hour incubation of L1210 cells in RPMI 1640 with 10uM HN2 resulted in approximately 4 log cell kill which also compares well with the value noted in figure 1A.

The clonogenic assay is more accurate in determining drug cytotoxicity than methods of continous incubation. The number of L1210 cells is only able to increase by approximately 2 logarithms which is, therefore, the maximum detectable cell kill in inhibition of growth studies. In contrast, the clonogenic assay determines the number of viable cells that are able to form discrete colonies of 50 cells or more. The number of cells plated was increased so that at least 30 colonies were counted. This allows a more accurate estimate of logarithmic cell kill than by methods of inhibition of growth.

A second advantage of determining drug toxicity by clonogenic assay is that with some drug treatments cells are able to undergo one or two rounds of cell division before death ensues. In inhibition of growth studies these divisions would be counted and so obscure the true toxicity of the drug. In contrast, assaying the clonogenic potential of L1210 cells is determined by counting the number of

viable cells able to form colonies of 50 cells or more and those colonies consisting of less cells would be excluded from any calculations. Cells expsed to an LD_{90} dose of x rays are able to continue to divide nearly normally until the cells in culture have divided once (Alexander 1969). In contrast, a number of investigators have shown that, after HN2 treatment, cells are able to continue to synthesise DNA, RNA and proteins but are unable to divide further (Alexander 1969; Brewer 1961).

The quality of the information obtained by growth inhibition studies is still valuable. In determinations of drug cytotoxicity, this method is very easy, quick and cheap to perform in contrast to clonogenic assays.
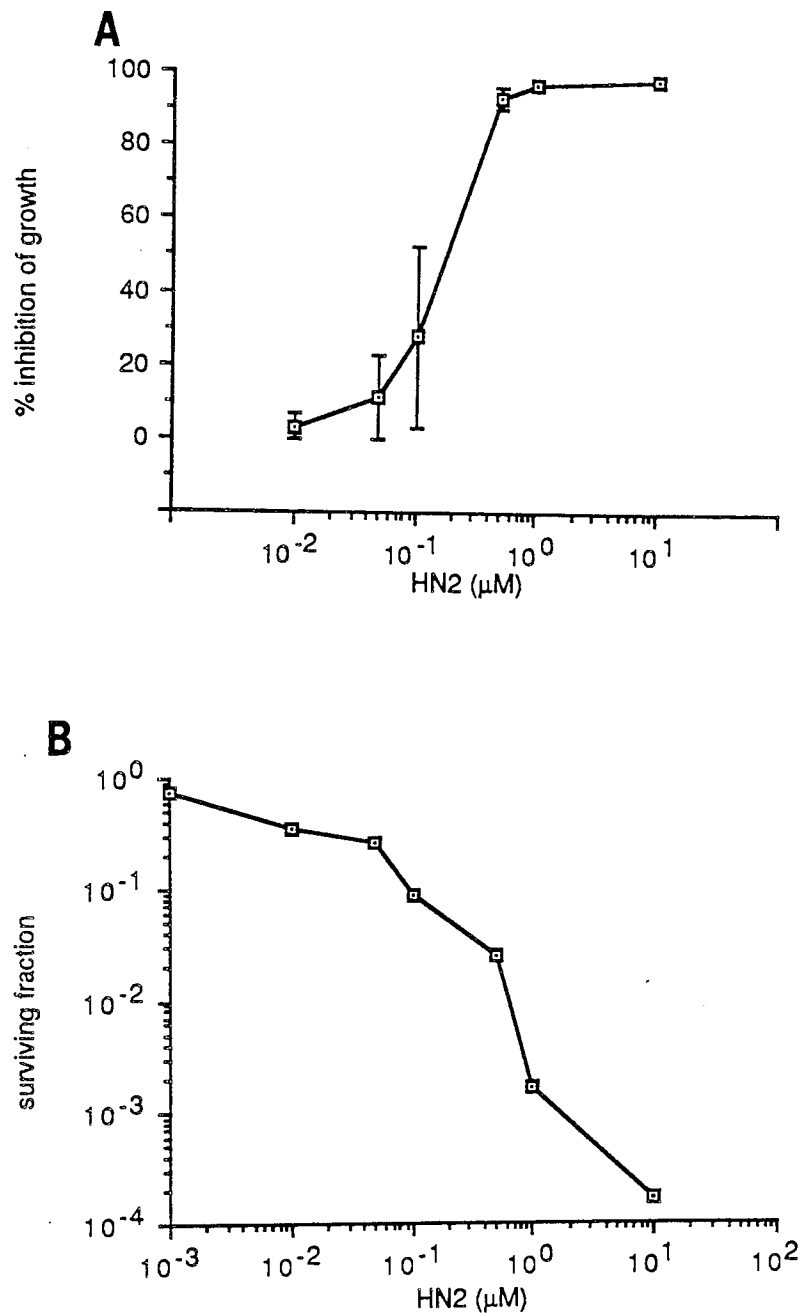
Figure 1. The cytotoxicity of HN2 of murine leukaemic L1210 cells *in vitro* determined by A) percentage inhibition of growth (n=3, mean±S.D.) or B) clonogenic potential (n=3).

3.1.2 <u>The</u> <u>effect</u> <u>of</u> <u>a</u> <u>4</u> <u>hour</u> <u>incubation</u> <u>with</u> <u>10uM</u> <u>nitrogen</u>
<u>mustard</u> <u>on</u> ^{86}<u>rubidium</u>$^+$ <u>influx</u> <u>in</u> <u>L1210</u> <u>cells.</u>

Introduction.

The inhibitory effect of HN2 on potassium transport into the cell has been identified by a number of investigators. Inactivation of $Na^+-K^+-Cl^-$ cotransport is thought to be responsible for this lesion (Doppler <u>et</u> <u>al</u> 1985; Grunicke <u>et</u> <u>al</u> 1985a; Wilcock <u>et</u> <u>al</u> 1988). The aim of this project is to understand better the nature and importance of this lesion in HN2 cytotoxicity.

Results.

The inhibition of ^{86}rubidium$^+$ influx in L1210 cells in RPMI 1640 after a 4 hour incubation with 10uM HN2 is demonstrated in Figure 2. The fractional uptake of ^{86}rubidium$^+$ was depressed by 45%. Ouabain-sensitive ^{86}rubidium$^+$ uptake was inhibited by only 10%. In contrast, bumetanide-sensitive ^{86}rubidium$^+$ uptake was inhibited by 75%. Passive uptake into the cell was unaffected by 10uM HN2.

Discussion.

Wilcock et al (1988) observed a selective inhibition of $Na^+-K^+-Cl^-$ cotransport by 10uM HN2 in L1210 cells incubated in a Kreb's-Ringer solution for 3 hours. Wilcock (1987) observed an inhibition of ^{86}rubidium$^+$ uptake after 4 hours incubation in RPMI 1640 with 10uM HN2, though no further investigations were carried out. We have observed that a 4 hour incubation with 10uM HN2 inhibited ^{86}rubidium$^+$ uptake into L1210 cells by a selective lesion of the $Na^+-K^+-Cl^-$ cotransport protein. Thus, these results are in good agreement with those of other investigators (Wilcock et al 1988; Grunicke et al 1985a; Doppler et al 1985) although in the studies of Wilcock et al (1988) the L1210 cells were incubated in a Kreb's-Ringer buffer.

The passive uptake of ^{86}rubidium$^+$ can be regarded as one indication of membrane integrity and is unaltered by a 4 hour incubation with 10uM HN2. This is in agreement with other methods of assessing membrane integrity and of cell viability such as exclusion of the vital dye Trypan Blue, internal concentrations of potassium and free calcium, internal pH and transmembrane potential (results 3.4.4. and 3.4.5.; Wilcock et al 1988). Thus, at the time of this lesion in potassium transport, cell integrity and viability are still maintained. Therefore, selective inhibition of $Na^+-K^+-Cl^-$ cotransport by 10uM HN2 may be of some importance in the cytotoxic mechanism of this drug.
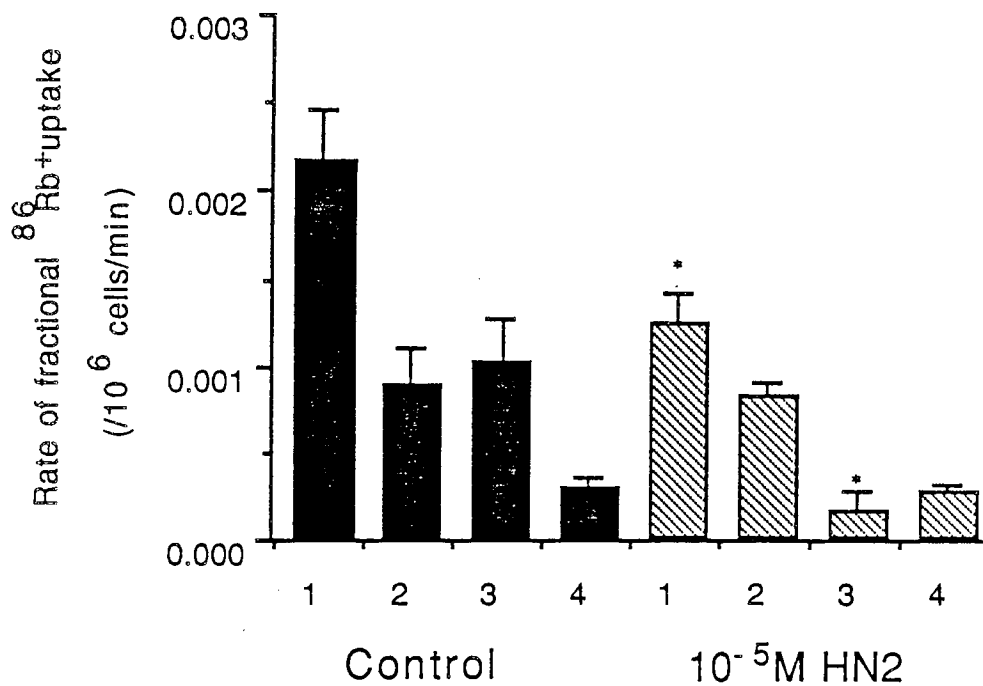
Figure 2. The effect of 10uM HN2 on the potassium ion transport systems of murine leukaemic L1210 cells after a 4 hour incubation in RPMI 1640. 1= total ^{86}rubidium$^+$ flux, 2= Na^+/K^+ ATPase pump flux, 3= $Na^+-K^+-Cl^-$ cotransport flux, 4= passive flux. (n=3, mean±S.D.). Significant difference from control value, $*$ = $p<0.005$ (student t-test).

3.2.1 The effect of HN2 on ^{86}rubidium$^+$ influx in Human red blood cells.

Introduction.

The selective inhibition of $Na^+-K^+-Cl^-$ cotransport observed in L1210 cells incubated with 10uM HN2 (results 3.1.2.) may be due to a direct inactivation of the cotransporter by the formation of cross-links by HN2. Alternatively, it may be due to either interference with the control of the activity of the cotransporter (i.e. alteration of cAMP levels or internal calcium) or because of new gene expression of the cell in response to treatment with HN2. I decided to investigate the effects of HN2 on potassium transport in human erythrocytes. Red blood cells are enucleate and so any interference in potassium transport by new gene expression can be disregarded.

It was assumed that the potassium transport systems of human red blood cells are similiar to those found in the nucleated murine leukaemic L1210 cells. In human erythrocytes $Na^+-K^+-Cl^-$ cotransport was identified by its sensitivity to loop diuretics, of which bumetanide is more potent than furosemide (Ellory and Stewart 1982). However, the linkage of Cl^- to Na^+-K^+ movements has proved to be difficult to establish as Cl^- fluxes via the Cl^-/HCO_3 exchanger are so fast that they obscure Cl^- fluxes via cotransport (Wiater and Dunham 1983; Duhm and Goebel 1984). Na^+ and K^+ movements in human erythrocytes do, nonetheless,

conform to cotransport involving Cl^- (Duhm and Goebel 1984). There is also evidence for K^+-Cl^- cotransport in human erythrocytes (Kaji 1986).

Results.

^{86}Rubidium$^+$ uptake into human red blood cells was determined (figure 3). The Na^+/K^+ ATPase pump is responsible for 75±2% of ^{86}rubidium$^+$ influx into human erythrocytes. The $Na^+-K^+-Cl^-$ cotransporter accounts for 19±2% of ^{86}rubidium$^+$ uptake, the remainder is due to the passive flux of ions down their concentration gradient.

The effect of 2,1,0.5 and 0.1mM HN2 on ^{86}rubidium$^+$ uptake was investigated over a 4 hour time period (Figure 4). An unequivocal inhibition of ^{86}rubidium$^+$ uptake was only observed with 2mM HN2. This inhibition did not appear to increase with time as ^{86}rubidium$^+$ uptake was depressed by 44±5% after 1 hour and 48±12% after 4 hours incubation with 2mM HN2.

Incubation of red blood cells, at a 5% haematocrit, with 1mM HN2 did not produce a clear inhibition of ^{86}rubidium$^+$ uptake (Figure 5). Incubation of red blood cells at a 5% hematocrit for 4 hours with 1mM HN2 resulted in a 28±6% inhibition of ^{86}rubidium$^+$ influx in contrast to 19±4% with a 50% hematocrit.

Haemolysis was determined by measuring the absorbance of the supernatant at 540nm (Table 1). This value was used as an indicator of membrane integrity. Treatment of red blood cells with either 2 or 1mM HN2 did not cause any significant increase in haemolysis during the time period investigated.

In Figure 6 the effect of 2mM HN2 on the different potassium transport systems of human erythrocytes was investigated. There was no significant difference in the inhibition of either the Na^+/K^+ ATPase pump (46±7) or $Na^+-K^+-Cl^-$ cotransport (35±8) in the first hour of incubation of human erythrocytes with 2mM HN2. However, significant differences were observed between the inhibition of Na^+/K^+ ATPase pump activity and $Na^+-K^+-Cl^-$ cotransport 2 hours after exposure to 2mM HN2. Four hours after administration of 2mM HN2 to human erythrocytes the Na^+/K^+ ATPase pump activity was inhibited by 35±9% and $Na^+-K^+-Cl^-$ cotransport by 64±13% (p<0.05). Passive uptake of $^{86}rubidium^+$ was significantly inhibited by 2mM HN2 at each of the times investigated.

Discussion.

Potassium transport systems in the red blood cell have been pharmacologically defined. The Na^+/K^+ATPase pump was responsible for much of potassium transport in the red blood cell (75%) whilst $Na^+-K^+-Cl^-$ cotransport accounted for 15% of total uptake into the erythrocyte. The red blood cells from different individuals displayed a wide variation in ^{86}rubidium$^+$ uptake. Thus, results are expressed as a percentage of the untreated ^{86}rubidium$^+$ uptake.

HN2 inhibited ^{86}rubidium$^+$ influx into red blood cells only at very high concentrations (2mM) which were unpharmacological. A haematocrit of 50% represented approximately $5x10^7$cells/ml, a factor of 10 greater than the cell density employed in investigating ^{86}rubidium$^+$ influx in L1210 cells. Thus, it was decided to reduce the haematocrit by a factor of 10 from 50% to 5% but increase the activity of ^{86}rubidium$^+$ from 0.1uCi/sample to 0.5uCi. This, it was hoped, would increase the sensitivity of the assay system and enable us to use lower concentrations of HN2. Incubation of erythrocytes with 1mM HN2 under these conditions did not produce any significant increase in the inhibition of ^{86}rubidium$^+$ uptake. Thus, it was decided to continue these experiments employing the original conditions.

Cellular integrity was assayed by measuring the absorbance of the supernatant at 540nm. This value is an estimate of level of haemoglobin in the supernatant that has leaked from the erythrocytes and so an indicator of haemolysis. This parameter was not significantly changed by incubation of red blood cells with either 2 or 1mM HN2 over 4 hours (Table 1). This indicates that membrane integrity was maintained in spite of treating erythrocytes with such high concentrations of HN2.

In human erythrocytes all potassium transport systems were equally inhibited $\frac{1}{2}$ and 1 hour after exposure to 2mM HN2 but at later times (2,3 and 4 hours) the level of inhibition of $Na^+-K^+-Cl^-$ cotransport was significantly greater than that of the Na^+/K^+ ATPase pump. It is possible that the inhibition of potassium ion transporters in the first hour after the administration of HN2 represents the direct alkylation of these potassium transport systems by HN2.

HN2 is a very reactive compound. In unbuffered water the half-life for chloride production at 37^OC has been reported as 87 seconds (Spurgin 1983). Williamson et al (1966) reported that, employing this assay, the half-life for the alkylating ability of HN2 in a 0.1M phosphate buffer (pH 7.4, 37^OC) was approximately 1 hour. The half-life of the alkylating ability of HN2 was determined colormetrically by its rection with 4-(4'-nitrobenzoyl)pyridine (Friedman

and Boger 1961). This method will detect both uncyclized and cyclized HN2 and any monofunctional alkylating agents derived therefrom. Therfore, it is of some surprise that a difference in the levels of inhibition in the two potassium transport systems was not observed until 2 hours after exposure to 2mM HN2. Thus, the progressive inhibition of $Na^+-K^+-Cl^-$ cotransport in human red blood cells by 2mM HN2 may not be due to the direct inactivation of the cotransporter by the formation of cross-links by HN2.

The decrease in passive flux may be due to the inhibition of K^+-Cl^- cotransport identifued by Kaji (1986). Papahadjopoulos et al (1973) reported that sodium diffusion was dependent on the fluidity of the lipid bilayer. Therefore, the decrease in passive flux may also be a consequence of changes in membrane fluidity induced by such a high concentration of HN2. Though Grunicke et al (1985a) observed no change in membrane fluidity of Ehrlich cells treated with 0.1mM HN2, in these experiments a higher concentration of HN2 (2mM) was tested on human erythrocytes.

Assuming that the $Na^+-K^+-Cl^-$ cotransporter was similiar in both human erythrocytes and murine leukaemic L1210 cells; the data from red blood cells would indicate that the selective inhibition of $Na^+-K^+-Cl^-$ cotransport in L1210 cells by 10uM HN2 is unlikely to be a consequence of either a direct alkylation of the cotransporter, new gene expression or the release of proteins from internal organelles such as lysosomes. It is possible that changes

in the organisation of the cytoskeleton, the control mechanisms of the cotransporter or membrane fluidity are responsible for the progressive inhibition of the $Na^+-K^+-Cl^-$ cotransporter in L1210 cells and human erythrocytes.
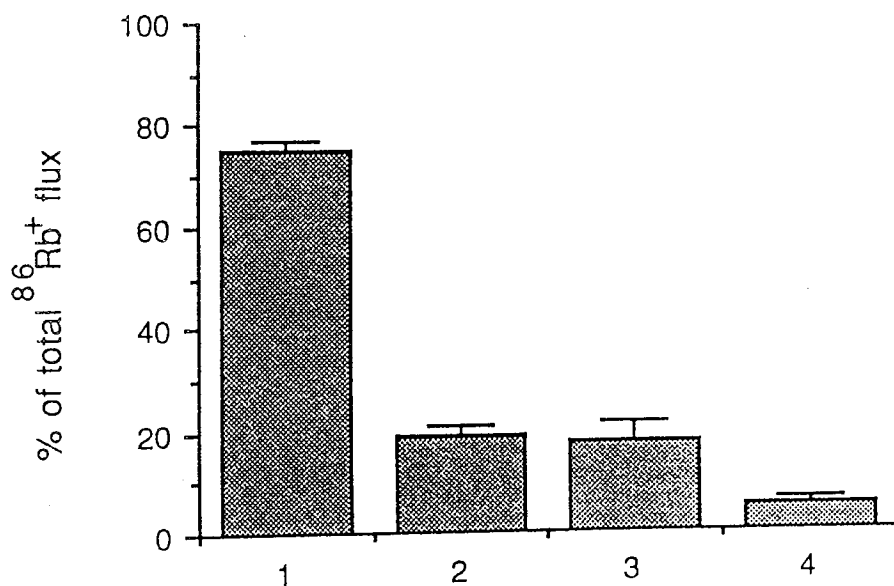
Figure 3 The effect of of potassium transport inhibitors on the ^{86}rubidium$^+$ flux of human erythrocytes incubated in a phosphate buffer. 1= total flux, 2= Na$^+$/K$^+$ ATPase pump flux, 3= Na$^+$-K$^+$-Cl$^-$cotransport flux, 4= passive flux. (n=3, mean±S.D.).
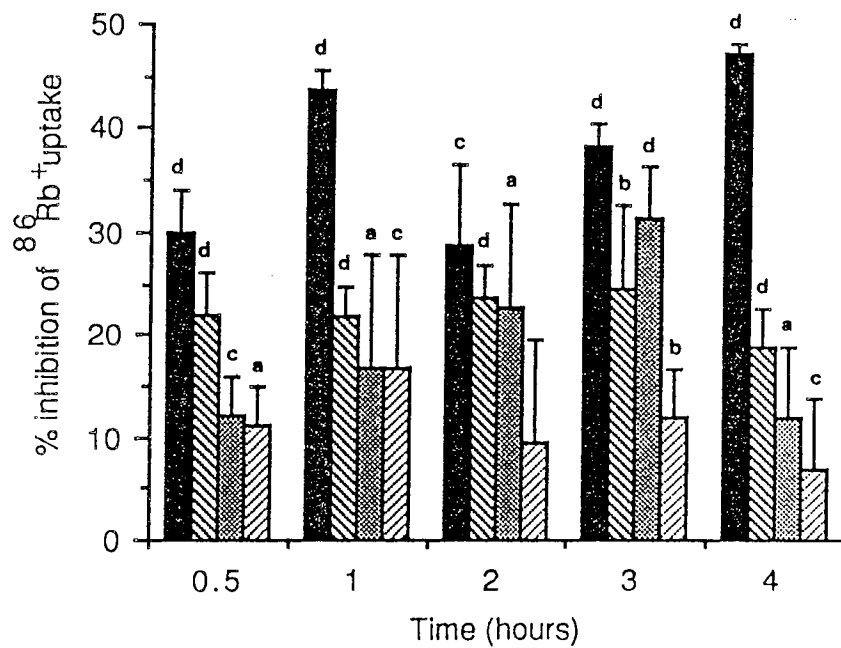
Figure 4. The percentage inhibition of ^{86}rubidium$^+$ uptake in human eryhtrocytes by 4 concentrations of HN2. ■ =2mM, ▨ =1mM, ▨ =0.5mM, ▨ =0.1mM HN2.(n=3, mean±S.D.). Significant difference from control value, a = p<0.05; b = p<0.01; c = p<0.005; d = p<0.001 (student t-test).

Time (hours)	control	2mM HN2	1mM HN2
0.5	0.056±0.040	0.075±0.040	0.015±0.005
3	0.020±0.002	0.020±0.008	0.027±0.019
4	0.018±0.006	0.025±0.014	0.024±0.010

Table 1.   The haemolysis of human erythrocytes incubated
with either 2 or 1mM HN2 for up to 4 hours. Haemolysis was
determined by measuring the absorbance of the supernatant
at 540nm.   (n=3, mean±S.D.).
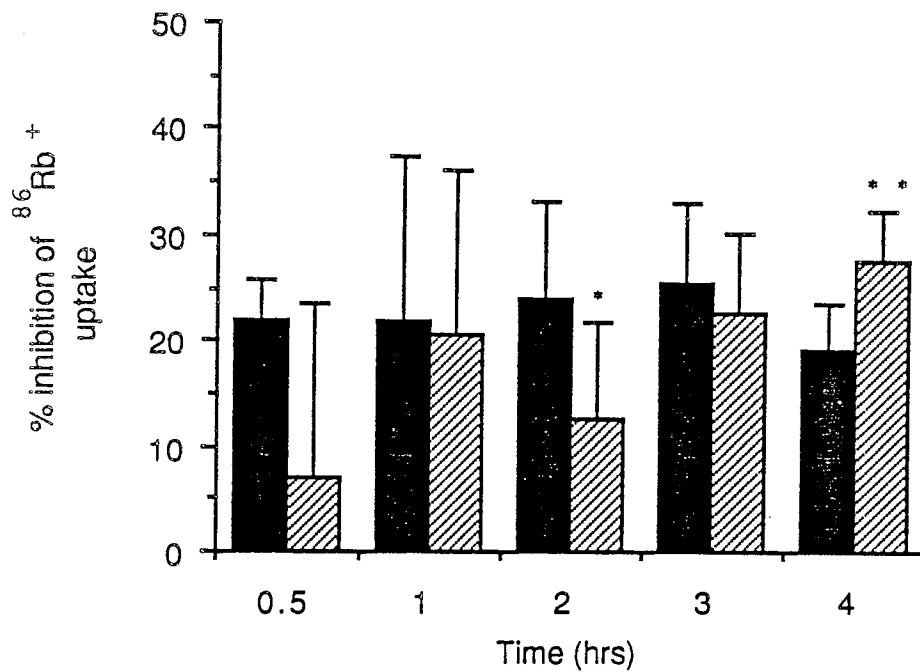
Figure 5. The effect of the density of human erythrocytes on the inhibition of ^{86}rubidium$^+$ uptake by 1mM HN2. ■ =50% haematocrit, ▨ =5% heamatocrit. (n=3,mean±S.D.). Significant difference between the two values, * = p<0.1, ** = p< 0.05 (student t-test).
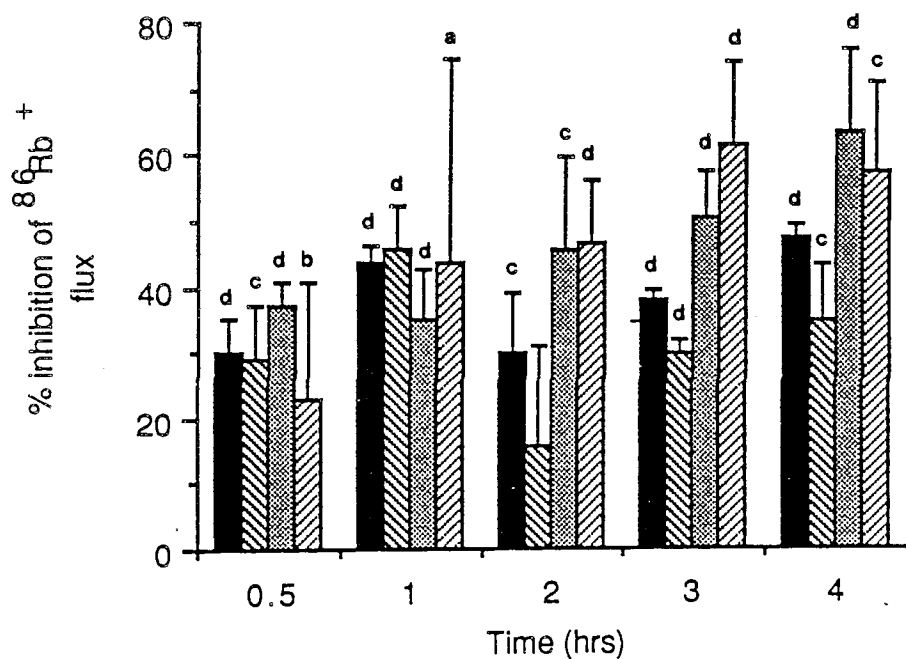
Figure 6. The inhibition of ^{86}rubidium$^+$ uptake by potassium transport systems in human erythrocytes by 2mM HN2. ■ = total flux, ▨ = Na$^+$/K$^+$ATPase pump flux, ▨ = Na$^+$-K$^+$-Cl$^-$ cotransport flux, ▨ = passive flux. (n=3, mean±S.D.). Significant difference from control values, a = $p<0.05$, b = $p<0.01$, c = $p<0.005$, d = $p<0.001$ (student t-test).

3.2.2. The nature of potassium transport inhibition by 1mM nitrogen mustard in the murine leukaemic L1210 cell line.

Introduction.

Potassium transport was inhibited by ¦8% immediately after the addition of 1mM HN2 in the murine leukaemic L1210 cell line (Wilcock 1987; Wilcock and Hickman 1988). However, no further experiments were performed in order to determine if any single potassium ion transporter was responsible for this inhibition or whether it was totally non-selective in comparision to the inhibition observed at 10uM. The high concentration of HN2 and the rapid onset of this interference in ion transport would indicate that this phenomenon was a consequence of the direct alkylation of ion transport proteins by HN2. It was of interest to determine if inhibition of a single ion transport system was responsible for this observation so that the alkylation sensitivities of the two major potassium transport systems (Na^+/K^+ ATPase pump and the $Na^+-K^+-Cl^-$ cotransporter) could be compared in L1210 cells.

Results.

The effect of a 10 minute incubation of L1210 cells with 1mM HN2 in RPMI 1640 is displayed in Figure 7. The fractional uptake of ^{86}rubidium$^+$ was depressed by 27% (p<0.01).The ouabain-sensitive flux was inhibited by 26% (p<0.05) and the bumetanide-sensitive by 28% (p<0.05). Passive uptake of ^{86}rubidium$^+$ into L1210 cells was reduced by 43% (p<0.05).

Discussion.

Treatment of L1210 cells with 1mM HN2 for 10 minutes resulted in a small inhibition of the initial rates of ^{86}rubidium$^+$ uptake which was due to the non-selective inhibition of both the Na$^+$/K$^+$ ATPase pump and the Na$^+$-K$^+$-Cl$^-$ cotransporter. However, millimolar HN2 is an extremely high concentration and may inactivate both the potassium transport systems such that any difference in the alkylation sensitivities between them may be obscured. An increase in the level of inhibition of the Na$^+$-K$^+$-Cl$^-$ cotransporter compared to that of the Na$^+$/K$^+$ ATPase pump was observed 2 hours after the exposure of human red blood cells to 2mM HN2 (results 3.2.1). Therefore, changes in potassium transport into L1210 cells should have been investigated at later times after the addition of 1mM HN2. However, Doppler et al (1985) reported that a 4 hour incubation with 100uM HN2

completely abolished ^{86}rubidium$^+$ transport into Ehrlich cells.

In contrast to the selective inhibition of Na$^+$-K$^+$-Cl$^-$ cotransport exerted by 10uM HN2, no inhibition of ^{86}rubidium$^+$ transport in L1210 cells treated with either 100uM of the monofunctional agent Me-HN1 or 10uM HN2 was observed (Wilcock 1987). The selective inactivation of Na$^+$-K$^+$-Cl$^-$ cotransport appeared to be a drug-specific phenomenon and may be a consequence of a direct interaction between the cotransporter and HN2. Me-HN1 is a monofunctional analog of HN2 and is unable to cross-link molecules. This was the reason given for its inability to inhibit ^{86}rubidium$^+$ uptake in L1210 cells (Wilcock 1987). However, 100uM Me-HN1 is not as toxic as 10uM HN2 (2 log and 4 log cell kill). Though a 3 hour incubation of L1210 cell with 10uM chlorambucil (an equitoxic concentration with 10uM HN2 as assayed by growth inhibition studies) did not inhibit ^{86}rubidium$^+$ uptake, the rate of immonium ion formation is much slower. Thus, any inhibition in potassium ion transport may be observed at later times after the addition of chlorambucil.

This result may suggest that the Na$^+$/K$^+$ ATPase pump and the Na$^+$-K$^+$-Cl$^-$ cotransporter are equally sensitive to alkylation by HN2. The inhibtion of potassium transport observed in L1210 cells after a 4 hour incubation with 10uM HN2 may therefore have a number of possible explanations.

It may be due to new gene expression or the release of proteins from internal organelles such as lysosomes. However, experiments with ennucleated human erythrocytes (section 3.2.1) demonstrated that Na^+-K^+-Cl^- cotransport was more sensitive to inhibition by HN2 than the Na^+/K^+ ATPase pump. This suggested that the inhibition of the Na^+-K^+-Cl^- cotransporter by HN2 was unlikely to be a consequence of new protein synthesis or release of proteins from internal organelles such as lysosomes. It should also be noted that only early times (10 minutes) after the addition of very high concentrations of HN2 (1mM) were investigated and caution must be exercised in the interpretation of these results. It is possible that, as in human erythrocytes (results 3.2.1), an increase in the inhibition of Na^+-K^+-Cl^- cotransport may be observed at later times after the addition of 1mM HN2 to L1210 cells.
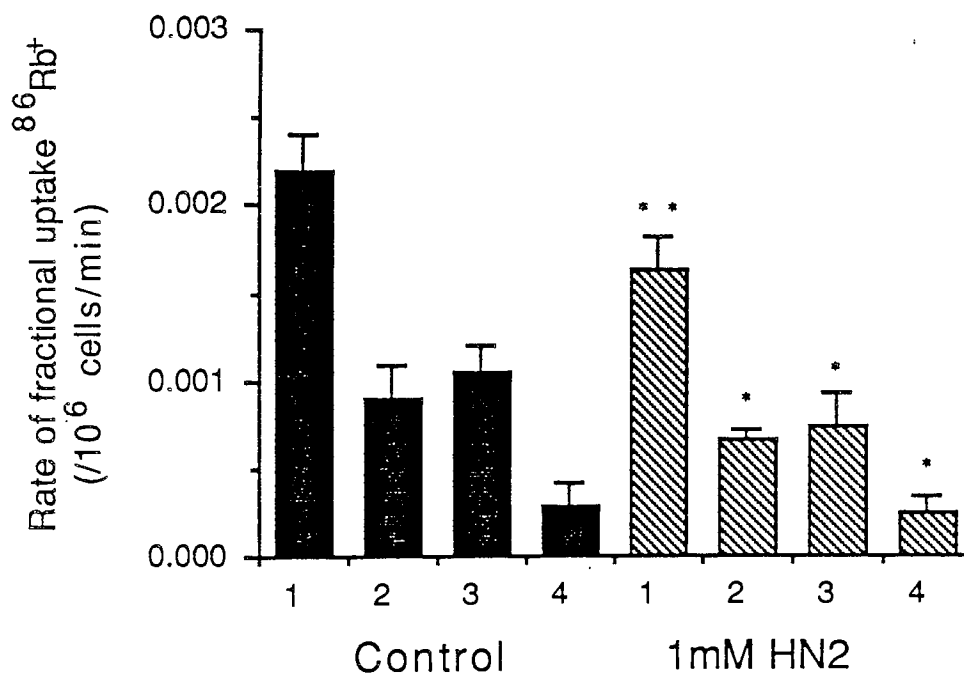
Figure 7. The inhibition of ^{86}rubidium$^+$ uptake by potassium transport systems in murine leu..aemic L1210 cells by 1mM HN2. 1 = total flux, 2 = Na$^+$/K$^+$ATPase pump flux, 3 = Na$^+$-K$^+$-Cl$^-$ cotransport flux, 4 = passive flux. (n=3, mean±S.D.). Significant difference from control values, * = p<0.05; ** = p<0.01 (student t-test).

3.3.1 <u>The</u> <u>effect</u> <u>of</u> <u>the</u> <u>imposition</u> <u>of</u> <u>Na$^+$-K$^+$-Cl$^-$ cotransport</u>
<u>inhibition</u> <u>on</u> <u>alkylating</u> <u>agent</u> <u>toxicity.</u>

Introduction.

The importance of the selective inhibition of Na$^+$-K$^+$-Cl$^-$ cotransport by HN2, reported by a number of investigators (Wilcock <u>et</u> <u>al</u> 1988; Doppler <u>et</u> <u>al</u> 1985; results 3.2.1), in the process of cell death induced by HN2 is unclear. Bumetanide, at concentrations which inhibited Na$^+$-K$^+$-Cl$^-$ cotransport activity, was not cytotoxic to L1210 cells (Wilcock <u>et</u> <u>al</u> 1988) or Ehrlich cells (Doppler <u>et</u> <u>al</u> 1985). Wilcock <u>et</u> <u>al</u> (1988) concluded that the selective inhibition of the cotransporter was a not the primary lesion of HN2 but may be of importance as a secondary but vital lesion in cells committed to death

Therefore, the inhibition of Na$^+$-K$^+$-Cl$^-$ cotransport by bumetanide and its effects on HN2 and Me-HN1 toxicity was investigated. This was in order to determine the importance of inhibition of cotransport activity in the process of cell death. In contrast to HN2, the survival curve of Me-HN1, a monofunctional analogue of HN2, exhibits a large shoulder region. This can be considered to be indicative of cellular damage that is insufficient to induce cell death. However, an alternative explanation is that the the alkylating agents are inactivated by reactive centers in the cell (e.g. glutathione SH groups; Alexander 1969). Furthermore, Me-HN1 had no effect on the activity of the cotransporter (Wilcock <u>et</u> <u>al</u> 1988). Thus, the effect of coincubation of

Me-HN1 treated L1210 cells with bumetanide was investigated to ascertain if the sub-lethal damage induced by Me-HN1 was made lethal by the concomitant inhibition of $Na^+-K^+-Cl^-$ cotransport.

The incubation of rat 9L brain tumour cells for twenty minutes in a X3.5 strength hypertonic solution was not cytotoxic. However, if this treatment was repeated after exposure to a potentially lethal dose of X-ray radiation cell survival was reduced (van Ankeren and Wheeler 1985). This phenomenon was due to an altered balance between the expression of DNA damage and its repair (van Ankeren and Wheeler 1985). Wilcock (1987) demonstrated that L1210 cells shrunk when incubated with bumetanide, an inhibitor of the $Na^+-K^+-Cl^-$ cotransporter. Incubation of Ehrlich ascites tumour cells with hypertonic media also resulted in a decrease in cell volume (Geck and Pfeiffer 1985). It was considered that bumetanide and hypertonicity could be analogous treatments and thus it was decided to investigate whether incubation of L1210 cells in hypertonic media could enhance the cytotoxicity of HN2.

The aim of these experiments is to investigate whether cytotoxicity of alkylating agents is enhanced by a concomitant inhibition of $Na^+-K^+-Cl^-$ cotransport. This would test the hypothesis that inhibition of $Na^+-K^+-Cl^-$ cotransport is a causative, but not independent lesion, which is responsible for cell death.

Results.

In Figure 8 the effect of incubation of 0.1mM bumetanide on HN2 toxicity is demonstrated. There is no significant difference between the toxicity curves of L1210 cells continously incubated over 72 hours with HN2 only and those incubated with 0.1mM bumetanide also.

In Figure 9 the effect of incubation of 0.1mM bumetanide on Me-HN1 toxicity is displayed. Continous incubation for 72 hours of L1210 cells with 0.1mM bumetanide did not cause any significant change in Me-HN1 toxicity.

The effect of incubations of either 1 hour or 20 minutes with various strenghts of hypertonic RPMI 1640 media on the survival of L1210 cells was investigated (Figure 10). A solution of X3.5 was found to be significantly toxic at both times investigated but lower strength solutions (X2 and X1.5) were not.

The effect of incubation in an hypertonic solution on HN2 toxicity was investigated (Figure 11). L1210 cells were treated with HN2 and incubated for 1 hour in an isoosmotic media. The cells were then washed twice and incubated in a X2 strength solution of RPMI 1640 for 1 hour after which they were returned to an isoosmotic RPMI 1640 media with 10% horse serum. After a 72 hour incubation at 37°C, cell numbers were determined and percentage growth inhibition calculated. Exposure of cells to a hypertonic media did not alter the cytotoxicity of HN2.

Discussion.

Treatment of L1210 cells with a concentration of bumetanide (0.1mM), which inhibited $Na^+-K^+-Cl^-$ cotransport, was not cytotoxic (Figures 8 and 9). Therefore, the inactivation of $Na^+-K^+-Cl^-$ cotransporter is in itself not a cytotoxic lesion and cannot explain the antitumour activity of HN2.

The toxicity of either HN2 or Me-HN1 was unaltered by the coincubation of L1210 cells with 0.1mM bumetanide (Figures 8 and 9). These results suggest that inhibition of the $Na^+-K^+-Cl^-$ cotransporter is not an important lesion in either HN2 or Me-HN1 induced cell death, which is in good agreement with Grunicke et al (1985a). However, Grunicke et al (1985a) investigated the effect of 200uM furosemide at only one concentration of HN2 (2uM) which completely inhibited the cotransporter.

No increase in cytotoxicity was observed when L1210 cells were incubated for 1 hour in a X2 hypertonic media after exposure to HN2 (Figure 11). This result is in contrast to the report of van Ankeren and Wheeler (1985) that incubation in a X3.5 hypertonic media enhanced the cytotoxicity of irradiated 9L/Ro rat brain tumour cells. It is possible that the difference in strength of the hypertonic media used in these experiments may account for the inability to reproduce the phenomenon reported by van Ankeren and Wheeler (1985). However, a twenty minute

incubation of L1210 cells with X3.5 RPMI 1640 media was significantly toxic.

HN2 has been classified as a radio-mimetic drug. One of the criteria for this classification is that the sensitivity of cells can be altered by treatments after HN2 exposure. In contrast to irradiated cells there was no increase in survival of lymphoma cells incubated for 24 hours at 30°C after HN2 treatment (Goldenberg and Alexander 1965). Alexander (1969) thought that that the term radiomimetic was unhelpful as these treatments exhibited a great diversity in their modes of cell death. This was further emphasised as hypertonicity was able to enhance cytotoxicity by irradiation but not, under the conditions described, after exposure to HN2.

L1210 cells shrunk when incubated with bumetanide (Wilcock 1987). This parameter was not investigated after exposure to hypertonic media but Ehrlich ascites shrunk and were unable to regain their cell volume in hypertonic media (Geck and Pfeiffer 1985). However, Geck and Pfeiffer (1985) did observe an increase in $Na^+-K^+-Cl^-$ cotransport activity of Ehrlich cells incubated in hypertonic media. Thus, whether the exposure of L1210 cells to either hypertonicity or bumetanide were analogous treatments required further investigation.

It has been proposed that the cytotoxic action of HN2 may be exerted through a variety of lesions (Wheeler 1967),

of which the inhibition of the $Na^+-K^+-Cl^-$ cotransporter may be one. In contrast, Sakomoto and Elkind (1969) suggested that HN2 acted through a "single hit" inactivation, citing in evidence the exponential nature of the survival curve. An alternative interpretation of such a curve may be that the cell kill of HN2 is also dependent on cell number (Alexander and Mikulski 1961). However, inhibition of $Na^+-K^+-Cl^-$ cotransport by bumetanide did not enhance the cytotoxicity of either HN2 or Me-HN1. Therefore, if HN2 cytotoxicity is a multi-modal process, then inhibition of the $Na^+-K^+-Cl^-$ cotransporter was not an essential lesion for cell death. The hypothesis that inhibition of $Na^+-K^+-Cl^-$ cotransport was an important secondary lesion in cell death induced by HN2 or Me-HN1 did not appear to be substantiated by these results.
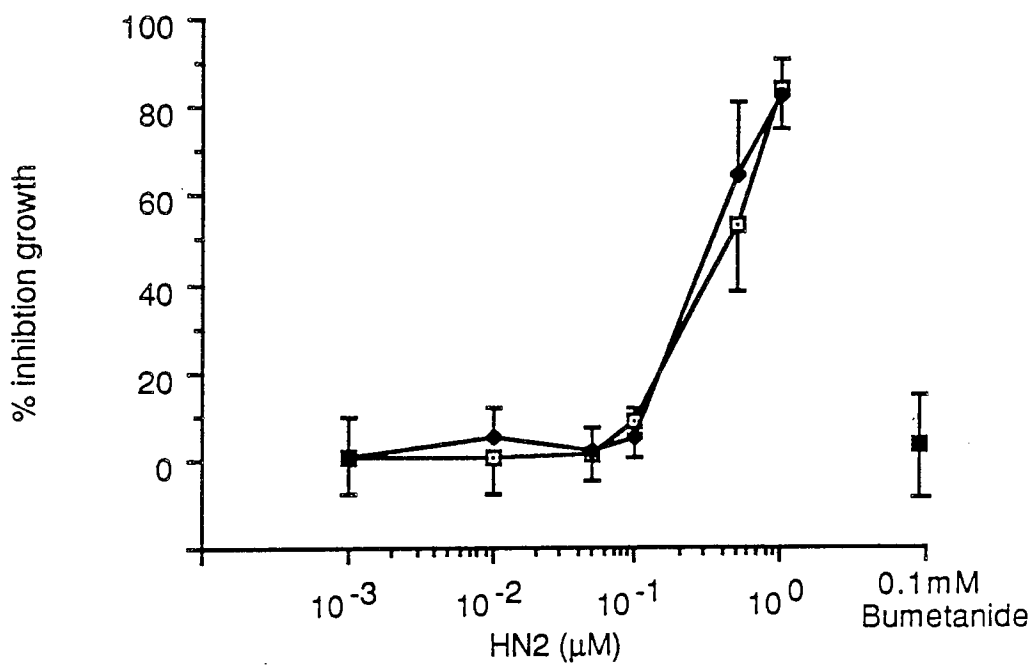
Figure 8. The inhibition of growth of HN2 treated L1210 cells incubated for 72 hours with or without 0.1mM bumetanide. ▫ = HN2 only, ◆ = HN2 + 0.1mM bumetanide, ■ = 0.1mM bumetanide. (n=3, mean±S.D.).
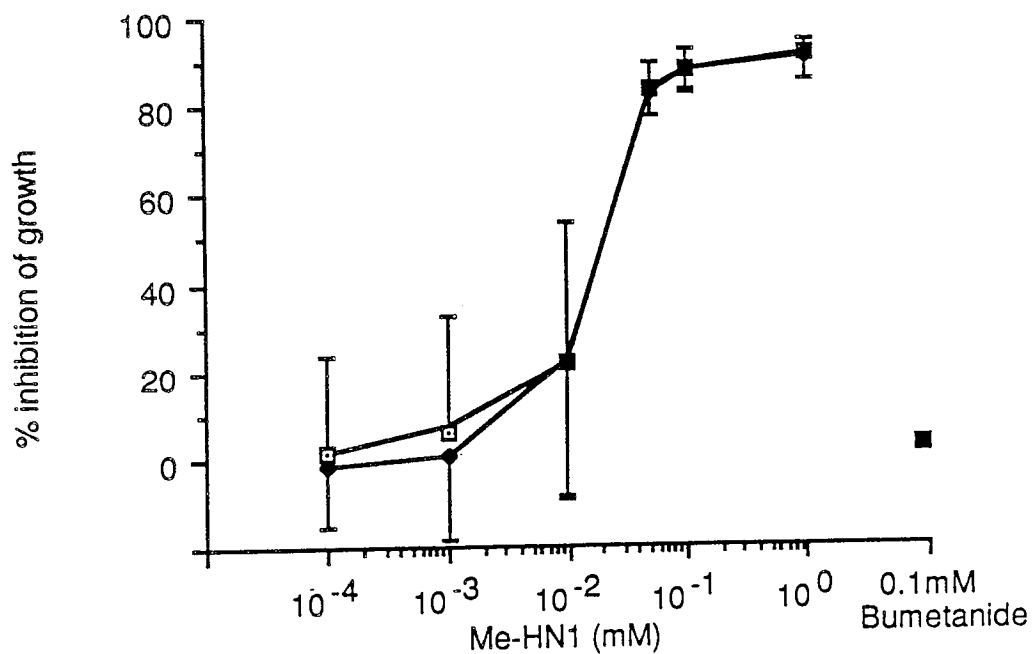
Figure 9. The inhibition of growth of Me-HN1 (the monofunctional analog of HN2) treated cells incubated for 72 hours with or without 0.1mM bumetanide. ● = Me-HN1 only, ◻ = Me-HN1 and 0.1mM bumetanide, ■ = 0.1mM bumetanide. (n=3, mean±S.D.).
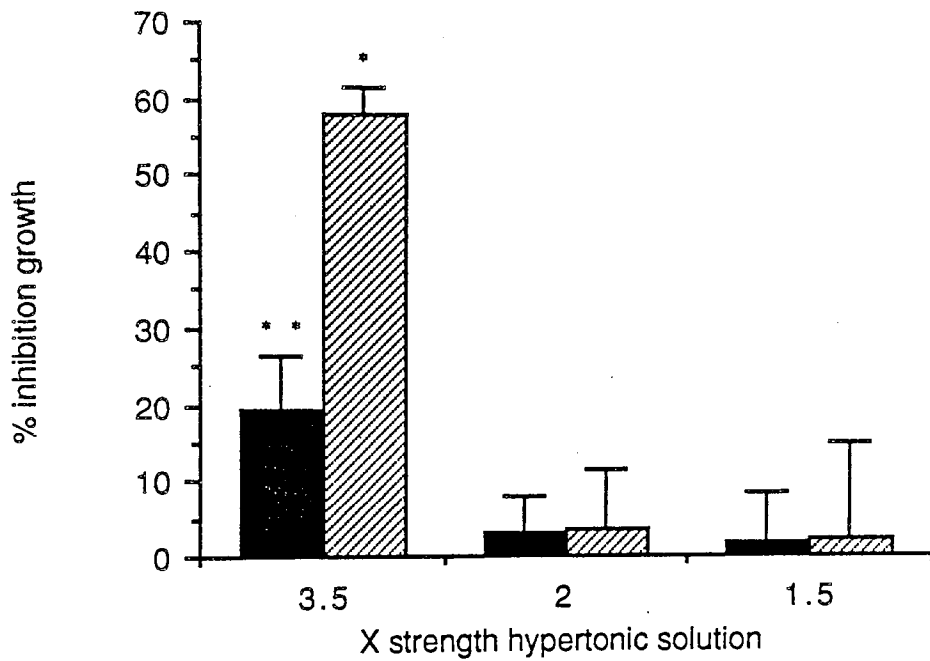
Figure 10. The inhibtion of cell growth of murine leukaemic L1210 cells incubated for either 20 or 60 minutes with va.ious strengths of RPMI 1640 media. ■ = 20 minutes, ▨ = 60 minutes. (n=3,mean±S.D.). Significant difference from control values, ** = p<0.005; * = p<0.001 (student t-test).
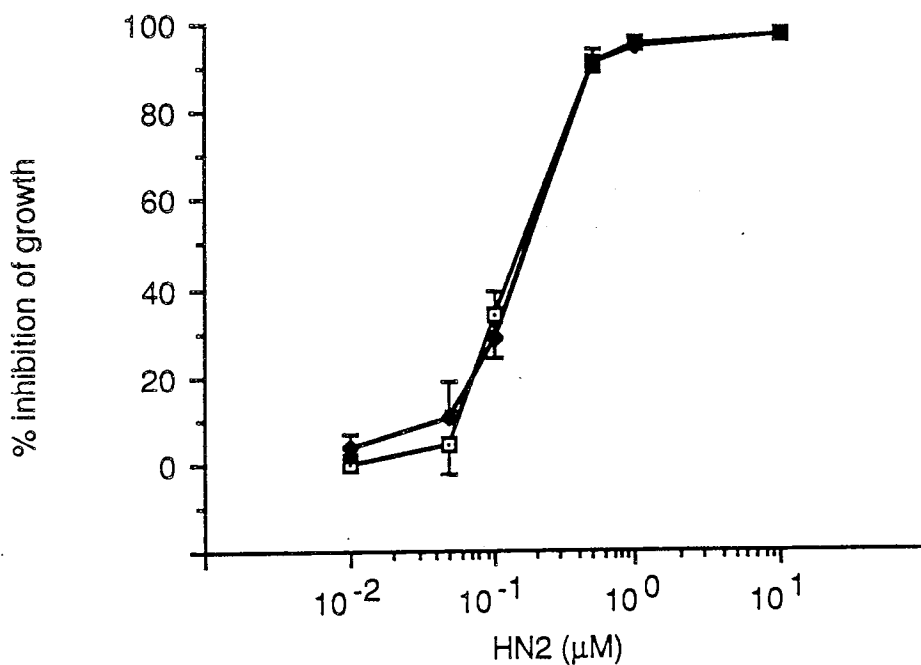
Figure 11. The effect of hypertonic RPMI 1640 media on the cytotoxicity of HN2 in murine leukaemic L1210 cells. L1210 cells were incubated for 60 minutes with HN2 in isotonic RPMI 1640 media, washed twice and incubated for 60 minutes in a X2 strength hypertonic RPMI 1640 media. L1210 cells were then resuspended in normal RPMI 1640 media with 10% horse serum and inhibtition of growth measured after 72 hours. -□- = HN2 only, -◆- = HN2 and hypertonic media. (n=3, mean±S.D.).

3.3.2 The toxicity of nitrogen mustard in a furosemide-resistant L1210 cell line.

Introduction.

A furosemide-resistant cell line was prepared that was able to grow in concentrations of furosemide (5mM) which were completely growth inhibitory to parental L1210 cells. It was considered that under these conditions the Na^+-K^+-Cl^- cotransporter might be completely inactive. The sensitivity of the furosemide-resistant cell line to HN2 was compared to the normal L1210 cell line. I wished to investigate the importance of the Na^+-K^+-Cl^- cotransporter as a site of cytotoxic interaction of HN2 and whether resistance to the toxic effects of furosemide in L1210FR cells was coincident with the expression of HN2 resistance.

Results.

In figure 12 the growth curves of L1210 and L1210FR in the absence or presence of 5mM furosemide are displayed. The doubling time of L1210 cells is approximately 12 hours. The doubling time of L1210FR cells grown without 5mM furosemide is approximately 15 hours, and in the presence of 5mM furosemide it is approximately 18 hours.

In figure 13 the inhibition of growth of both L1210 and L1210FR cells, after a 72 continous incubation, is compared. The IC_{50} of furosemide of L1210 cells is approximately 2mM, but in L1210FR it is 6mM. L1210 cells are unable to grow in

5mM or greater concentrations of furosemide. L1210FR cells are able to grow in concentrations of furosemide which are not permissive for L1210 cells.

The cytotoxicity of HN2 was compared for L1210 and L1210FR cells grown in either the presence or absence of 5mM furosemide (Table 2). The IC_{50} of HN2 for the three conditions was not significantly different.

Discussion.

The L1210FR cell line was able to grow in 5mM concentrations of furosemide with a doubling time of approximately 18 hours. At this concentration of furosemide the parental L1210 cell line was unable to proliferate. This would indicate that the $Na^+-K^+-Cl^-$ cotransporter had either mutated so that it was no longer pharmacologically sensitive to furosemide or it was functionally inactive in the L1210FR cell line.

HN2 was no more cytotoxic to the furosemide-resistant cell line than to normal L1210 cells. However, no further experiments were performed in order to determine the type of the resistance of the $Na^+-K^+-Cl^-$ cotransporter in the L1210FR cell line and it is possible that this was a purely pharmacological change rather than a functional one. Wilcock (1987) defined the absence of $Na^+-K^+-Cl^-$ cotransport in the HN2-resistant L1210 cell line only on the basis of the lack of an inhibitory effect of furosemide on

^{86}rubidium$^+$ uptake. Grunicke et al (1985b) simply noted that there was no inhibition of ^{86}rubidium$^+$ uptake in a HN2-resistant cell line and made no attempt to define the status of the Na$^+$-K$^+$-Cl$^-$ cotransporter, defined pharmacologically in wild-type cells, as a pharmacological or functional mutant.

L1210FR cells expressed a longer doubling time when grown both in the presence or absence of furosemide. van Putten and Lelieveld (1971) noted that HN2 was more cytotoxic to rapidly proliferating cells than quiescent cells. Thus, one may consider that HN2 should have been less cytotoxic to the slower growing L1210FR cell line. However, the small difference in the doubling times between L1210 and L1210FR cells may not have been sufficient to result in a difference in the toxicity of HN2.

Wilcock et al (1988) suggested that the cotransporter was not necessarily the primary target for HN2, but that its inhibition might act as a secondary but vital lesion in a cell which had accumulated other damage. Clearly, the loss of its activity, defined in terms of its pharmacological sensitivity, did not modulate HN2 toxicity. However, this result unfortunately remains equivocal in the absence of an analysis of the reason for the loss in sensitivity to the loop diuretic furosemide.
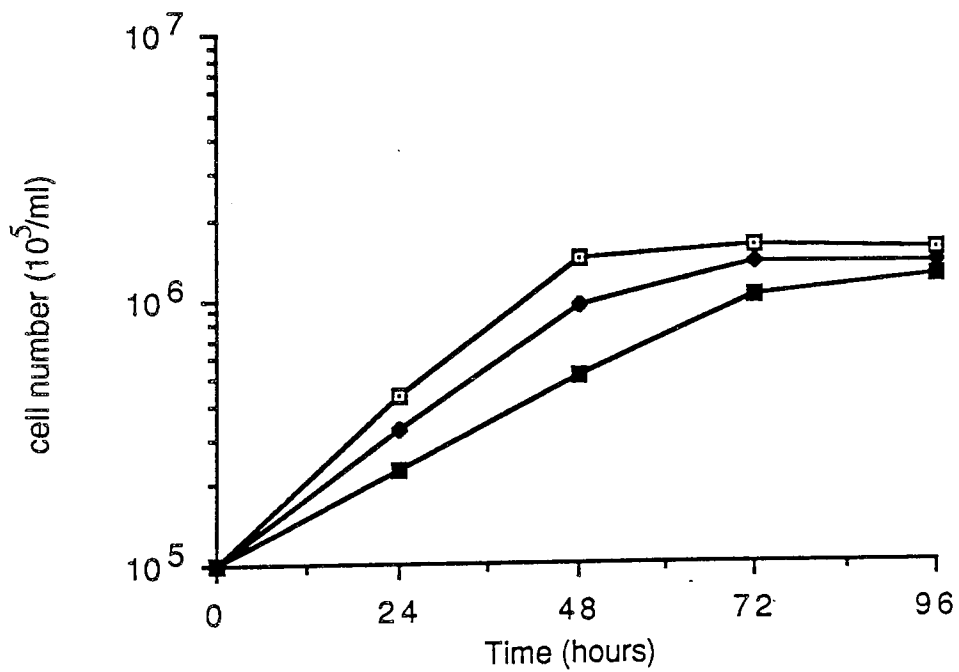
Figure 12. A comparision of the growth curves of L1210 and L1210FR cells. ⊡ = L1210 cells, ◆ = L1210FR cells, = ▪ L1210FR cells grown in 5mM furosemide.
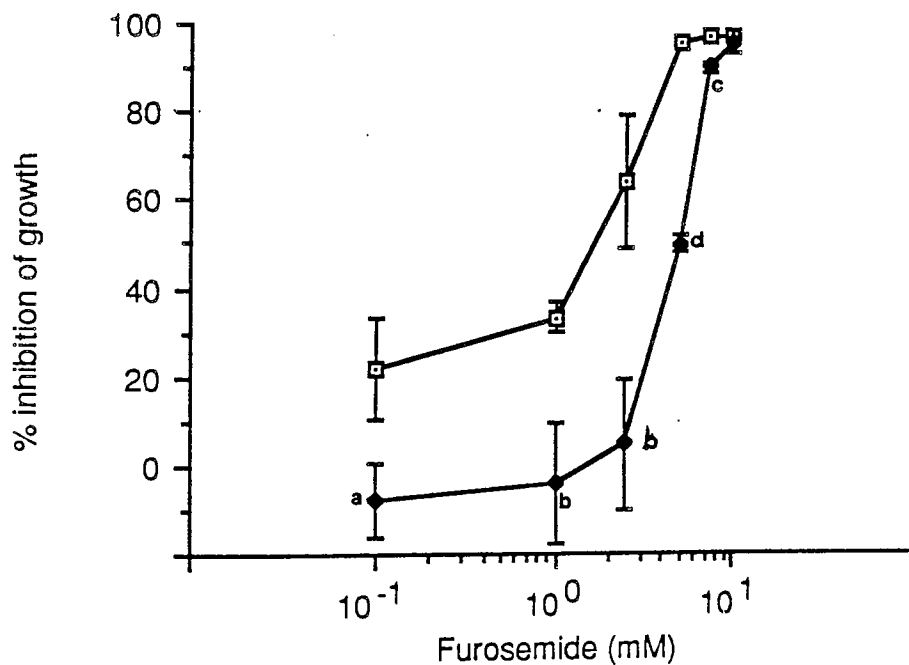
Figure 13. A comparision of the cytotoxicity, determined by the inhibition of growth, of furosemide to either L1210 (-□-) or L1210FR (♦) cells. (n=3, mean±S.D.). Significant difference betwen 1210 and L1210Fr values, a = p<0.05, b = p<0.01, c = p<0.005, d = p<0.001 (student t-test).

Cell line	L1210	L1210FR	L1210FR
Furosemide	----	----	5mM
HN2 (μM)	% Inhibition of growth		
Control	0±0	33±12	71±6
10	99±0.5	99±0.7	99±1
1	98±0.6	99±0.7	98±2
0.5	98±0.6	98.5±1	95±1.3
0.1	69±1	71±11	59±12
0.05	28±4	39±17	35±6
0.01	6±13	2±20	18±21

Table 2. A comparision of HN2 toxicity in the L1210 and furosemide-resistant L1210FR cell lines. L1210FR cells were grown either with or without 5mM furosemide. Toxicity was determined by inhibition of growth studies. (n=3, mean±S.D.).

3.4.1 ^{86}Rubidium$^+$ influx in L1210 cells treated with nitrogen mustard over 24 hours.

Introduction.

Na$^+$-K$^+$-Cl$^-$ cotransport in Ehrlich ascites was inhibited 4 hours after exposure to HN2. This lesion was concomitant with inhibition of cell growth by HN2 (Grunicke et al 1985a). In L1210 cells Na$^+$-K$^+$-Cl$^-$ cotransport was selectively inhibited 4 hours after exposure to 10uM HN2. However, no inhibition of ^{86}rubidium$^+$ influx was observed 4 hours after exposure to 1 and 0.5uM HN2 (Wilcock et al 1988). Though all three concentrations of HN2 inhibited cell growth by >90%, they were not equitoxic as determined by clonogenic assay (results 3.1.1). However, Wilcock et al (1988) did not investigate if ^{86}rubidium$^+$ uptake was inhibited at later times after exposure to lower concentrations of HN2. I wished to investigate whether the lesion in potassium ion transport was not only a function of HN2 concentration but also time, after exposure to HN2.

Results.

4 hours after treatment of L1210 cells with 10uM HN2 ^{86}rubidium$^+$ influx into cells was inhibited by 41±5% (p<0.001; Figure 14). ^{86}Rubidium$^+$ influx into L1210 cells was similiarly inhibited at 12 and 24 hours after exposure to 10uM HN2. Treatment of L1210 cells in RPMI 1640 for 4 hours with either 1 or 0.5uM HN2 did not inhibit ^{86}rubidium$^+$

influx. ^{86}Rubidium$^+$ influx was unaffected by 1uM HN2. Slight increases in ^{86}rubidium$^+$ influx were observed at 12 (121±15%, p<0.05) and 24 (119±11%, p<0.05) hours after exposure to 0.5uM HN2. However, at the other time points investigated there were no significant changes in ^{86}rubidium$^+$ influx into L1210 cells.

Discussion.

The inhibition of ^{86}rubidium$^+$ uptake into L1210 cells 4 hours after treatment with 10uM HN2 was demonstrated to be as a consequence of the selective inhibition of the $Na^+-K^+-Cl^-$ cotransporter (section 3.1.2). As ^{86}rubidium$^+$ influx was inhibited up to 24 hours after exposure to 10uM it was reasonable to assume that the inhibition of $Na^+-K^+-Cl^-$ cotransport was maintained, though this was not investigated further.

Grunicke et al (1985a) observed a recovery in furosemide-sensitive ^{86}rubidium$^+$ uptake 12 hours after exposure of Ehrlich ascites to 2uM HN2 but this was followed by a gradual decline due to an increase in dead cells. This suggests that some repair mechanism of damage to ion transport systems exists in Ehrlich but not L1210 cells.

Wilcock (1987) reported that, in contrast to 10uM HN2, a 4 hour incubation of L1210 cells with lower concentrations of HN2 (1 and 0.5uM) did not alter ^{86}rubidium$^+$ uptake. No significant inhibition of ^{86}rubidium$^+$ uptake was observed in

L1210 cells up to 24 hours after exposure to either 1 or 0.5uM HN2 (Figure 14). Thus, the inhibition of potassium ion transport was dependent only on the concentration of HN2 employed and not time after exposure.

The mode of cell death induced by the lower concentrations of HN2 may be different from that of 10uM HN2. The inhibition in potassium transport, in L1210 cells incubated with 10uM HN2, was concomitant with a decrease in cell volume (Wilcock et al 1988) which has been considered to be characteristic of apoptosis (Wyllie 1981); mitotic or some other sort of cell death may be induced in L1210 cells treated with the lower concentrations of HN2 and this may be characterised by no change in ^{86}rubidium$^+$ uptake.

The determination of ^{86}rubidium$^+$ fractional uptake produced a mean value for the influx of 10^6 cells. This method is unable to distinguish between different populations of cells. So some cells may have a severly reduced uptake of ^{86}rubidium$^+$ whilst others may express an enhanced activity. Thus, it is possible that two populations of cells exist after treatment with either 1 or 0.5uM HN2. This is further corroborated by the observation that a dying population of cells may exhibit various forms of cell death (Allen 1987).
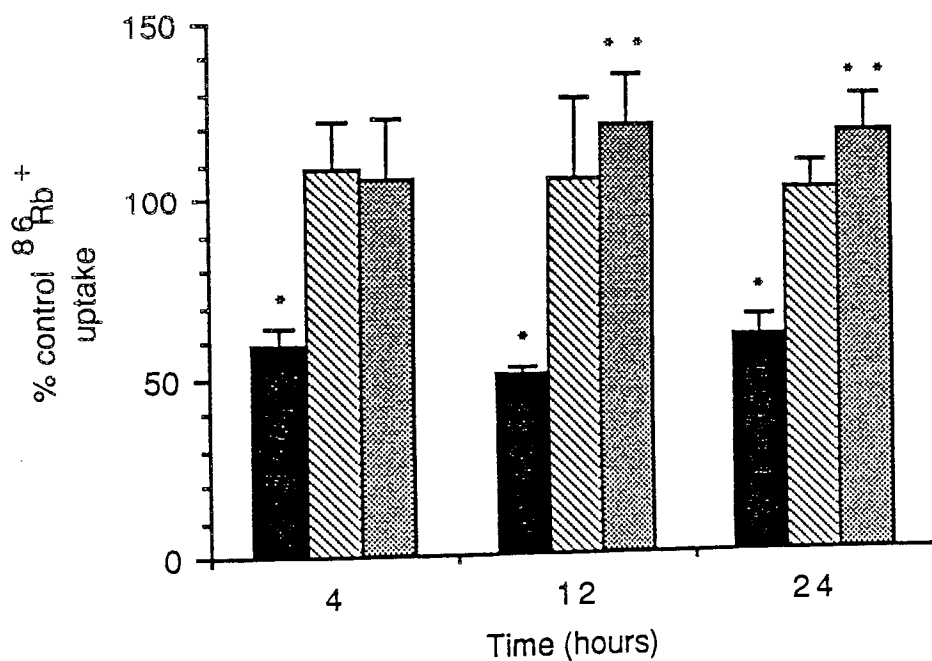
Figure 14. The change in ^{86}rubidium^{+} uptake in murine leukaemic L1210 cells 24 hours after treatment with various concentrations of HN2. ■ = 10μM HN2, ◩ = 1μM HN2, ▨ =0.5μM HN2. (n=3, mean±S.D.). Significantly different from control values, ** = p<0.05; * = p<0.001 (student t-test).

3.4.2. Volume changes of L1210 cells after treatment with nitrogen mustard over 72 hours.

Introduction.

The plasma membrane is permeable to water. Thus, volume is determined by the osmolarity of the cell, the external milieu and the consequent flow of water. The internal potassium concentration of cells is high (120-140mM) compared to sodium ions (20mM) and changes in the ratio of these ions can result in alterations in cell volume. The most familiar theory of cell volume regulation is the pump-leak hypothesis (Tosteson and Hoffman 1960). This states that an osmotic balance is sustained by the Na^+/K^+ATPase pump in opposition to the passive leak of ions from the cell down a concentration gradient.

Geck et al (1980) proposed that cell volume is also maintained and regulated by the antagonistic action of the Na^+/K^+ATPase pump and $Na^+-K^+-Cl^-$ cotransporter. Any interference in the transport of potassium ions across the plasma membrane may result in cell volume changes. Inhibition of Na^+/K^+ATPase pump activity by ouabain will result in an increase in cell volume whilst the opposite is true if $Na^+-K^+-Cl^-$ cotransport activity is depressed by diuretic drugs (Geck et al 1980).

Changes in cell volume are often observed in cell death and can be used to distinguish apoptosis from necrosis (Wyllie 1981). Apoptosis is characterised by a decrease in

cell volume and necrosis and secondary necrosis by an increase (Introduction 1.6.2.). Thus, an investigation of the steady-state volume of L1210 cells may indicate the type of cell death induced by the three concentrations of HN2.

Results.

No significant volume changes, as measured by Coulter counter, were observed for 24 hours after treatment with 10uM HN2 (Figure 15B). Volume was reduced by 20% (from 908±45 to 751±16 flitres, p<0.001) at 36 hours and remained so during the time course investigated. Treatment of L1210 cells with 1 and 0.5uM HN2 resulted in an increase in volume by 12 hours which is in contrast to 10uM HN2. This increase in cell volume reached a maximum at 48 hours for cells treated with 1uM (3214±236 flitres, p<0.001) and 0.5uM HN2 (2958±166 flitres, p<0.001). After 48 hours there was a steady reduction in the cell volume measured so that by 72 hours cells treated with 1uM HN2 were at 1970±404 flitres and with 0.5uM HN2 were at 1644±372 flitres.

The volume of L1210 cells was also determined by measurement of the equilibration of the radio-isotopes of $[^3H]H_2O$ and $[^{14}C]$-inulin carboxylic acid (Figure 15A). A significant decrease in cell volume was observed 4 hours after incubation with 10uM HN2 (77±4% of control volume, p<0.001). Cell volume was maintained at this level until 48

hours when a drastic reduction was noted (31±7.5%). Cell volume did not alter significantly until 48 hours after treatment with 1uM HN2 when an 18% reduction from control values was observed (82±2%, p<0.001). No significant alteration in cell volume was noted when L1210 cells were treated with 0.5uM HN2.

Discussion.

Incubation of L1210 cells with 10uM HN2 resulted in a decrease in volume within 4 hours, assayed by equilibration of radio-isotopes, but no significant reduction was observed until 36 hours when measured with a Coulter Counter. The cell volume of L1210 cells after exposure to 1 and 0.5uM HN2, assayed by radio-isotope equilibration, showed little change. In contrast, cell volume, measured by the Coulter Counter, showed significant increases within 12 hours. Wilcock (1987) also noted that changes in cell volume after incubation with 10uM HN2 were not detected with a Coulter Counter. The discrepancies observed in the estimation of cell volume may be a consequence of the different methods used to estimate cell volume. The distribution of $[^3H]-H_2O$ and $[^{14}C]$-inulin carboxylic acid, markers of total water and extracellular space, produced mean values for the volume of 10^6 cells. In contrast, only particles that were above the lower threshold were registered by the Coulter Counter.

The reduction of L1210 cell volume, assayed by equilibration of radio-isotopes, 4 hours after exposure to 10uM HN2 was concomitant with an inhibition of ^{86}rubidium$^+$ uptake (results 3.4.1) which was due to the selective inactivation of the $Na^+-K^+-Cl^-$ cotransporter. These results are in good agreement with those presented by Wilcock et al (1988) who also reported that the selective inhibition of $Na^+-K^+-Cl^-$ cotransporter by loop diuretics resulted in a similiar reduction in cell volume.

In the early stages of apoptosis a decrease in cell volume is observed (Wyllie 1981), but the process by which this occurs is unknown (Lockshin and Beaulaton 1981). It is possible that the lesion in $Na^+-K^+-Cl^-$ cotransport observed with 10uM HN2 and the consequent reduction in cell volume is an early marker of apoptotic cell death. The lesion in potassium ion transport occurred at a time when there were no observable changes in internal calcium and pH, ATP levels or transmembrane potential (Wilcock et al 1988; results 3.4.4.-3.4.5.).

An increase in the volume of L1210 cells, measured with a Coulter counter, was observed when treated with either 1 or 0.5uM HN2. The two concentrations of HN2 may have induced mitotic cell death in L1210 cells as ATP and calcium levels were maintained until well after the increase in cell volume (results 3.4.4 and 3.4.5). This result is in good agreement with other investigators who have shown an

increase in cell volume, assayed by Coulter counter, after exposure to HN2 (Doppler et al 1985; Alexander 1969). Doppler et al (1985) proposed that the the increase in cell volume may be due to a G_2/M block in the cell cycle. In a later section (results 3.4.3) it is reported that treatment of L1210 cells with 1 and 0.5uM HN2 resulted in a G_2/M block in the cell cycle but no specific block was observed for cells treated with 10uM HN2. Thus, the increase in cell volume observed after exposure to 1 and 0.5uM HN2 may have been due to sterilization whilst 10uM HN2 may have induced an alternative mode of death, possibly apoptosis, in L1210 cells.

However, the volumes of L1210 cells treated with 1 and 0.5uM HN2 showed no significant increase when assayed by radio-isotope equilibration in contrast to cell volumes measured by a Coulter Counter. All three types of cell death (apoptosis, necrosis and sterilization) may be observed in a population of dying cells and this situation may have occurred in L1210 cells treated with 1 and 0.5uM HN2. Therefore, the increase in cell volume of one population of dying cells may have been measured by a Coulter Counter. In contrast, radio-isotope equilibration is a technique in which an average value of the sample tested is obtained and this may be a mean of two populations of dying cells. In an attempt to determine the volume of individual populations of cells, forward light scatter (FLS)

of L1210 cells in a flow cytometer was used to estimate cell size. However, the results obtained were inconclusive.
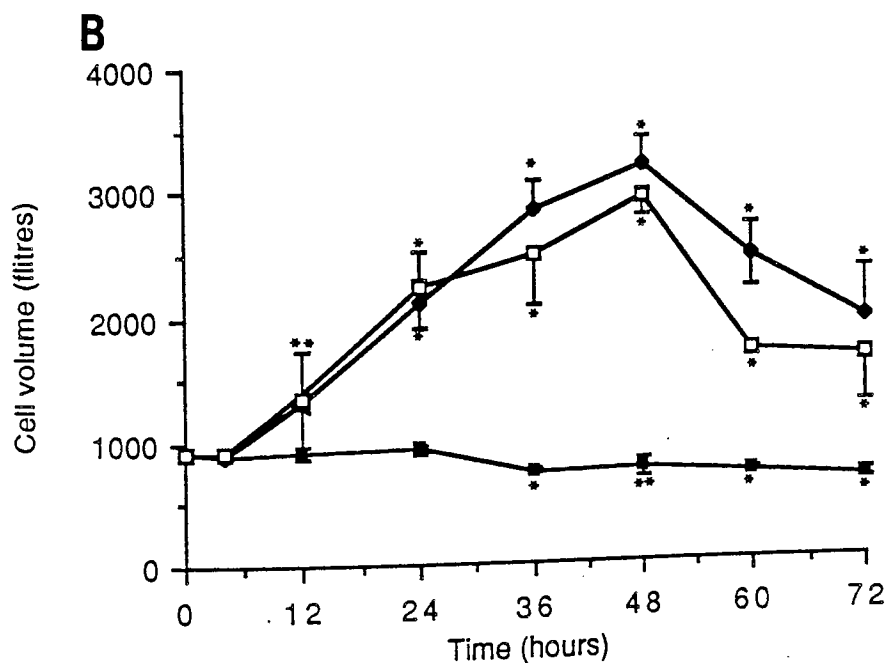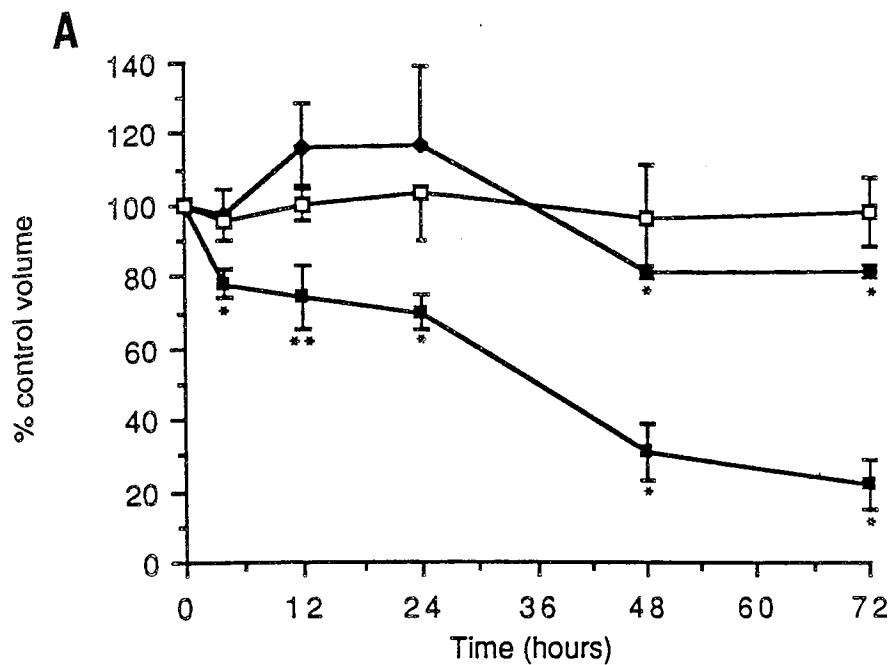
Figure 15. The change in the volume of murine leukaemic L1210 cells over 72 hours after treatment with HN2. Cell volume was determined in A) by radio-isotope equilibration and in B) by Coulter Counter. ■ = 10μM HN2, ◆ = 1μM HN2, □ = 0.5μM HN2. (n=3, mean±S.D.). Significantly different from control values, ** = $p < 0.005$; * = $p < 0.001$ (student t-test).

3.4.3. <u>Cell cycle analysis of L1210 cells after nitrogen mustard treatment.</u>

Introduction.

Na^+-K^+-Cl^- cotransport was selectively inhibited 4 hours after exposure to 10uM HN2 but not after 1 and 0.5uM (results 3.4.1.). No significant inhibition of [86]rubidium[+] influx was observed in L1210 cells up to 24 hours after exposure to either 1 or 0.5uM HN2. It was considered that the type of cell death induced in L1210 cells by 10uM HN2 may be different from that observed at lower concentrations (i.e. 1 and 0.5uM HN2) and that this may account for the lack of inhibition of [86]rubidium[+] influx. Therefore, analysis of the cell cycle, by flow cytometry, of L1210 cells exposed to HN2 may reveal differences between the different concentrations of HN2 employed and the points in the cell cycle at which cells were inhibited (introduction 1.2.2).

Results.

DNA histograms of untreated L1210 cells exhibited clear peaks and very little debris was observed. Cells treated with 10uM HN2 appeared to become inhibited in whichever stage of the cell cycle they were in (Table 3). However, L1210 cells treated with either 1 or 0.5uM HN2 exhibited a G_2/M block. The number of L1210 cells in G_1 dropped from 33% to 21% when exposed to 1uM HN2 for 4 hours. However, by

12 hours nearly all cells were in either S or G_2/M. This was maintained up to 24 hours. A similiar pattern was observed for L1210 cells treated with 0.5uM HN2.

Discussion.

The DNA histograms of L1210 cells exposed to HN2 did not exhibit clear G_2/M peaks so that I was unable to calculate the percentage of cells in the S phase of the cell cycle. Thus, the results were presented in the form of percentage of cells in either G_1 or S and G_2/M. The method used to prepare and fix the samples may have contributed to the poor quality of the DNA histograms. Alternatively, the treatment of L1210 cells with HN2 may have resulted in changes which would make the analysis of the cell cycle difficult. Extensive DNA damage is typical of HN2 treatment and investigators have noted the appearance of multinuclear cells and nuclear fragmentation (Cobb 1960). However, samples of untreated L1210 cells exhibited good DNA histograms; 33% of cells were in G_1 of the cell cycle which is a similiar percentage reported by McGowan et al (1984).

Some cautious and qualitative conclusions may be drawn from these results. The treatment of L1210 cells with 10uM HN2 resulted in the blockade of cells in whichever phase of the cell cycle they were in. In contrast, 12 hours after treatment with lower concentrations of HN2 (1 and 0.5uM) a clear G_2/M block was observed. Brox et al (1980) reported

that treatment of RPMI 6410 human lymphoblastoid cells with melphalan resulted in a G_2/M block. However, an increase in the cytotoxicity of melphalan by a longer exposure of the drug resulted in a S phase block.

Thus, it is possible that the inhibition of $Na^+-K^+-Cl^-$ cotransport by 10uM HN2 is a consequence of a different mode of cell death than that induced by lower concentrations of HN2. Mitotic cell death was defined by Hendry and Scott (1987) as when the cell was unable to divide due to excessive nuclear damage though there may be no overt immediate morphological or functional changes. It is possible that mitotic cell death may be induced by the lower concentrations of HN2 whereas at 10uM there may be some other mechanism. The pattern of cell death induced by the three concentrations of HN2 was investigated further in the following sections.

		Cell	Cycle
Treatment	Time (hrs)	G_1	$S+G_2/M$
Control	————	33.5	66.5
10μM HN2	4	41.0	59.0
	12	36.0	64.0
	24	48.0	52.0
1μM HN2	4	27.0	73.0
	12	7.0	93.0
	24	2.0	98.0
0.5μM HN2	4	23.0	78.0
	12	2.0	98.0
	24	2.0	98.0

Table 3. Cell cycle analysis of L1210 cells exposed to 3 concentrations of HN2 (10, 1 and 0.5μM) at 4, 12 and 24 hours. Values are mean of 2 independent experiments (n=2).

CELL NUMBER

RELATIVE DNA CONTENT
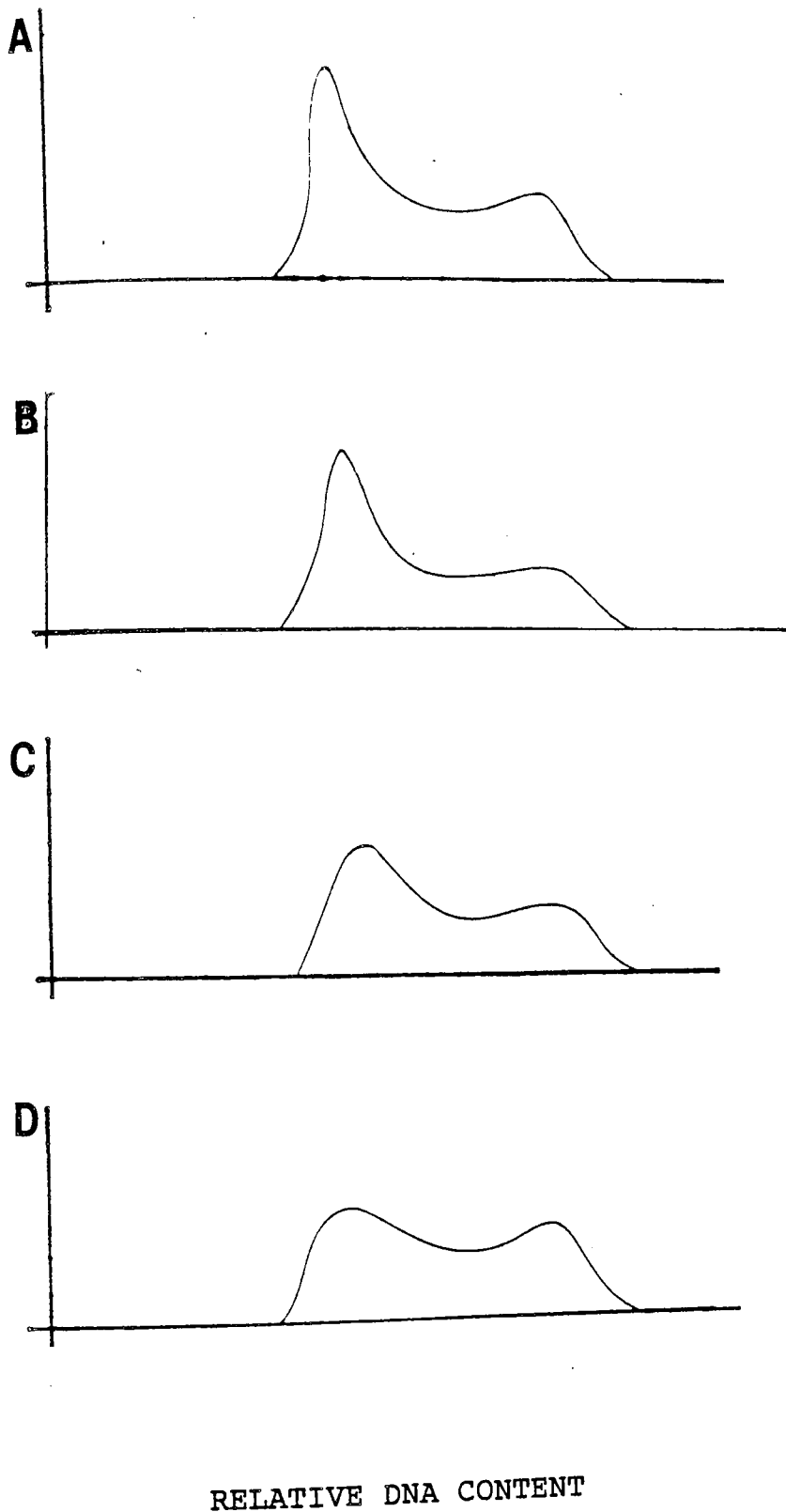
Figure 16A. Traces of representitive DNA histograms of L1210 cells 4 hours after exposure to HN2. A) Untreated L1210 cells, B) 10μM HN2, C) 1μM HN2, D) 0.5μM HN2.
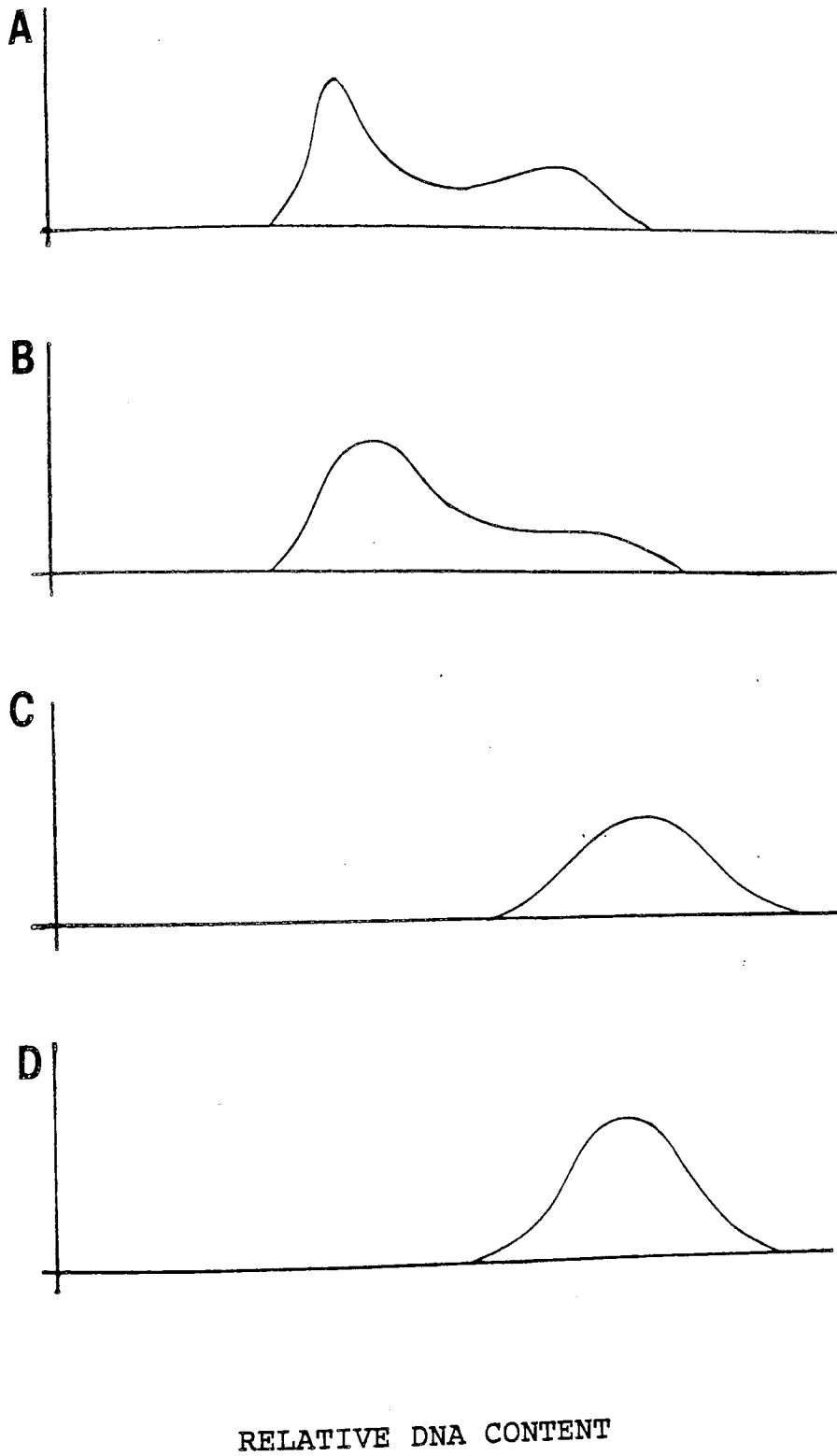
Figure 16B. Traces of representitive DNA histograms of L1210 cells 24 hours after exposure to HN2. A) Untreated L1210 cells, B) 10μM HN2, C) 1μM HN2, D) 0.5μM HN2.

3.4.4 <u>Assessment</u> <u>of</u> <u>L1210</u> <u>cell</u> <u>viability</u> <u>by</u> <u>trypan</u> <u>blue</u> <u>exclusion</u> <u>and</u> <u>by</u> <u>the</u> <u>estimation</u> <u>of</u> <u>cellular</u> ATP <u>after</u> <u>exposure</u> <u>to</u> <u>nitrogen</u> <u>mustard.</u>

Introduction.

$Na^+-K^+-Cl^-$ cotransport was selectively innibited after a 4 hour incubation of 10uM HN2 but was unaffected at this time by either 1 or 0.5uM HN2 (results 3.4.1). Though all three concentrations of HN2 inhibited cell growth by >90%, they were not equitoxic as determined by clonogenic assay. In order to determine the importance of the selective inhibition of $Na^+-K^+-Cl^-$ cotransport in the process of HN2 cytotoxicity, it was necessary to attempt to ascertain the types of cell death observed at the three concentrations of HN2 employed.

The loss of membrane integrity is the last stage in the process of cell death (Bhuyan <u>et</u> <u>al</u> 1976; Roper and Drewinko 1976) and can be assayed by the ability of the cell to exclude vital dyes such as trypan blue. Determination of trypan blue viability is an indicator of the rate of cell death and allows the correlation of changes in the biochemistry and morphology of the cell with the onset of cell death.

ATP is the source of energy within the cell and is destroyed by ATPases upon cell death (Mills and Thomas 1969). Rapid falls in cellular ATP are characteristic of necrosis (Wyllie 1981), the result of which is loss of

ionic homeostasis and consequent high amplitude swelling of the cell leading to the rupture of the plasma membranes. In contrast, metabolic energy is necessary for the process of apoptosis and ATP levels and ion homeostasis are not lost until the onset of secondary necrosis (Wyllie 1981). Therefore, the determination of the ATP levels may expose the pattern, and any differences, of cell death induced in L1210 cells treated with the three concentrations of HN2 (10, 1 and 0.5uM).

Results.

Cell viability was assayed over 72 hours after HN2 treatment by the exclusion of the vital dye trypan blue (Figure 16). Cell viability was maintained up to 12 hours after 10uM HN2 treatment but by 24 hours only 63±14% of cells were viable. This value declined so that by 72 hours after exposure to 10uM HN2 only 8±5% of cells were able to exclude trypan blue dye. 90% or greater of L1210 cells exposed to 1 and 0.5uM HN2 were able to maintain viability until after 36 hours. The number of cells able to exclude trypan blue then declined, so that 46±12% and 55±8% of L1210 cells were viable 72 hours after exposure to 1 and 0.5uM HN2.

The ATP levels of those cells able to exclude the vital dye trypan blue was assayed (Table 4). A signifiacnt increase in cellular ATP was observed (123±6%, p<0.05) in

L1210 cells 4 hours after exposure to 10uM HN2. However, significant decreases in ATP levels were determined at 12 (73±12, p<0.01) and 24 hours (77±10%, p<0.01), but these returned to control levels 48 and 72 hours after the administration of 10uM HN2. Significant increases in ATP were determined in L1210 cells up to 48 hours after treatment with 1uM HN2. No change in cellular ATP was observed in L1210 cells exposed to 0.5uM HN2 except at 48 hours after exposure when a significant increase was determined (155±37%, p<0.05).

Discussion.

The basis of the assay of ATP levels is the release of light by luciferin. Firefly luciferase enzyme catalyses the activation of luceferin by ATP and its subsequent oxidation. Light is emitted upon the transition of luceferin to its ground state which is detected by the luminometer. Thus, light emission is proportional to ATP concentration.

The selective inhibition of Na^+-K^+-Cl^- cotransport occurred after 4 hours incubation with 10uM HN2 and before any changes in cellular ATP levels, the exclusion of trypan blue or changes in cell membrane potential, both measures of membrane integrity (Wilcock et al 1988). The results presented here are in good agreement with those reported by Wilcock et al (1988). The maintenance of ATP levels and the lack of any significant inhibition of Na^+/K^+ATPase pump

(Wilcock et al 1988; results 3.1.2) in L1210 cells, 4 hours after exposure to 10uM HN2, indicated that cell death did not occur by a process of early cellular necrosis characterised by a depletion of cellular ATP and a consequent loss in ion homeostasis.

Significant decreases in cellular ATP levels were observed in L1210 cells 12 and 24 hours after exposure to 10uM HN2. These reductions in cellular ATP did not resemble the early massive depletion of ATP characteristic of necrosis although a significant population of cells may have undergone secondary necrosis. The return of cellular ATP levels to control values in the 20% of cells able to exclude trypan blue at 48 hours after exposure to 10uM HN2 may represent a population of cells that have experienced a slower rate of cell death or had become sterilized (i.e. mitotic cell death).

In L1210 cells treated with 1uM HN2 increases in cellular ATP were observed but no changes occurred in cells exposed to 0.5uM. An increase in cellular ATP has been reported in L1210 cells exposed to 10uM HN2 (Wilcock et al 1988) and cells treated with adriamycin (Hickman, unpublished observation). The increase in ATP may represent an attempt by the cell to repair drug-induced cellular damage.
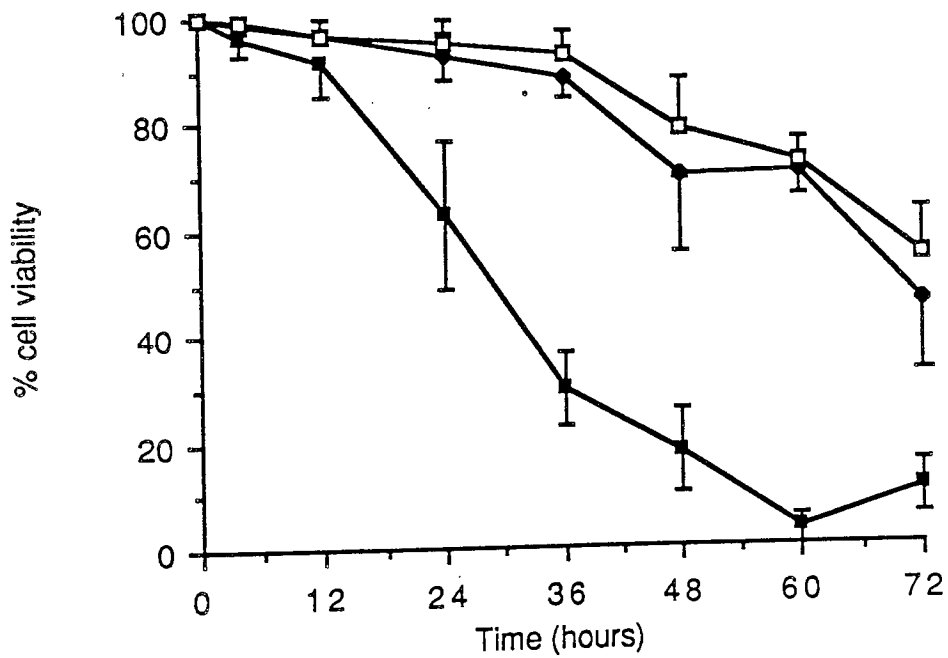
Figure 17. Viability, assayed by trypan blue exclusion, of murine leukaemic L1210 cells over 72 hours after treatment with HN2. ■—■ = 10μM HN2, ◆—◆ = 1μM HN2, □—□ = 0.5μM HN2. (n=3, mean±S.D.).

HN2	10μM HN2	1μM HN2	0.5μM HN2
Time (hours)	% Control ATP levels		
4	123 ± 6^a	134 ± 20^a	100 ± 6
12	73 ± 12^b	153 ± 2^d	129 ± 25
24	77 ± 10^b	141 ± 32^a	103 ± 11
48	102 ± 21	114 ± 7^a	155 ± 37^a
72	71 ± 40	117 ± 43	133 ± 50

Table 4. The effect on ATP levels in viable L1210 cells after exposure to HN2 (10, 1 and 0.5μM). ATP levels are expressed as a percentage of controls. (n=3, mean±S.D.). Significant diffrence from control value, a=p<0.05; b=p<0.01; c= p<0.005; d= p<0.001 (student t-test).

3.4.5 Changes in internal calcium concentrations in L1210 cells after exposure to nitrogen mustard.

Introduction.

The internal calcium concentration of viable cells is maintained at between 10 and 200nM. The external concentration of calcium ions is approximately 1mM so that the low intracellular concentration of free calcium ions is maintained against a 10,000 fold transmembrane concentration gradient. Intracellular calcium concentrations are regulated by either the active extrusion of calcium or by sequestration in intracellular organelles such as mitochondria and endoplasmic reticulum.

Increases in internal calcium have been identified as a common pathway of various types of cell death (Schanne et al 1979). Disruption of the plasma membrane (Schanne et al 1979) or release from intracellular stores (Jewell et al 1982) would lead to rises in internal calcium. The mechanisms by which calcium rises are induced are also dependent on the type of cell death (introduction 1.6.4). The loss of ATP or membrane integrity in necrotic cells would lead to the loss of cellular calcium homeostasis. However, in apoptotic cells rises in calcium, from intracellular stores, appear to be pivotal in the control of cell death. The concentration of free intracellular calcium in L1210 cells was assayed using the internal flourochrome Quin-2 in order to investigate the type of, and any

diffrences, in cell death induced by three concentrations of HN2 (10,1 and 0.5uM HN2).

Results.

Internal concentration of free calcium ions in untreated L1210 cells was 106±15nM (Figure 17). However, 10uM HN2 resulted in no increase in intracellular calcium levels until 48 hours (161±33nM, p<0.001) and 72 hours (254±129nM, p<0.005). No rises in internal calcium were observed with 1 and 0.5uM HN2 over the 72 hours investigated.

Discussion.

Internal calcium was assayed by measuring the flourescence of the dye Quin-2. The dye is loaded into cells by incubation with the acetoxymethyl ester of quin-2. Non-specific esterase enzymes hydrolyse the ester to the tetracarboxylic acid form which is impermeable and free in the cytoplasm. Calcium ions are bound by quin-2 with 1:1 stoichiometry and the fluorescent signal is increased five-fold on binding of calcium ions (Tsien et al 1982).

Tsien et al (1982) noted an internal calcium concentration, measured using the fluorescent probe quin-2, in resting mouse and pig lymphocytes of 120nM. A similiar concentration of free internal calcium was determined in

L1210 cells (Keyes et al 1987). These values are in good agreement with the calcium levels observed in untreated L1210 cells.

No change in internal free calcium was observed in L1210 cells exposed to 1 or 0.5uM HN2 and only at 48 and 72 hours after treatment with 10uM HN2 were increases noted. A number of investigators have proposed that sustained increases in cytosolic calcium represented a common pathway in cell death (Schanne et al 1979; Orrenius et al 1989; McConkey et al 1988). In contrast, Lemasters et al (1987) observed no increases in internal calcium in hepatocytes following ATP depletion by "chemical hypoxia". Nieminen et al (1988) proposed that depletion of cellular ATP was sufficient to induce cell death and that rises in internal calcium were a consequence of the increased permeability of the plasma membrane. However, no reductions in cellular ATP were observed in L1210 cells except at 12 and 24 hours after 10uM HN2 (results 3.4.4). It is possible that a rapid depletion of cellular ATP could have resulted in an increased membrane permeability and consequent rise in intracellular calcium.

In the systems employed by the above investigators, the loss of cell viability (measured by tryan blue) was a rapid event (>50% within 6 hours). In contrast, the loss of membrane integrity was a comparatively slow event (60% loss by 24 hours) in L1210 cells treated with HN2. Changes in

intracellular homeostasis (i.e. calcium and ATP levels) may only occur in a small population of the cells at any one time and so may not be detected by the methods employed. However, rises in internal calcium were observed in L1210 cells within 2 hours of treatment with 10uM adriamycin even though cell viability was maintained (Keyes et al 1987).

Wilcock (1987) reported that calcium levels were unaffected in L1210 cells treated for 3 hours with 10uM HN2, which is in good agreement with the results presented here. A selective inhibition of the $Na^+-K^+-Cl^-$ cotransporter was observed 4 hours after exposure of L1210 cell to 10uM HN2 though no rises in internal calcium were reported. Therefore, the rises in cytosolic calcium do not appear to be responsible for the selective inhibition of $Na^+-K^+-Cl^-$ cotransport by 10uM HN2.
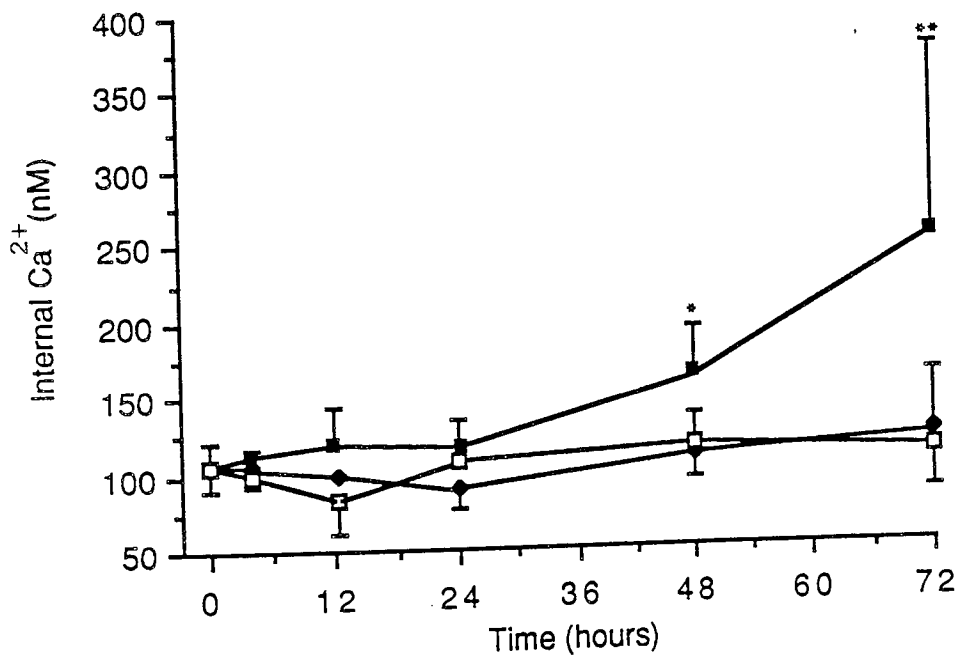
Figure 18. Concentration of free internal calcium in murine leukaemic L1210 cells over 72 hours after treatment with HN2. Cytosolic calcium concentrations were determined by the flourescence of the internal flourochrome Quin-2. ─■─ = 10μM HN2, ─◆─ = 1μM HN2, ─□─ = 0.5μM HN2. (n=3, mean±S.D.). Significantly different from control values, ** = p<0.005; * = p<0.001 (student t-test).

3.4.6 Analysis of scanning electron micrographs of L1210 cells treated with nitrogen mustard.

Introduction.

Protrusions at the surface of the plasma membrane, known as blebs, have been observed in cells following exposure to a wide variety of toxic compounds (Lemasters et al 1987; Mesland et al 1981). Blebbing of the cell surface occurs before any change in membrane permeability and is initially reversible. The formation of blebs probably reflects a change in cytoskeletal organisation as compounds that react directly with cytoskeleton, such as cytochalasin B, can induce blebs (Mesland et al 1981). The appearance of blebs has been attributed either to changes in internal calcium (Jewell et al 1982) or to a depletion of cellular ATP (Nieminen et al 1988). It has been proposed that the final event in cell death is the rupture of large membrane blebs (Lemasters et al 1987).

Scanning electron micrography was employed to study the appearance of possible blebs and the surface of L1210 cells after exposure to HN2. This was in order to ascertain the pattern of cell death induced by the three concentrations of HN2 (10, 1 and 0.5uM).

Results.

Scan electron micrographs were taken of L1210 cells at various time points after treatment with HN2. The surface of untreated cells appeared very bumpy and uneven but there were few proturbences and processes (Figures 19, 20 and 21). Up to 12 hours after treatment with 10uM HN2 there was very little change in the appearance of L1210 cells (Figure 19). The formation of a few blebs on the surface of the cells was apparent within 4 hours but did not represent more than a third of the cells observed. At 24 hours, and time points after, extensive formation of blebs and an appearance of large membrane holes was observed.

There was little change in the appearance of L1210 cells treated with 1uM HN2 until 48 hours though they did seem smoother than controls (Figure 20). At 48 hours a small percentage of the cells observed exhibited the formation of small blebs on their surface. By 72 hours, in a number of cells observed, the formation of blebs was well advanced and was typical of apoptotic bodies.

The membrane surface of L1210 cells appeared smooth and rounded after treatment with 0.5uM HN2 (Figure 21). Not until 48 hours any blebs were observed and these were present in only a small percentage of cells.

Discussion.

In L1210 cells exposed to HN2, no changes in cytosolic calcium, determined by the fluorescence of the internal dye Quin-2, were noted which correlated with the appearance of blebs (results 3.4.5). This is in contrast to the report that increases in the internal calcium of hepatocytes were responsible for the formation of blebs (Jewell et al 1988). However, in hepatocytes treated with cyanide and iodoacetate no rises in free internal calcium were observed, but depletion of cellular ATP was concomitant with the appearance of blebs (Lemasters et al 1988). Twenty-four hours after exposure to 10uM HN2 a 25% inhibition of cellular ATP was measured in L1210 cells able to exclude trypan blue (results 3.4.4). The reduction in cellular ATP was an average value for the population of cells. Therefore, there may have been an extensive depletion of ATP in those L1210 cells in which blebbing was observed.

The disruption of cytoskeletal organisation either by compounds such as cytochalasin B (Mesland et al 1981) or by modification of SH-groups (Boobis et al 1989) could lead to plasma blebbing. HN2 and tris(chloroethyl)amine (HN3), at millimolar concentrations, have been shown to alkylate the proteins of the red blood cell cytoskeleton (Wildenhauer et al 1980; Levy 1965). 2 and 1mM HN3 can protect cells from echinocytic transformations (Wildenhauer et al 1980). Thus, a similiar ability of HN2 to cross-link cytoskeletal

proteins of L1210 cells could have altered their organisation and led to plasma blebbing which is independent of calcium. In comparision to the $t\frac{1}{2}$ of the alkylating ability of HN2 (~1 hour; Williamson et al 1966) the onset of cell blebbing was a much slower process and therefore unlikely to have been solely due to cytoskeletal reorganisation by HN2.

Figure 19. Scanning electron micrographs of L1210 cells over 72 hours after exposure to 10µM HN2. A) Control L1210 cells (magnification x2,000); B) 4 hours (magnification x2,000), C) 12 hours (magnification x2,000), D) 24 hours (magnification x1,500), E) 48 hours (magnification x4,000) and F) 72 hours (magnification x1,500) after exposure to 10uM HN2.

Figure 20. Scanning electron micrographs of L1210 cells over 72 hours after exposure to 1 µM HN2. A) Control L1210 cells (magnification x2,000); B) 4 hours (magnification x2,000), C) 12 hours (magnification x1,500), D) 24 hours (magnification x1,000), E) 48 hours (magnification x2,000) and F) 72 hours (magnification x3,000) after exposure to 1uM HN2.

Figure 20. Scanning electron micrographs of L1210 cells over 72 hours after exposure to 0.5 µM HN2. A) Control L1210 cells (magnification x2,000); B) 4 hours (magnification x1,500), C) 12 hours (magnification x1,500), D) 24 hours (magnification x2,000), E) 48 hours (magnification x1,000) and F) 72 hours (magnification x1,000) after exposure to 1uM HN2.
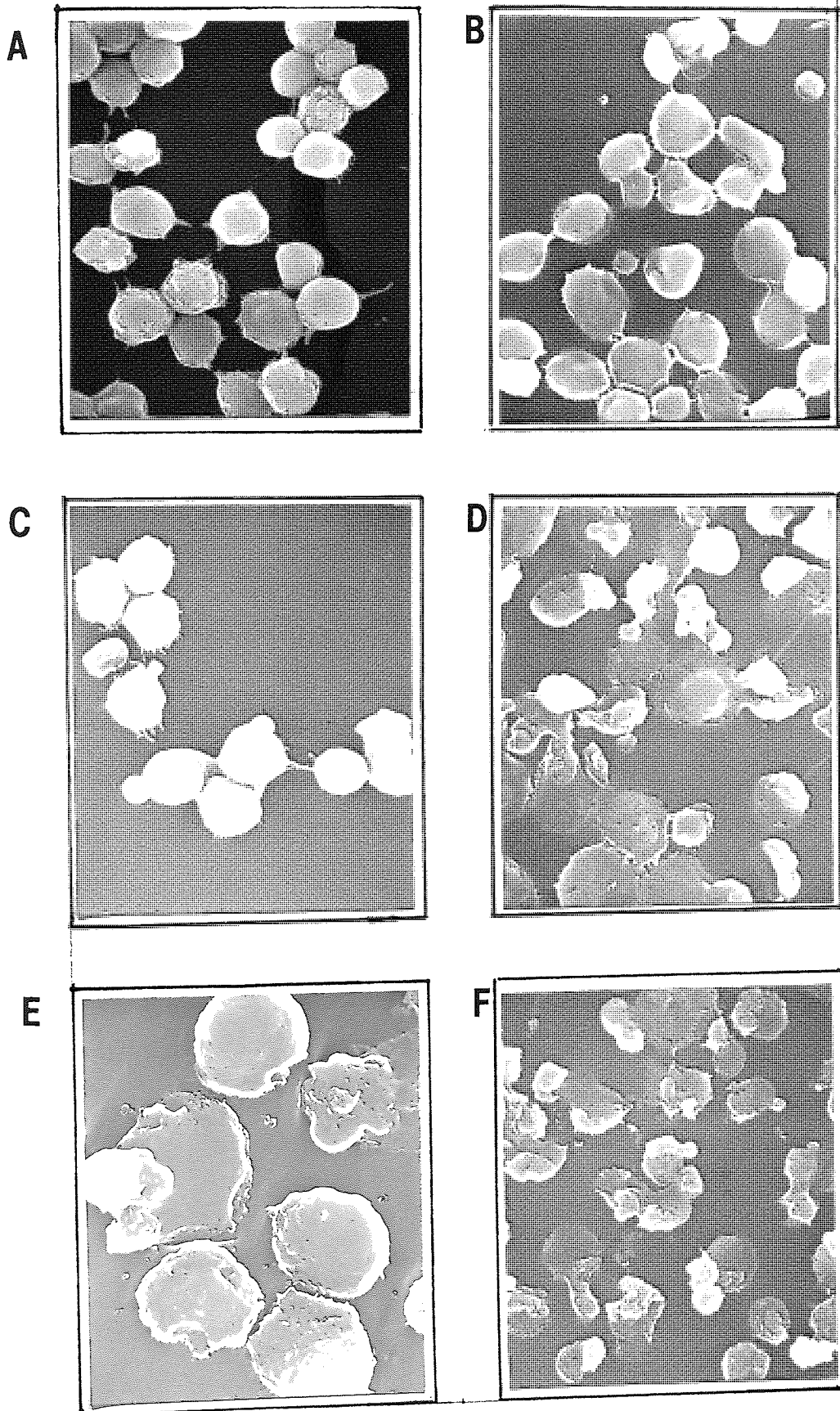
FIGURE 19.

FIGURE 20.
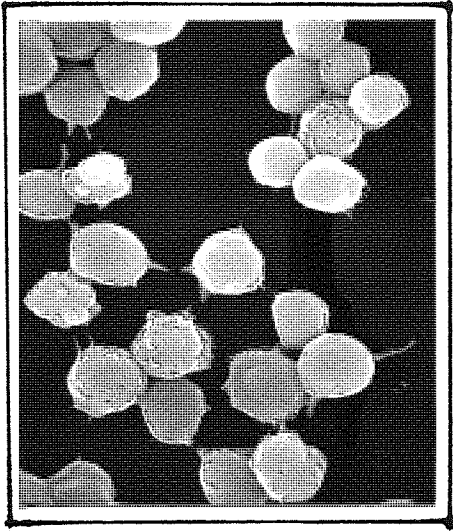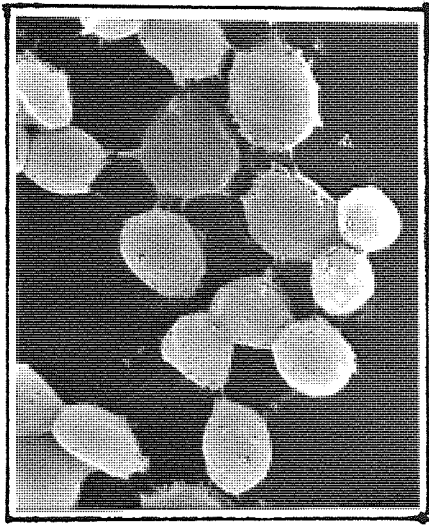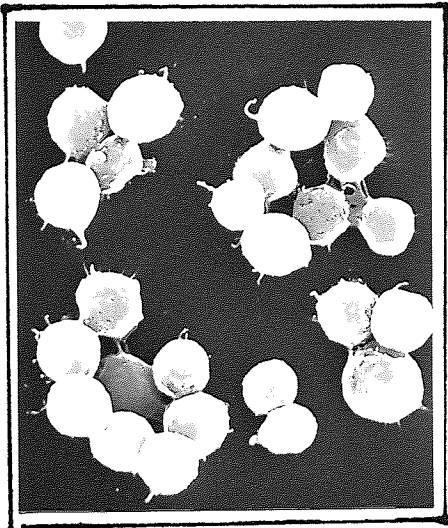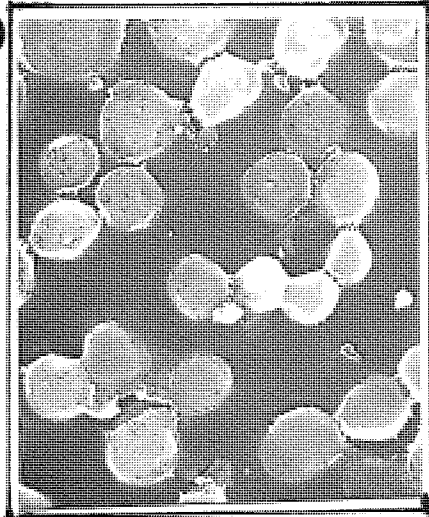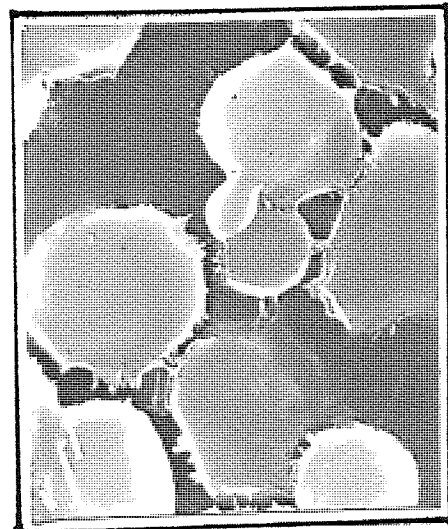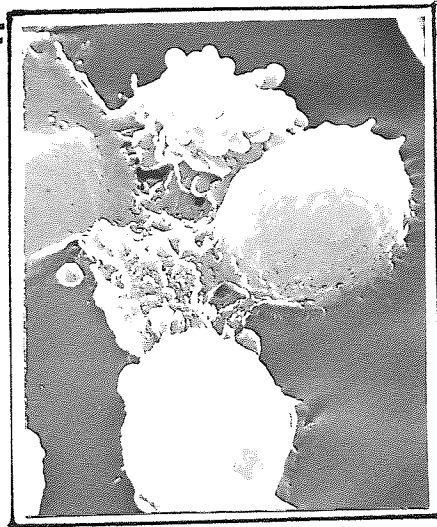
FIGURE 21.
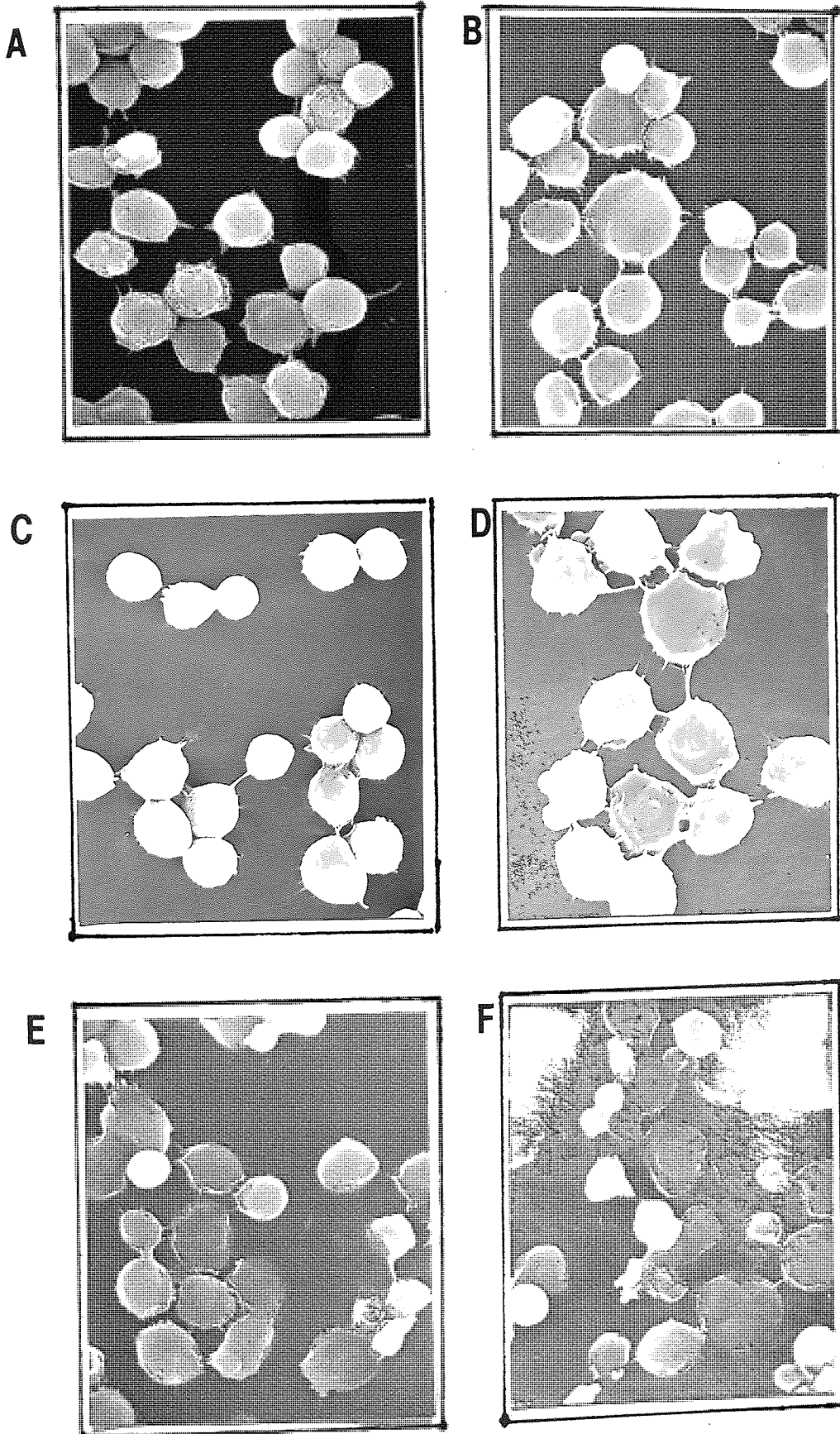
3.4.7 Analysis of transmission electron micrographs of

L1210 cells treated with nitrogen mustard.

Introduction.

Transmission electron micrographs are cross-sections of cells and so enable the investigator to observe and analyse changes in the appearance and structure of cellular organelles. There are two major classifications of cell death – apoptosis and necrosis (introduction 1.6.2.) and may be distinguished by their characteristic morphology (Wyllie et al 1980; Wyllie 1981).

I wished to determine if the lack of any potassium ion transport lesion in L1210 cells exposed to 1 and 0.5uM HN2 (results 3.4.1.) was a consequence of an alternative mechanism of cell death to that in cells treated with 10uM HN2 in which the volume reduction, brought about by the inhibition of the Na^+-K^+-Cl^- cotransporter, is compatible with the process of programmed cell death. By the analysis of transmission electron micrographs, the type of cell death induced by the three concentrations of HN2 (10,1 and 0.5uM) may be ascertained.

Results.

The nuclei of untreated L1210 cells exhibit little condensation of chromatin, although nucleoli are visible (Figure 22A). The cytoplasm of the cells is very granular and polysomes are found on the endoplasmic reticulum. Mitochondria are of between 0.5 and 0.7um in size but the internal organisation is difficult to discern.

There is little change in nuclear morphology four hours after exposure to 10uM HN2 (Figure 22B). In comparision to the controls the mitochondria do appear to have swollen ranging in size from 1-1.7um. However, there is little disaggregation of polysomes and swelling of the endoplasmic reticulum. In cells exposed to 10uM HN2 for 12 hours (Figure 22C) there is focal margination and extensive condensation of chromatin on the periphery of the nucleus. There is a scattering of dark grains in the center of the nucleus which may be the remnants of the dissolved nucleolus. Polysomes do not appear to be disaggregated and the endoplasmic reticulum is not distended. The size of mitochondria range from 0.6 to 1um in diameter and in many small vacoules can be observed.

The nucleus of cells 36 hours after exposure to 1uM HN2 appears to be lobed (Figure 22D). There is some condensation of chromatin on the periphery of the nucleus but it does not appear pyknotic. There are a large number

of mitochondria in close proximity which are of a similiar size to those in control cells (~0.9um).

The nucleus appears to be fragmented 72 hours after treatment of L1210 cells with 1uM HN2 (Figure 22E). However, there is very little condensation of chromatin but vacoules were observed in the nuclear fragments. Polysomes do not appear disaggregated from the endoplasmic reticulum. There are lipid vacoules of 1.6 and 1.2um in diameter in the cytoplasm. There are many mitochondria which, in comparision to controls, do not appear to have changed in size (~0.8um).

In L1210 cells, 48 hours after exposure to 0.5uM HN2, the nucleus exhibits little condensation of chromatin, though there is a nucleolus in one of the segments (Figure 22F). There are three separate nuclei, but these are probably lobes of the same nucleus, and there is vacuolation in this nucleus. The polysomes do not appear disaggregated and there is no distension of the endoplasmic reticulum. There are a large number of mitochondria which vary in size from 0.6 to 1.4um.

The nucleus appears as five separate units which may either be lobes or fragmentation of the nucleus 72 hours after exposure of L1210 cells to 0.5uM HN2 (Figure 22G). There is some condensation of chromatin on the periphery of the nucleus. The mitochondria are very faint and hard to

discern. Polysomes do not appear disaggregated and the endoplasmic reticulum is not distended.

Discussion.

Wyllie (1981) described the main morphological differences between necrotic and apoptotic cell death. Changes in the appearance of the nucleus are often used to define the two types of cell death (introduction 1.6.2). Necrosis can be identified by the pyknotic condensation of chromatin, whilst apoptosis is often characterised by the dense aggregation of chromatin as a cresentic cap in the nucleus. This phenomenon was not observed in any of the transmission electron micrographs taken of L1210 cells treated with HN2.

Kerr et al (1987) warned that in cells grown in suspension culture secondary necrosis rapidly follows apoptosis. Kerr et al (1987) proposed that the observation of nuclear fragments with dense aggregations of chromatin is one way of distinguishing the secondary necrosis of apoptotic cells from necrotic cells ab initio. The appearance of the nucleus in L1210 cells that have been exposed to 10uM HN2 for 12 hours is of necrosis, identified by the pyknotic clumping of chromatin on the periphery of the nucleus. However, the appearance of granulation in the nucleus may be the remnants of a dissolved nucleolus which is indicative of apoptosis. 72 hours after the

administration of the lower concentrations of HN2 (1 and 0.5uM) fragmentation of the nucleus and compacting of mitochondria was observed, typical of apoptosis. However, these fragments did not exhibit any dense aggregations of chromatin typical of apoptosis.

Apoptosis has been observed in malignant cell populations (Kerr et al 1972) and in cells treated with cytotoxic drugs (Searle et al 1975). This led Kerr et al (1987) to speculate that this may be a mechanism to maintain the genetic integrity of the tissue. However, Allen (1987) argued against the proposition that apoptosis was the mode of cell death induced by cytotoxic drugs as it was an evolutionary mechanism and unlikely to cover the reaction of cells to recently synthesised drugs. However, no evidence was offered to support this statement. Hume (1987) reviewed the different types of cell death observed in epithelial tissue. Type B dark cells were moribund cells with condensed chromatin but the mitochondria were enlarged. Kerr et al (1987) suggested that this cell death was probably a modification of necrosis.

Therefore, determination of the type of cell death induced by HN2, at the concentrations used, by the study of the morphology of L1210 cells is difficult. The problems are that secondary necrosis often rapidly follows apoptosis and whether apoptosis and necrosis are the only two types of cell death available for cells. Populations of moribund

cells also may display both necrotic and apoptotic cell death (Allen 1987). L1210 cells exposed to 10uM HN2 do appear to exhibit certain morphological characteristics of necrosis. However, ATP levels are maintained in the first 4 hours at least (Wilcock and Hickman 1988; results 3.4.4) and there is a decrease in cell volume which was maintained over 72 hours (results 3.4.2) which are not events compatible with necrosis. In L1210 cells treated with lower concentrations of HN2 (1 and 0.5uM) fragmentation of the nucleus and compaction of mitochondria is observed at later time points but there is little dense condensation of chromatin typical of apoptosis at any of the times investigated.

Figure 22. Transmission electron micrographs of L1210 cells. A) Control cells (magnification x9,100), B) 10μM HN2 for 4 hours (magnification x4,500), C) 10μM HN2 for 12 hours (magnification x4,500), D) 1μM HN2 for 36 hours (magnification x5,700), E) 1μM HN2 for 72 hours (magnification x3,400), F) 0.5μM HN2 for 48 hours (magnification x4,500), G) 0.5μM HN2 for 72 hours (magnification x5,700). 1 = Nucleus, 2 = Polysome, 3 = Rough endoplasmic reticulum, 4 = Mitochondria, 5 = Lipid vacoule, 6 = Nuclear vacoule, 7 = Nucleolus.

A

B

2μm

3μm

C

3μm

D

2μm

E

3μm

F

2μm

G
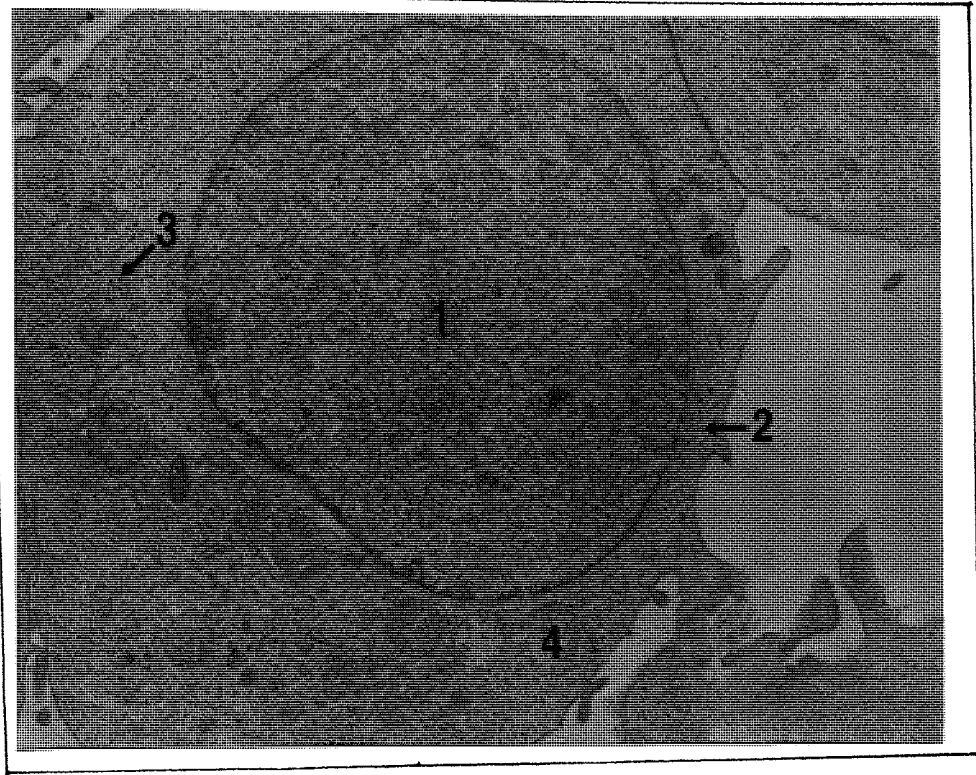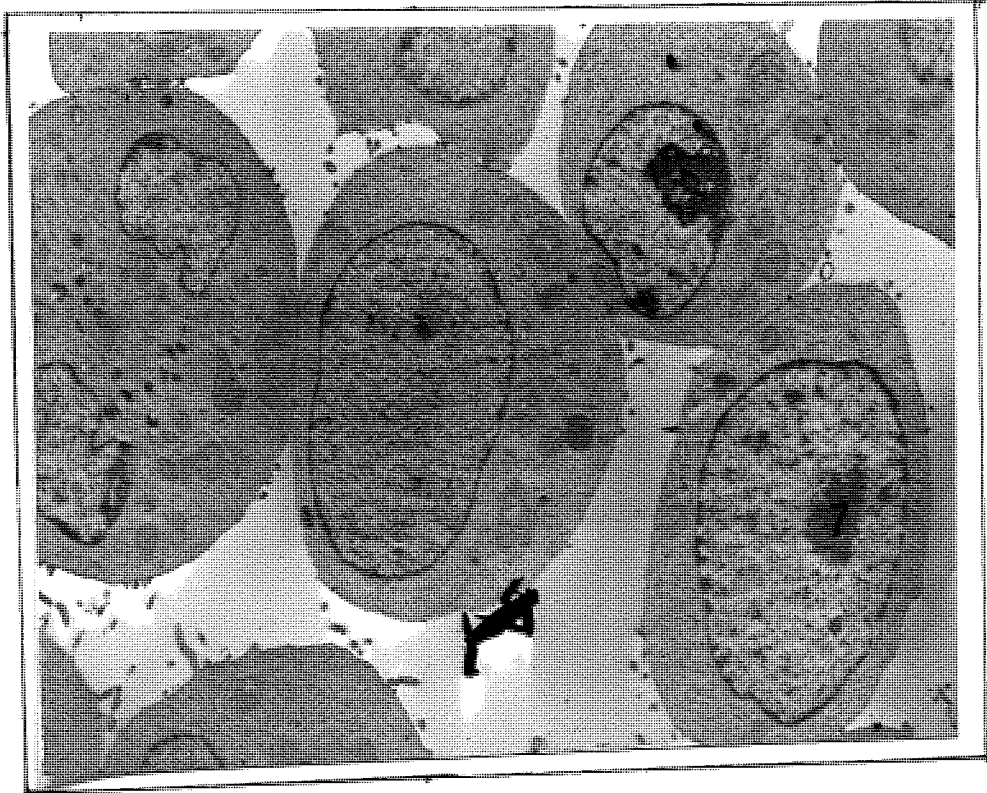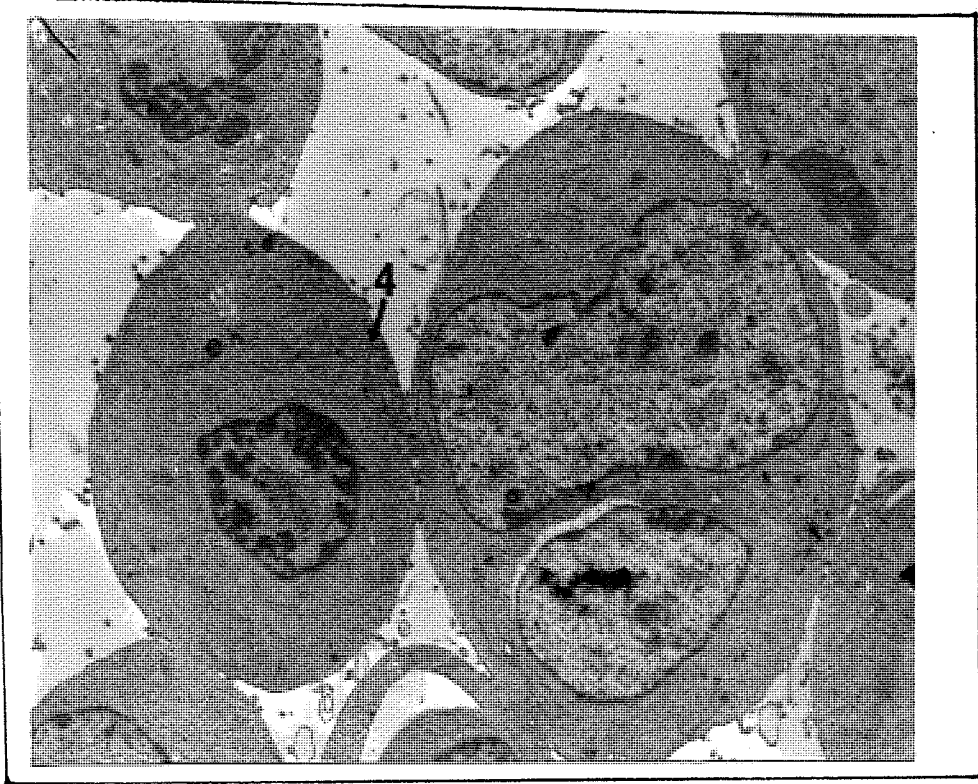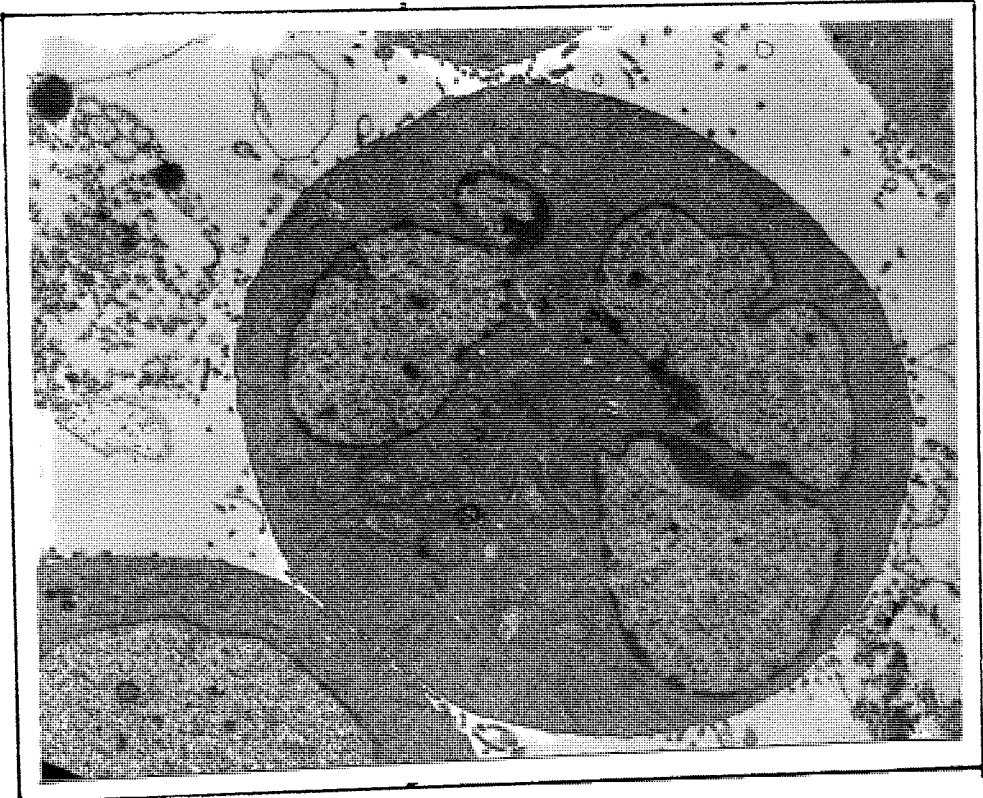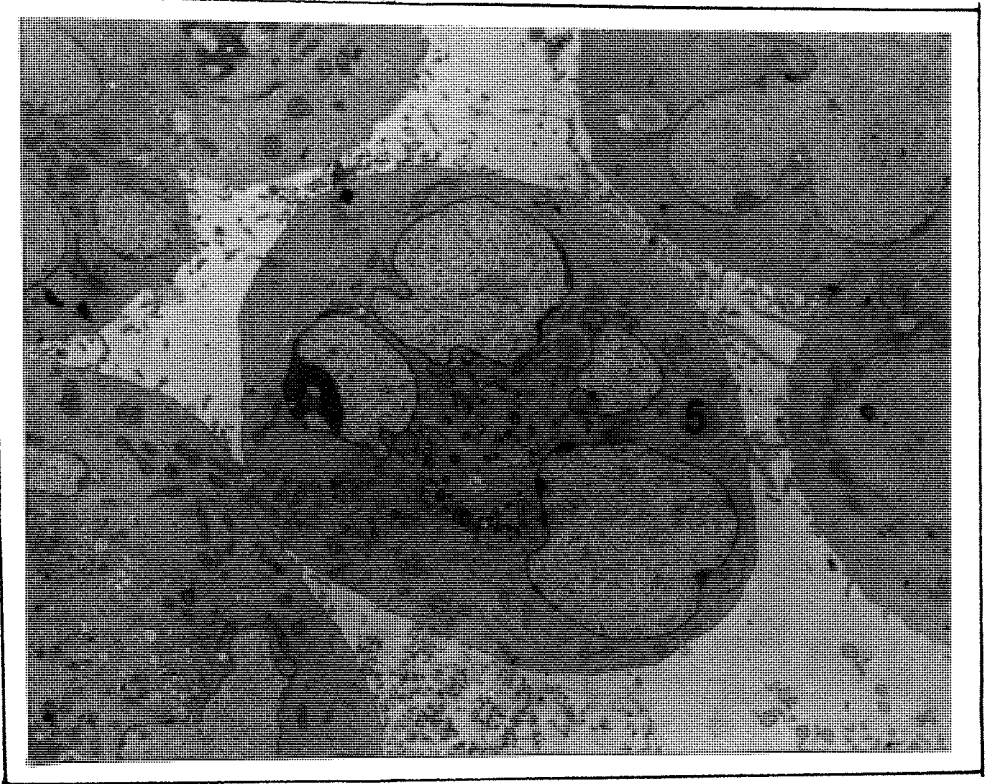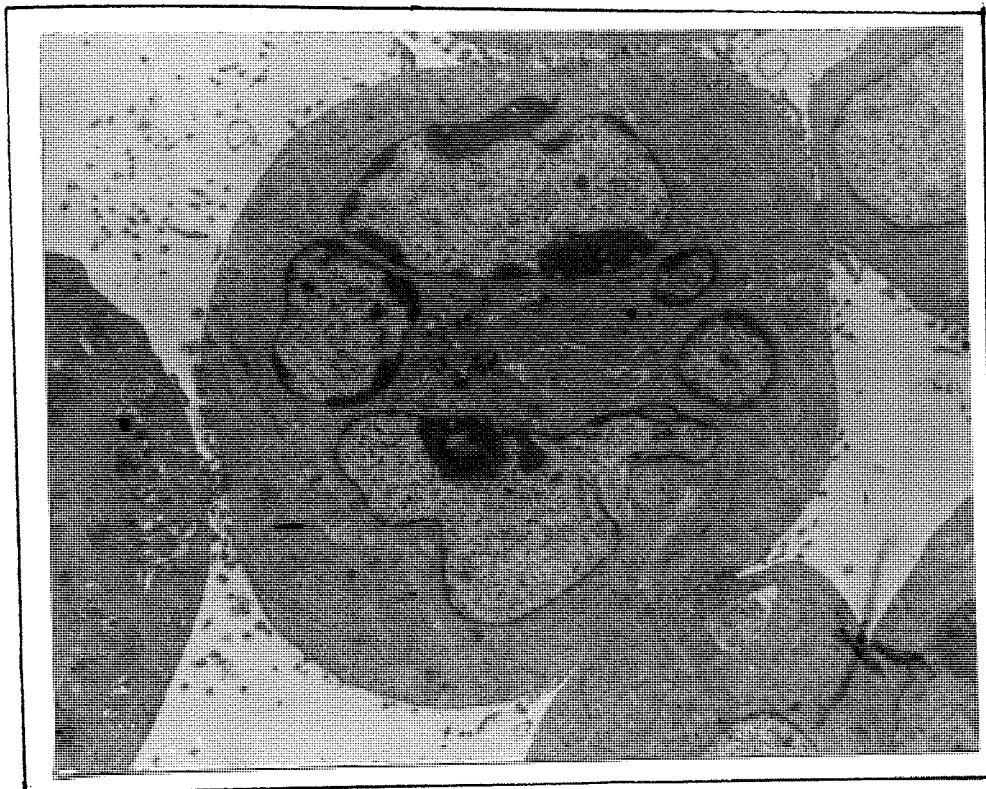
2μm

3.5 The effect on nitrogen mustard cytotoxicity to L1210 cells by coincubation with cycloheximide.

Introduction.

In contrast to necrosis, apoptosis is an active process in which the cell will synthesise proteins to induce death (Wyllie et al 1980). Administration of cycloheximide to rats before or after (up to 45 minutes) exposure to HN2 prevented cytotoxicity of gut epithelial cells (Lieberman et al 1970). However, in lymphoid cells, cycloheximide enhanced HN2 toxicity (Lieberman et al 1970) or offered only a transient protection (Weissberg et al 1978; Ben-Ishay and Farber 1975).

Thus, it was important to investigate whether cycloheximide could protect L1210 cells in vitro from the toxic effects of HN2. This would further enable us to classify the cell death induced by HN2 and the importance of protein synthesis in this process. Our understanding of the selective inhibition of $Na^+-K^+-Cl^-$ cotransport, exerted by 10uM HN2, would be enhanced if this lesion could be absolved by cycloheximide. This would indicate the nature of the lesion (i.e. a direct or indirect interaction) and whether it was important in the process of cell death.

Results.

The toxicity of L1210 cells incubated for various times with three concentrations of cycloheximide was investigated (Table 5). Incubation for 3 and 6 hours with 10ug/ml cycloheximide resulted in a slight depression of growth of L1210 cells but a significant inhibition was observed after 12 (40%, $p<0.001$) and 18 (44%, $p<0.005$) hours exposure. The growth of L1210 cells incubated with 5 and 2.5ug/ml cycloheximide was not significantly altered. However, exposure to cycloheximide for 12 and 18 hours did result in significant inhibition of cell growth at both concentrations.

Incorporation of $[^3H]$-leucine is used as a measure of protein synthesis. Cycloheximide inhibited protein biosynthesis in a concentration-dependent manner (Figure 23). Protein synthesis was depressed by 78% by 10ug/ml cycloheximide, 72% by 5ug/ml cycloheximide and 63% by 2.5ug/ml cycloheximide. This level of inhibition was maintained throughout the 3 hours of the experimental protocol.

The modulation of HN2 toxicity by the addition of 5ug/ml cycloheximide, 10 minutes after exposure to HN2, for 3 and 6 hours was investigated (Table 6). L1210 cells incubated for 3 hours with 5ug/ml cycloheximide did not show any significant change in their proliferative capability. However, the toxicity of 0.1, 0.05 and 0.01uM HN2 was

slightly but significantly increased by a 6 hour incubation with 5ug/ml cycloheximide (p<0.05). However, in this series of experiments incubation of L1210 cells with 5ug/ml cycloheximide did result in a slight but significant inhibition of cell growth (9±6%, p<0.05).

Incubation of L1210 cells with 10uM HN2 for 3 hours in a Kreb's-Ringer solution resulted in a 48% reduction in ^{86}rubidium$^+$ uptake (Figure 24). The addition of 5ug/ml cycloheximide, 10 minutes after exposure to HN2, did not alter this inhibition and incubation of cells with cycloheximide alone had no discernable effect on ^{86}rubidium$^+$ influx (Figure 24).

Discussion.

The toxicity of cycloheximide was investigated in order to determine both appropriate concentrations and incubation times to be used in the following experiments. Incubation of L1210 cells with 5ug/ml cycloheximide, unlike 10ug/ml, for 3 and 6 hours was found not to be toxic. Thus, 5ug/ml cycloheximide was used in subsequent experiments though there was a greater inhibition of protein synthesis with 10ug/ml cycloheximide. Cycloheximide is thought to prevent protein synthesis by the inhibition of the enzymatic transfer of pepitdyl tRNA from the acceptor to the peptidyl site of the ribosome.

Incubation of L1210 cells with cycloheximide after exposure to HN2 did not alter toxicity. A slight but significant increase in HN2 toxicity was observed when cells were incubated for 6 hours with 5ug/ml. However, this could be a consequence of a slight toxicity when cells were incubated with 5ug/ml cycloheximide alone. A number of investigators have shown that death of intestinal epithelial cells, induced in vivo with HN2, can be prevented (Lieberman et al 1970; Ben-Ishay and Farber 1975). Lieberman (1970) stated that both pretreatment and posttreatment (up to 45 minutes) with cycloheximide was sufficient to prevent cell death, but only if protein synthesis was inhibited by 80% or more. The concentration of cycloheximide used resulted in an inhibition of only 72% but higher concentrations of cycloheximide appeared cytotoxic to L1210 cells. Weissberg et al (1978) demonstrated that peripheral blood cells were protected from the cytotoxic action of HN2 by pretreatment with cycloheximide. However, only a transient protection was observed in bone marrow cells (Weissberg et al 1978; Ben Ishay and Faber 1975). Lieberman et al (1970) noted that treatment with cycloheximide enhanced the toxicity of HN2 to lymphoid cells. The possibility that cycloheximide may protect tumour cells from the toxic effects of HN2 was not investigated. Non-tumourgenic cell lines were more likely to accumulate in G_1 than transformed cell lines if treated with cycloheximide (Medrano and Purdee 1980). This could

explain the ability of cycloheximide to protect tissues from the cytotoxic action of HN2 and why a similiar protective action in the L1210 tumour cell line was not observed. Thus, the protective effects of cycloheximide observed in normal tissues (Lieberman et al 1970) may not be repeated for tumour cell lines. Under the conditions described, protein synthesis does not appear to be involved in HN2-induced death in L1210 cells. However, a more rigorous regime of cycloheximide treatment may have protected the L1210 cells from the toxic effects of HN2.

Cycloheximide did not absolve the inhibition of $Na^+-K^+-Cl^-$ cotransport exerted by 10uM HN2. This suggests that this lesion is a consequence of a direct interaction between the cotransporter and HN2, possibly through the formation of cross-links or inactivation of processes that control cotransport activity. However, the concentration of cycloheximide used inhibited protein synthesis by only 72% and this lesion may be a consequence of a complex cascade of events that are initiated from the synthesis of a few proteins. Thus, it is possible that protein(s) produced may still have resulted in the inhibition of $Na^+-K^+-Cl^-$ cotransport. Alternatively, the inhibition of $Na^+-K^+-Cl^-$ cotransport may be due to the activity of lysosomal proteins. Treatment of L1210 cells with cycloheximide after exposure to HN2 did not protect them from the cytotoxic effects of HN2. Thus, it would appear unlikely that

cycloheximide could absolve the lesion of ^{86}rubidium$^+$ influx, if it were important in the process of cell death.
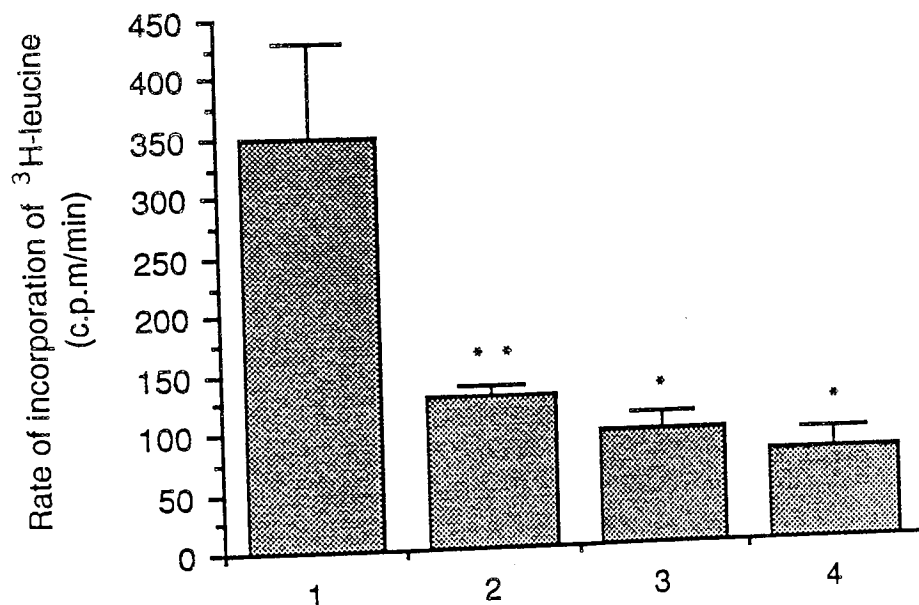
Figure 23. The incorporation of ^{3}H-leucine into murine leukaemic cells, measured as the increase in c.p.m. per minute, and the effect of the protein inhibitor cycloheximide (CHX). $1=$ untreated, $2=$ 10µg/ml CHX, $3=$ 5µg/ml CHX, $4=$ 2.5µg/ml. (n=3, mean±S.D.).Significantly different from control value, $** = p<0.005; * = p<0.001$ (student t-test).

Time incubated with CHX (hours)	3	6	12	18
CHX (µg/ml)	% Inhibition of growth			
10	13 ± 11^a	7 ± 5^b	40 ± 3^d	45 ± 11^c
5	1 ± 7	3 ± 6	37 ± 7^d	35 ± 13^c
2.5	-8 ± 11	-6 ± 9	20 ± 4^d	20 ± 7^c

Table 5. The cytotoxicity of 3 concentrations of cycloheximide (CHX; 10, 5 and 2.5µg/ml) incubated with L1210 cells for either 3, 6, 12 or 18 hours. Toxicity was determined by growth inhibition studies. (n=3, mean±S.D.). Significant difference from control growth, a= $p<0.1$; b= $p<0.05$; c= $p<0.005$; d= $p<0.001$ (student t-test).

Time incubated with CHX (5µg/ml)	0	3 hours	6 hours
HN2 (µM)	% Inhibition of growth		
control	0±0	3±6	8±6*
10	99±1	99±3	99±1
1	97±3	95±4	97±3
0.5	95±1	98±1	97.5±1
0.1	26±6	29±6	37±6*
0.05	16±2	23±11	33±9*
0.01	8±6	17±11	24±8*

Table 6. The toxicity of HN2, determined by the inhibtion of growth, in L1210 cells and the effect of incubation of cells with 5µg/ml cycloheximide (CHX) for 3 and 6 hours. Toxicity was determined by inhibition of growth studies. (n=3, mean±S.D.). Significant difference from control values, * = p<0.05 (student t-test).
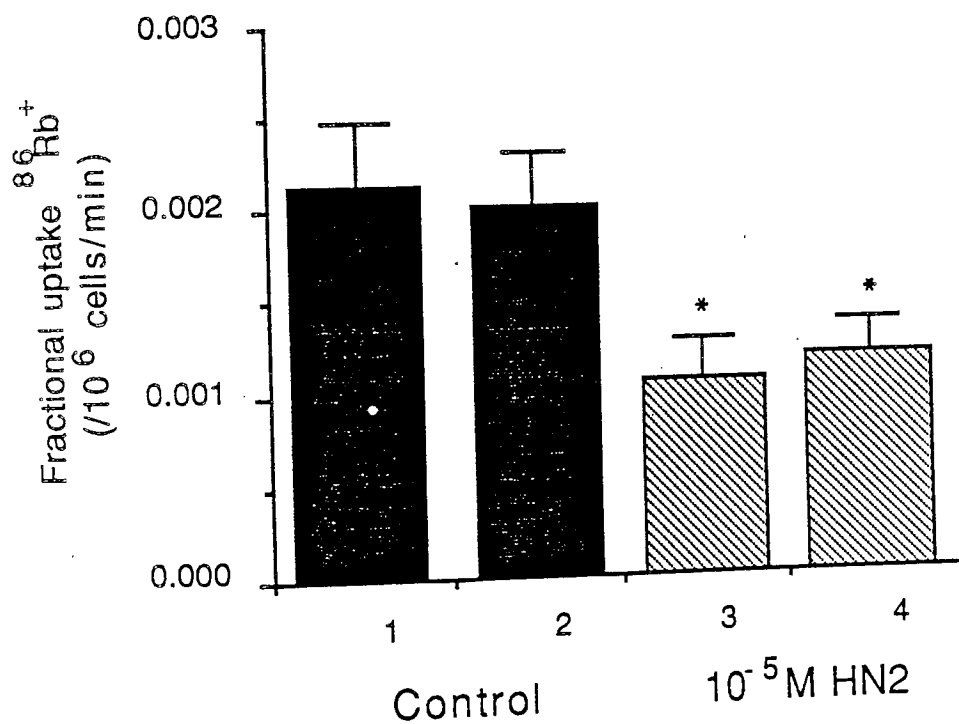
**Figure 24.** The inhibition of [86]rubidium[+] uptake into L1210 cells, in a Kreb's-Ringer solution, by a 3 hour incubation with 10μM HN2 and the effect of a co-incubation with cycloheximide (5μg/ml). 1 = untreated L1210 cells, 2 = 5μg/ml cycloheximide, 3 = 10μM HN2, 4 = 10μM HN2 and 5μg/ml cycloheximide. (n=3, mean±S.D.). Significant difference from control values, * = $p < 0.01$ (student t-test).

3.6. The effect on nitrogen mustard cytotoxicity to L1210 cells by the coincubation of L-azetidine carboxylic acid.

Introduction.

Azetidine carboxylic acid (ACA) is an analog of the non-essential amino acid proline. In contrast to proline, which is a five-membered ring with a single amino group, ACA is a four-membered ring. Duncan and Hershey (1987) reported that a 9 hour incubation of HeLa cells with 10mM ACA resulted in a 70% inhibition of protein synthesis. ACA is able to replace proline in rabbit haemoglobin (Baum et al 1975). However, incorporation of ACA instead of proline into protein structures could lead to conformational changes and consequent inactivation of the protein (Berman et al 1969). Amino acid analogs are also able to induce heat shock proteins (hsp) (Thomas and Mathews 1984).

I decided to investigate the effect of incubation of L1210 cells with ACA on HN2 cytotoxicity. A concentration of any agent which completely inhibited protein production was of course toxic to L1210 cells: I, therefore, first investigated the effect of ACA alone and then in combination with HN2 in L1210 cells. L1210 cells, in response to HN2 exposure, may synthesise two different classes of protein. Proteins which induce cell death may be produced at concentrations of HN2 where extensive cytotoxicity is observed; but at lower concentrations, where there is little

cell death, proteins may be synthesised in order to repair any sub-lethal damage. The incubation of L1210 cells with ACA would result in protein production being aberrant as proline would be substituted by ACA and so the possible importance of new protein synthesis in HN2-induced cell death could be determined.

It has been demonstrated that amino acid analogs, if incorporated into protein, can induce hsps (Thomas and Mathews 1984). Hsps have been reported to to be involved in the cellular responses to stress and injury and may play some role in the process of cell death (introduction 1.6.6). Thus, it was also of interest to investigate whether hsps were of importance in any alteration of HN2 cytotoxicity by ACA in the murine leukaemic L1210 cell line.

Results.

Growth inhibition studies were used to determine the toxicity of L-azetidine carboxylic acid (ACA) (Figure 25). The IC_{50} of L1210 cells incubated for 72 hours with ACA was 1.6mM, for 18 hours it was 7.6mM and for 12 hours it was 9.6mM. In the following experiments L1210 cells were incubated with 5mM ACA for 12 and 18 hours as it was not significantly toxic (Figure 25).

In Figure 26 a 12 hour incubation with 5mM ACA, added 10 minutes after the addition of HN2, resulted in a slight but significant increase in the toxicity of 0.1uM (49±13%,

p<0.05). However, if the cells were incubated for 18 hours with ACA a significant increase in toxicity was observed. The inhibition of growth of 0.1uM HN2 increased from 10±17% to 73±6% (p<0.001), 0.05uM HN2 from 1±4% to 54±11% (p<0.001) and 0.01uM HN2 from 4±2 to 23±14 (p<0.05).

Incubation of L1210 cells with 5mM ACA for 12 hours increased the synthesis of the 70 kDa heat shock protein by 450% and the 90 kDa heat shock protein by 240% (Figure 27). An incubation of 18 hours with 5mM ACA increased the levels of the 70 kDa protein by 1,180% and the 90 kDa protein by 450% (Figure 28). A third protein of approximately 110kDa was induced by 12 hour incubation with 5mM ACA, but this was only observed on one of the 2 fluorographs. Incubation of L1210 cells with HN2 (0.1,0.05 and 0.01uM HN2) and 5mM ACA for 12 and 18 hours resulted in similiar increases in the synthesis of the 70 kDa and 90 kDa proteins (Figure 27 and 28).

Discussion.

L-histidinol is an analog of the histidine amino acid. Like ACA, l-histidinol can be incorporated into protein structures resulting in possible conformational changes and, in common with other amino acid analogs such as ACA, it is able to induce hsp production (Thomas and Mathews 1984). L-histidinol, like ACA, has often been used to investigate the effects of amino acid analogs in various biological systems.

Warrington et al (1984) demonstrated that incubation of L1210 cells with l-histidinol, an analog of the amino acid histidine, increased the cytotoxicity of 5-flourouracil (FUra) and 1-ß-d-arabinofuranosylcytosine. However, animals survived treatment with toxic doses of FUra if l-histidinol was administered also. Warrington and Fang (1989) demonstrated that l-histidinol enhanced the selectivity and efficacy of alkylating agents (BCNU, CisDDp and cyclophosphamide) and daunomycin in mice bearing the murine lymphocytic leukaemia P388 cell line. Stolfi et al (1987) observed that l-histidinol did protect $CD8F_1$ mice from the toxic effects of FUra but did not improve the therapeutic efficiency of the drug against $CD8F_1$ breast tumours.

Warrington et al (1984) proposed that this phenomenon was due to "differential cell cycle arrest". Histidine deprivation by l-histidinol would cause normal cells to become arrested in G_O whilst tumour cells would be able to continue in the cell cycle. Thus, normal but not tumour cells would be protected from the cytotoxic action of proliferative-dependent anticancer drugs. Warrington and Fang (1985) claimed that normal murine marrow cells did show a transient, but reversible, inhibition of proliferation unlike L1210 cells in response to l-histidinol. This hypothesis does explain the increased selectivity exhibited in vivo by FUra. However, no explanation is suggested for the increased sensitivity of L1210 cells to FUra. Why should FUra, in the presence of

l-histidinol, be more cytotoxic to cells progressing normally in their cell cycle?

Other investigators have since questioned the validity of this hypothesis of "differential cell cycle arrest" (Eldstein and Heilburn 1988; Warrington et al 1987; Warrington 1986). Eldstein and Heilburn (1988) compared the level of protection conferred by l-histidinol on both proliferating and resting hematopoietic stem cells and marrow granulocyte precursors. The order of protection predicted by the theory of cell cycle arrest would be: resting CFU-S>CFU-GM> proliferating CFU-S. However, the observed level of protection was proliferating CFU-S>CFU-GM> resting CFU-S. Warrington (1986) demonstrated that the cytotoxicity of anticancer agents for various malignant cells was increased by l-histidinol, despite the differing cell cycle effects induced by l-histidinol. L-histidinol inhibited the cycle progression of P815 mastocytoma cells treated with vinblastine, yet an increase in cytotoxicity was observed (Warrington et al 1987). This evidence argues against the proposition that l-histidinol confers an increased cytotoxicity by a purely cell kinetic mechanism.

Incubation of L1210 cells with 5mM ACA for 12 and 18 hours resulted in the production of 2 proteins. Using Rf values these proteins have a molecular weight of ~70 kDa and ~90 kDa. These results are in poor agreement with Watowich and Morimoto (1988) who observed that a 4 hour incubation of Hela cells with 5mM ACA induced production of 78 and 70 kDa

proteins. This may be explained by the inherent inaccuracy of using Rf values to determine molecular weight and the lack of positive controls. However, there is no consensus amongst investigators as to the type of hsps induced by incubation with amino acid analogues. Duncan and Hershey (1987) noted that in HeLa cells, treated with 10mM ACA, production of 70 and 90 kDa hsps was enhanced. Li and Laszlo (1985) indicated that 70,87 and 110 kDa hsps were induced by incubation of Chinese hamster HA-1 cells with 2.5mM ACA.

The relationship between the co-incubation of L1210 cells with 5mM ACA and the increase in the cytotoxicity of HN2 was dependent on, as far as I was able to ascertain, two factors. The first of these was the level of DNA damage induced by HN2. An 18 hour incubation of L1210 cells with 5mM ACA enhanced cytotoxicity more in those cells treated with 0.1uM than 0.01uM HN2. The second factor was the length of time that L1210 cells were exposed to ACA. The toxicity of 0.05uM HN2 was further increased if L1210 cells were incubated for 18 hours rather than 12 hours with 5mM ACA.

However, the relationship between the increase in HN2 cytotoxicity and the production of these 70 and 90 kDa proteins was unclear. Enhanced hsp expression in L1210 cells, incubated with 5mM ACA alone, was not toxic. There were no obvious changes between the levels of hsps induced by ACA in control cells and those treated with HN2.

However, a relationship between levels of damage induced by HN2, enhanced hsp expression and the duration of the incubation period with ACA may account for this phenomenon of enhanced cytotoxicity.

Duncan and Hershey (1987) demonstrated that a 9 hour incubation of HeLa cells with 10mM ACA inhibited protein synthesis by 70%. Proteins, necessary for the repair of sub-lethal damage induced by 0.1, 0.05uM and 0.01uM HN2, may not have been synthesised in L1210 cells incubated with ACA, so resulting in cell death. However, no investigations were performed to ascertain if any reduction in protein synthesis was observed in L1210 cells incubated for 12 or 18 hours with 5mM ACA.

Cells can be protected from lethal exposure to heat shock by previous exposure to non-lethal elevated temperatures (Henle and Leeper 1975) or other inducers of hsps (Hahn and Li 1982). Li and Laszlo (1985) reported that Chinese hamster fibroblasts (HA-1), pretreated with non-toxic concentrations of ACA (5mM), were more sensitive to exposure to lethal heat shock and proposed that the pretreatment of cells with ACA resulted in aberrant copies of proteins being synthesised which were unable to protect the cell from any subsequent trauma. It is possible that this theory may also explain the increased toxicity of HN2, in L1210 cells, when coincubated with ACA. Proteins may be synthesised that are able to either protect L1210 cells from the toxic effects of low doses of HN2 or repair such damage.

However, in the presence of ACA, aberrant copies of the protein(s) are synthesised and a consequent increase in toxicity is observed.

Insufficient evidence has been presented to allow a clear hypothesis to be constructed in order to explain the enhanced cytoxicity of HN2 by the incubation of L1210 cells with ACA. This phenomenon may be explained by the induction of hsps or the inhibition or aberrant protein synthesis. In order to understand better this phenomenon it would be of interest to induce hsps by some other method (i.e. non-lethal heat shock) and so eliminating the possible role of aberrant protein copies. In this way a clearer understanding of the involvement and role of hsps in cell death may emerge.
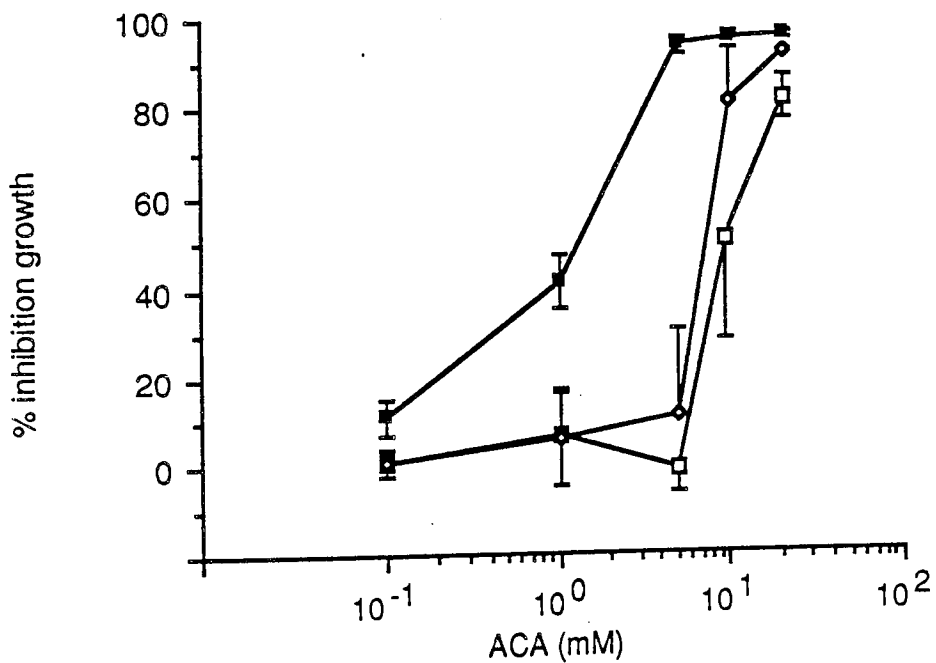
Figure 25. The inhibition of growth of murine leukaemic L1210 cells <u>in</u> <u>vitro</u> by incubation with various concentrations of L-azetidine-2 carboxylic acid (ACA) for 12 hours (-□-), 18 hours (-○-) and 72 hours (-■-). (n=3, mean±S.D.).
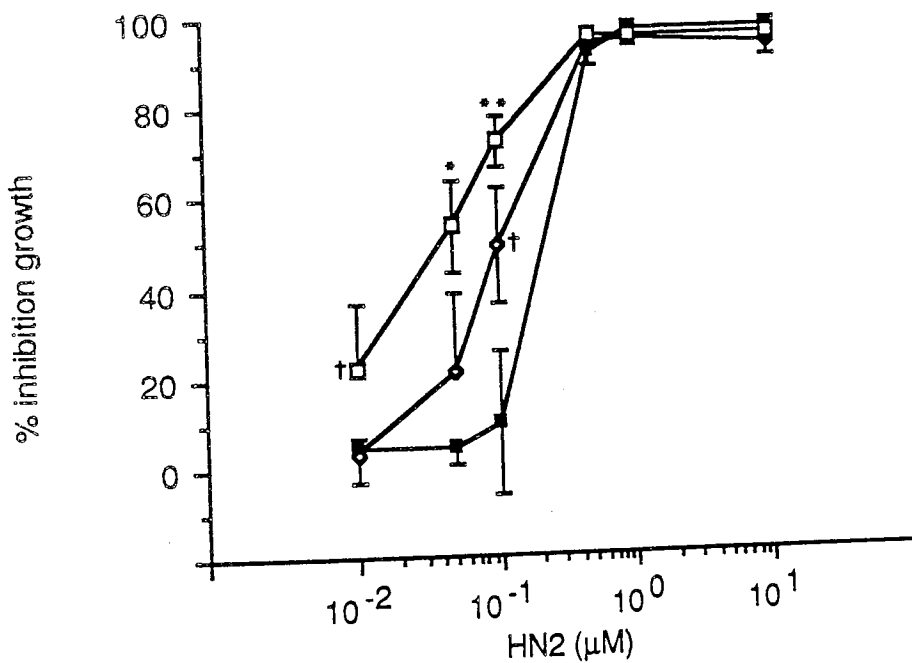
Figure 26. The effect of a 12 or 18 hour incubation of murine leukaemic L1210 cells with 5mM ACA on the toxicity of HN2. ━■━ = control HN2 cytotoxicity, ━◆━ = HN2 cytotoxicity after a 12 hour incubation with 5mM ACA, ━□━ = HN2 cytotoxicity after a 18 hour incubation with 5mM ACA. Significantly different from control cytotoxicity, † = $p < 0.05$; ** = $p < 0.005$; * = $p < 0.001$ (student t-test).

A

LANE NUMBER

molecular weight markers (kDa)

205 —

116 —
97 —

66 —

45 —

→ 90 kDa
→ 70 kDa

B

% control synthesis

800

600

400

200

0

5mM ACA

0.1µM HN2 + 5mM ACA

0.05µM HN2 + 5mM ACA
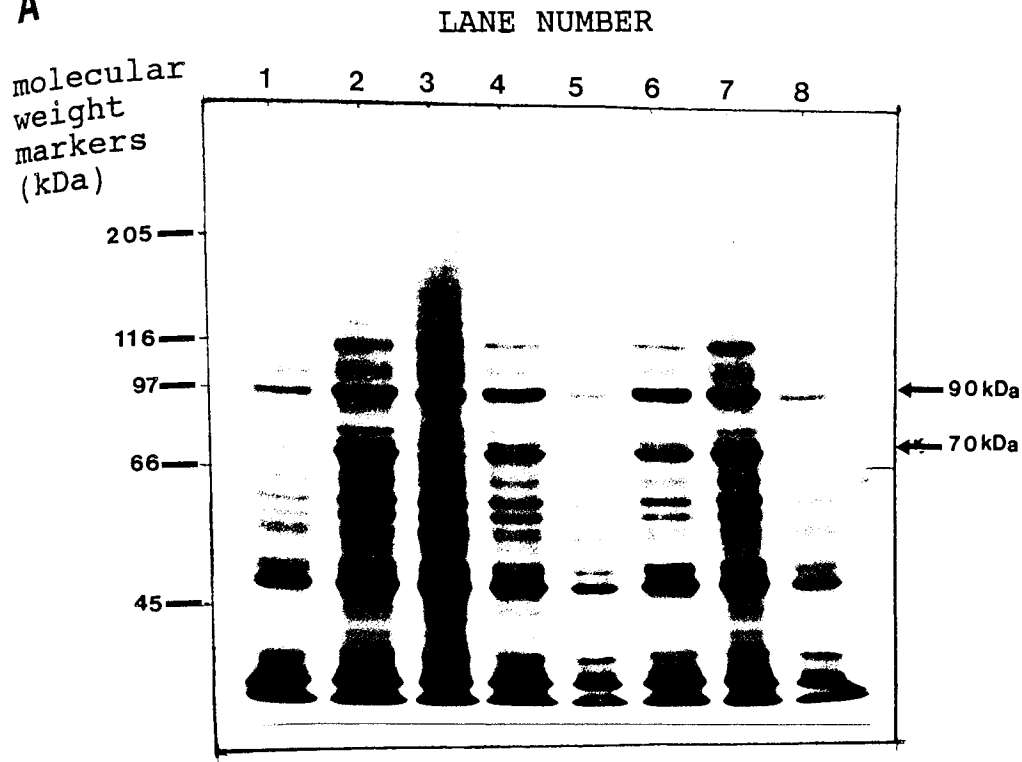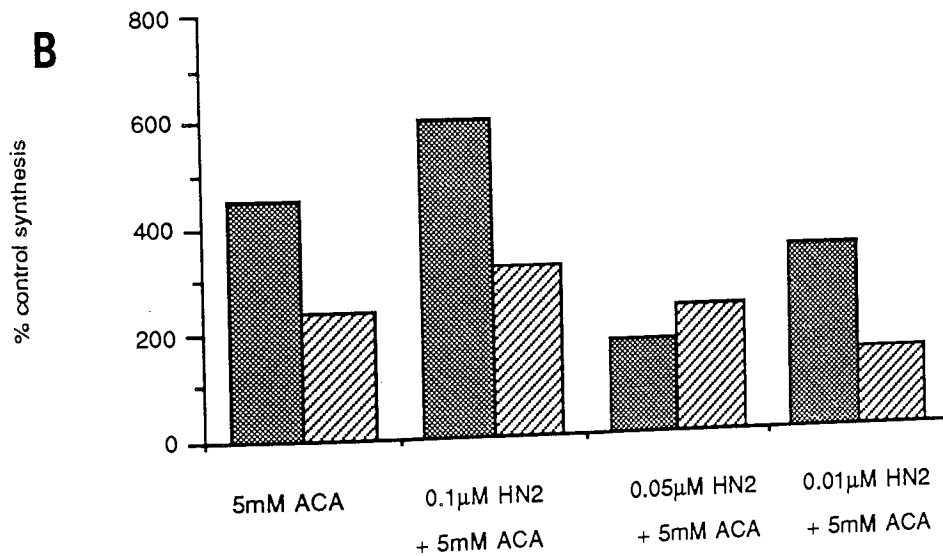
0.01µM HN2 + 5mM ACA

Figure 27. A) Fluorograph of L1210 cells pulse labelled with ^{35}S-methionine 12 hours after exposure to HN2 with or without 5mM ACA. Lanes: 1) control, 2) 5mM ACA, 3) 0.1µM HN2 4) + 5mM ACA, 5) 0.05µM HN2, 6) + 5mM ACA, 8) 0.01µM HN2, 7) + 5mM ACA. B) The increase of 70 (▓) and 90 (▨) kDa protein synthesis, induced by a 12 hour incubation with 5mM ACA, in comparision to control and HN2-treated L1210 cells determined by scanning densitometry of fluorographs. Mean of two independent experiments.

A

molecular
weight
markers
(kDa)

LANE NUMBER

B
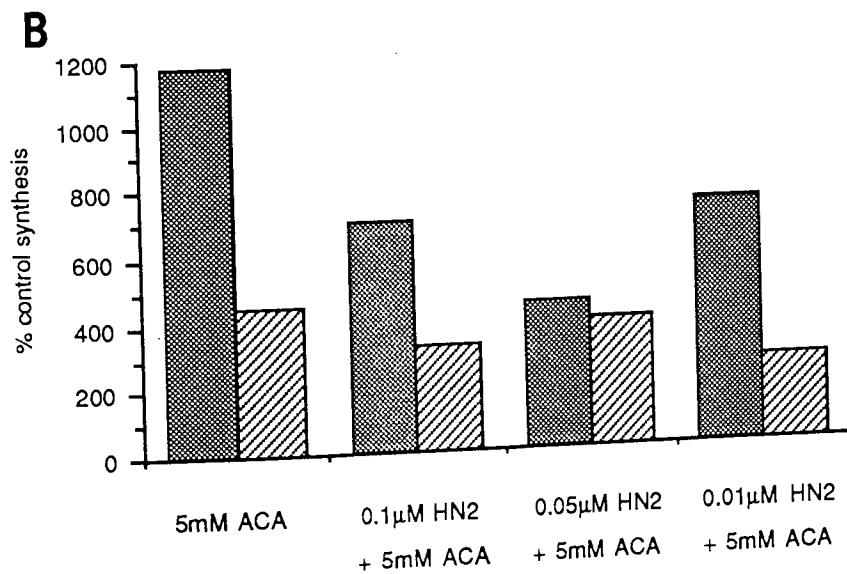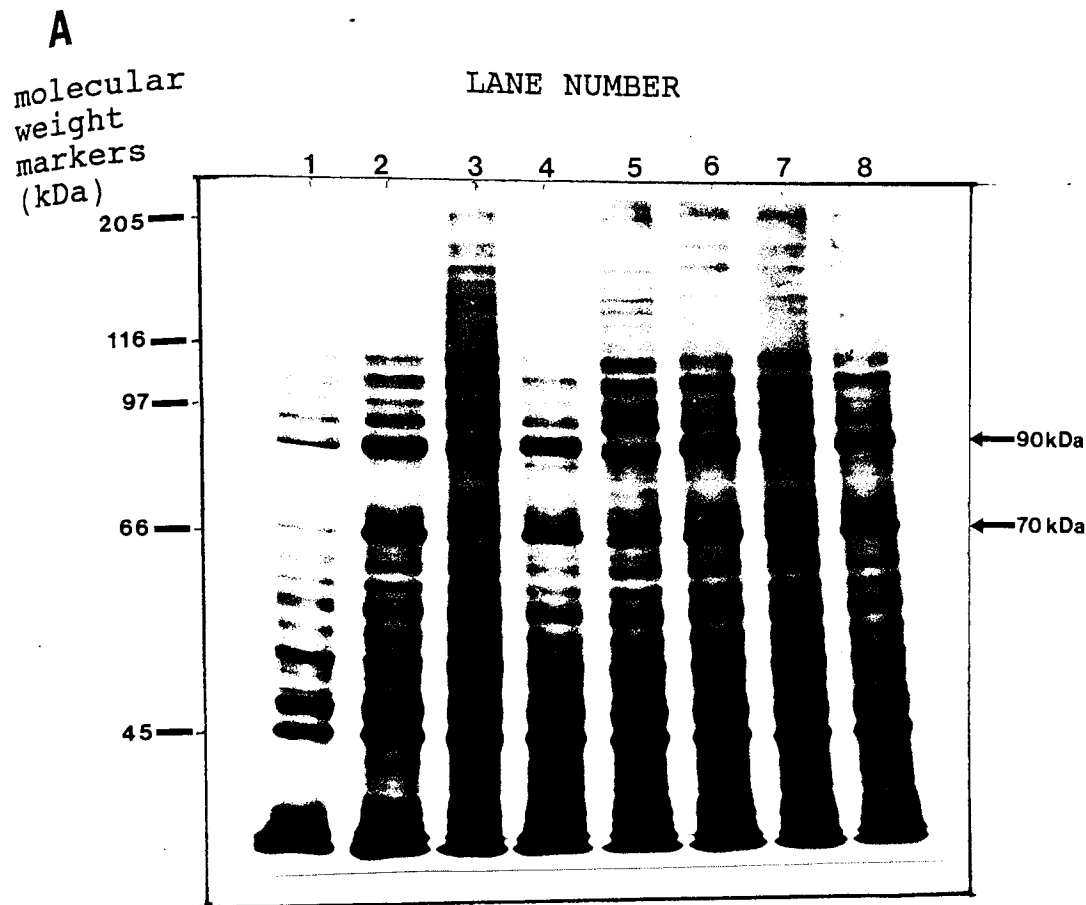
Figure 28.   A) Fluorograph of L1210 cells pulse labelled with ^{35}S-methionine 18 hours after exposure to HN2 with or without 5mM ACA.   Lanes: 1) control, 2) 5mM ACA, 3) 0.1µM HN2 4) + 5mM ACA, 5) 0.05µM HN2, 6) + 5mM ACA, 7) 0.01µM HN2, 8) + 5mM ACA. B) The increase of 70 (▧) and 90(▨) kDa protein synthesis, induced by an 18 hour incubation with 5mM ACA, in comparision to control and HN2-treated L1210 cells determined by scanning densitometry of fluorographs. Mean of two independent experiments.

5. GENERAL DISCUSSION.

DNA has often been considered the prime target of the cytotoxic action of HN2 (introduction 1.2.2). However, it has been proposed that HN2 may exert its cytotoxicity in a multimodal manner and that other cellular lesions may be equally important (Wheeler 1967). A clear understanding of how HN2 induces cell death has not emerged despite the fact that HN2 has been employed in cancer chemotherapy for over 40 years.

Peters (1947) suggested that the interaction of HN2 with the plasma membrane could lead to cell death. Since that time a number of investigators have demonstrated that HN2 inhibited ion transport across the plasma membrane (Ihlenfeldt et al 1981; Baxter et al 1982; Grunicke et al 1983; Doppler et al 1985; Wilcock et al 1988). The transport of ions is crucial in the maintainance of the internal cellular homeostasis and has been implicated in the control of cell proliferation. Thus, the inhibition of ion transport processes may be of some importance in HN2-induced cell death.

The incubation of murine leukaemic L1210 cells with 10uM HN2 for 3 hours resulted in the selective inhibition of the $Na^+-K^+-Cl^-$ cotransporter (Wilcock et al 1988). The aim of this present study was to understand better the nature and role of this lesion in the process of HN2-induced cell death. Wilcock et al (1988) reported that the selective inhibition of the $Na^+-K^+-Cl^-$ cotransporter occurred before

any changes in membrane potential, cellular ATP levels and the ability of the cell to exclude the vital dye trypan blue. It was concluded that the inhibition of potassium transport by 10uM HN2 appeared to follow lethal damage to the cell but preceded cell death. The role of this selective inhibition of Na^+-K^+-Cl^- cotransport as a lesion involved in either the cytotoxic action of HN2 or in the process of cell death was an area that merited further investigation.

The Na^+-K^+-Cl^- cotransporter in L1210 cells was unaffected after incubation with cytotoxic concentrations of either chlorambucil (Wilcock 1987) or the monofunctional analogue of HN2 (Wilcock et al 1988). Therefore, Wilcock et al (1988) suggested that the selective inhibition of the Na^+-K^+-Cl^- cotransporter was a drug-specific lesion and that it may be a consequence of a direct alkylation of the cotransporter, rather than a reflection of the onset of cytotoxicity. A non-selective inhibition of cellular potassium transport systems was observed after a 10 minute incubation of L1210 cells with a very high concentration (1mM) HN2 (results 3.2.2). This indicated that both the Na^+/K^+ATPase pump and the Na^+-K^+-Cl^- cotransporter were equally sensitive to alkylation by HN2 and, therefore, the selective inhibition of the Na^+-K^+-Cl^- cotransporter observed with 10uM HN2 may not have been a consequence of a direct interaction of HN2. However, some caution must be exercised in the interpretation of this result as the high

concentration of HN2 used may have obscured any differences in the alkylating sensitivities of the two potassium transport systems.

The inhibition of the Na^+-K^+-Cl^- cotransporter was observed 4 hours (in RPMI 1640 media) after exposure to 10uM HN2. This also would argue against a direct alkylation of the Na^+-K^+-Cl^- cotransporter especially as HN2 is a very reactive molecule with $t\frac{1}{2}$ in PBS at $30^{\circ}C$ of approximately 1 hour (Williamson et al 1966) and Ross et al (1978) observed maximum cross-linking of DNA within 30 minutes of treating L1210 cells with HN2. It has been proposed that the slow onset in the inhibition may be due to the slow alkylation of the Na^+-K^+-Cl^- cotransporter in some hydrophobic domain in which the rate of reactivity of HN2 is reduced (Wilcock et al 1988). However, it is unlikely that the formation of the aziridinium ring, which is a necessary step in the alkylation reaction of HN2, could occur in a hydrophobic environment.

In the first hour after human erythrocytes were treated with 2mM HN2 a non-selective inhibition of potassium transport was observed (results 3.2.1). However, between 2 and 4 hours after exposure to 2mM HN2 there was a significantly greater inhibition of the Na^+-K^+-Cl^- cotransporter than of the Na^+/K^+ATPase pump. Due to the rapid loss in the alkylating ability of HN2, the initial non-selective inhibition of ^{86}rubidium$^+$ uptake may be the consequence of the direct alkylation of the potassium

transport systems. The resultant increase in the level of inhibition of the Na^+-K^+-Cl^- cotransporter observed in human erythrocytes may reflect the situation reported in L1210 cells treated with 10uM HN2. Thus, the greater sensitivity of the Na^+-K^+-Cl^- cotransporter to HN2 may be due to changes in internal homeostasis or the organisation of the plasma membrane or cytoskeleton.

Garay (1982) reported that increases in cellular cAMP and calcium inhibited Na^+-K^+-Cl^- cotransport in human erythrocytes. An increase in cAMP was observed in Walker carcinoma cells treated with HN2 (Tisdale and Phillips 1975). A similiar increase in either cAMP or internal calcium may account for the enhanced inhibition of Na^+-K^+-Cl^- cotransport observed in human erythrocytes incubated with 2mM HN2 (results 3.2.1). However, changes in cAMP or cytosolic calcium in murine leukaemic L1210 cells are unlikely to explain the selective inhibition of Na^+-K^+-Cl^- cotransport by 10uM HN2. Wilcock and Hickman (1988) noted that dibutyryl cAMP had no effect on Na^+-K^+-Cl^- cotransporter activity in L1210 cell and no change in internal calcium concentrations was observed in L1210 cells incubated with 10uM HN2 (Wilcock 1987; results 3.4.5).

Wilcock et al (1988) reported that, in contrast to 10uM HN2, a 4 hour incubation of L1210 cells with a lower concentration of HN2 (1 and 0.5uM) did not inhibit potassium ion transport. No inhibition of $^{86}rubidium^+$ was observed after L1210 cells were incubated for 24 hours with either 1

or 0.5uM HN2 (results 3.4.1). This would argue against the thesis that the inhibition of $Na^+-K^+-Cl^-$ cotransport was concentration dependent and therefore the consequence of a direct alkylation. However, the inhibition of the $Na^+-K^+-Cl^-$ cotransporter may have been obscured by the response of the cell to toxic insult with the lower concentrations of HN2. This is further discussed below.

The effect of HN2 on potassium transport systems was investigated in human erythrocytes so that the control of the $Na^+-K^+-Cl^-$ cotransporter by either new gene expression in response to HN2 or the the release of lysosomal proteins could be discounted. First, the inhibition of the $Na^+-K^+-Cl^-$ cotransporter was greater than that of the Na^+/K^+ATPase pump in human erythrocytes 2 hours after treatment with 2mM HN2 (results 3.2.1). Secondly, cycloheximide, an inhibitor of protein synthesis, did not absolve the inhibition of ^{86}rubidium$^+$ uptake in L1210 cells incubated for 3 hours with 10uM HN2. The concentration of cycloheximide (5ug/ml) inhibited protein synthesis by 72% and a more rigorous regime may have resulted in no inhibition of potassium transport by 10uM HN2 (results 3.5.). These results indicate that the inhibition of the $Na^+-K^+-Cl^-$ cotransporter in L1210 cells after exposure to 10uM HN2 is unlikely to be due to new protein synthesis or the possible release of lysosomal proteins.

A number of investigators have proposed that cell death induced by HN2 may be a multi-modal process (Wheeler 1967; Tritton and Hickman 1984). If inhibition of the Na^+-K^+-Cl^- cotransporter was important then it would be expected that loop diuretics would be potent inhibitors of cell growth and/or would be cytotoxic. However, the complete inhibition of Na^+-K^+-Cl^- cotransport activity by loop diuretics did not result in the inhibition of cell growth (Wilcock et al 1988; Doppler et al 1985; Grunicke et al 1985a) indicating that it was not alone a site responsible for the antitumour activity of HN2. Wilcock et al (1988) considered that the sub-lethal damage induced by HN2, concomitant with the inhibition of Na^+-K^+-Cl^- cotransport, may result in cell death although it was not a lethal lesion in itself. Grunicke et al (1985a) demonstrated that furosemide did not alter the toxicity of 2uM HN2, but at this particular concentration of HN2 employed, Na^+-K^+-Cl^- cotransport was completely inhibited, so that the lack of effect may not be surprising.

In the experiments reported here, incubation of L1210 cells with bumetanide did not alter the cytotoxicity of HN2 over a range of concentrations (results 3.3.1.). As cytotoxic concentrations of Me-HN1 did not inhibit Na^+-K-$^+Cl^-$ cotransport, the addition of bumetanide may have provided two necessary sites of damage which would result in cell death. However, bumetanide did not enhance the cytotoxicity of Me-HN1 (results 3.3.1). Thus, the proposal

that the Na^+-K^+-Cl^- cotransporter may be a secondary lesion necessary for cell death induced by alkylating agents did not appear to be substantiated by these results.

van Ankeren and Wheeler (1985) demonstrated that the recovery of rat 9L brain tumour cells exposed to radiation-induced potentially lethal damage was inhibited by incubation in hypertonic media. However, no difference in HN2 cytotoxicity was observed in L1210 cells incubated with a X2 RPMI 1640 media (results 3.3.1) although this may be due to the lower strength of hypertonic media used. The recovery of cells exposed to potentially lethal radiation damage can be either enhanced or depressed by various post and pre-treatment schedules. However, such schedules do not necessarily enhance the cytotoxicity of "radio-mimetic" compounds such as HN2. Alexander (1969) proposed that these terms were unhelpful as it obscured the fact that each drug induced death in a variety of manners.

An L1210FR cell line which was able to grow in concentrations of furosemide which were completely cytotoxic to parental L1210 cells was prepared (results 3.3.2). It was assumed that under such conditions the Na^+-K^+-Cl^- cotransporter would be completely inhibited although no experiments were performed to determine if Na^+-K^+-Cl^- cotransport was pharmacologically insensitive to furosemide or functionally inactive. The question of whether resistance to furosemide was coincident with the expression of HN2 resistance was investigated in order to further

ascertain the importance of the $Na^+-K^+Cl^-$ cotransporter as a site of cytotoxic interaction of HN2. HN2 was equally cytotoxic to L1210 and L1210FR cells (results 3.3.2). Thus, an inactive $Na^+-K^+-Cl^-$ cotransporter did not appear to confer resistance upon L1210FR cells to HN2 although Wilcock (1987) demonstrated that an L1210 cell line resistant to HN2 did not express a pharmacologically active $Na^+-K^+-Cl^-$ cotransporter. However, in response to growing in toxic concentrations of furosemide, other ion transport systems may have compensated for the loss of $Na^+-K^+-Cl^-$ cotransport activity and so rendered the L1210 cells susceptible to HN2 toxicity. As HN2 toxicity in L1210 cells was unaltered by incubation with bumetanide, hypertonic media or resistance to furosemide, the proposition that the $Na^+-K^+-Cl^-$ cotransporter was a secondary lesion important in cell death did not appear to be substantiated by the results presented in this thesis (results 3.3.1 and 3.3.2).

Potassium transport was not inhibited in L1210 cells for up to 24 hours after treatment with either 1 or 0.5uM HN2. In contrast, the $Na-K^+-Cl^-$ cotransporter of L1210 cells was selectively inhibited 4 hours after exposure to 10uM HN2 (results 3.1.2 and 3.4.1). The lack of inhibition of potassium transport in L1210 cells treated with the lower concentrations of HN2 may have been due to differences in the pattern of cell death. This thesis was further confirmed by the cell cycle analysis, by flow cytometry, of L1210 cells exposed to HN2 (results 3.4.3). The imposition

of a non-specific cell cycle block was observed in L1210 cells exposed to 10uM HN2. In contrast, cells treated with either 1 or 0.5uM HN2 became blocked in either S or G_2/M, results comparable with those of Brox et al (1980).

Cell death may occur by a variety of pathways (introduction 1.6.1). The two best defined processes of cell death are necrosis and apoptosis and the criteria for distinguishing these have been presented by Wyllie (1981). A good criterion to distinguish these two types of cell death is the change in cell volume (Wyllie 1981). Necrosis is characterised by increases in cell volume whilst apoptotic cells become smaller.

In agreement with the results presented by Wilcock et al (1988), a decrease in the volume of L1210 cells was observed, when estimated by radio-isotope equilibration, which was maintained up to 72 hours after treatment with 10uM HN2 (results 3.4.2). Wilcock and Hickman (1988) also reported a decrease in the cell volume of L1210 cells incubated with loop diuretics at concentrations which completely inhibited $Na^+-K^+-Cl^-$ cotransport activity. Thus, the reduction in cell volume observed after exposure to 10uM HN2 may be characteristic of apoptosis. The mechanism by which apoptotic cells reduce their volume has not been identified (Lockshin and Beauloton 1981). Lochshin and Beauloton (1981) speculated that the loss of potassium ions from apoptotic cells may be due to the precipitation of major proteins or the contraction of cytoskeletal

components. Alternatively, the characteristic reduction in the volume of apoptotic cells may be due to the inhibition of ion transport processes such as the $Na^+-K^+-Cl^-$ cotransporter.

Allen (1987) suggested that both necrosis and apoptosis could be observed in a population of haemopoietic cells deprived of nutrient. Kerr (1987) noted that apoptotic cells underwent coagulative necrosis during which rapid increases in volume could be observed. Thus, two populations of cells, distinguished by their respective volumes, could exist in a sample of dying cells. This may explain the discrepancies in cell volume, according to the method of estimation, observed in L1210 cells treated with 1 and 0.5uM HN2 (results 3.4.2). Large increases were noted when cell size was estimated with a Coulter Counter; but no significant changes were observed when volume was assayed by radio-isotope equilibration. The Coulter Counter may have measured the volume of one particular population of cells which increased in size whilst radio-isotope equilibration gave an average value for the whole sample. Therefore, techniques such as flow cytometry should be employed which can identify the individual components in populations of cells committed to cell death.

The volume of L1210 cells treated with 10uM HN2 exhibited a reduced volume typical of apoptosis, but analysis of transmission electron micrographs demonstrated a pattern of chromatin clumping reminiscent of necrosis

(results 3.4.7). These contradictory observations may be reconciled by the possible existence of a number of different patterns of cell death, not only apoptosis and necrosis. In a review, Hume (1987) reported that there were at least four patterns of cell death in epithelial cells other than necrosis and apoptosis. These included type B dark cells which are characterised by condensed nuclei but swollen mitochondria and ghost cells which are surrounded by a discrete cell membrane but lack any nuclear remnants.

A number of investigators have observed rapid increases in cytosolic calcium after exposure to toxic insult (Schanne et al 1979; McConkey et al 1989; Lemaster et al 1987). In all these systems rapid losses in cell viability, determined by the exclusion of trypan blue dye, were observed. In contrast, no increases in internal calcium concentrations were observed until 48 hours after L1210 cells were treated with 10uM HN2 (results 3.4.5) and loss in cell viability was correspondingly slower (results 3.5). The slow onset of HN2-induced cell death may result in only a small proportion of cells exhibiting increases in cytosolic calcium at any one time and the technique employed (Quin-2 fluorescence) may not have been able to detect such small changes. Therefore, the rate at which cell death occurs, and also by implication the dose of the toxic insult, is of importance in observing changes in the morphology and biochemistry of the cell.

Wyllie et al (1984) proposed that apoptotic cell death was an active process which required new gene expression. Gut epithelial cells of Wistar rats were protected from the toxic effects of HN2 by treatment with the inhibitor of protein synthesis, cycloheximide (Lieberman et al 1970). Cycloheximide (5ug/ml) did not protect the murine leukaemic L1210 cells from the toxic effects of HN2 in vitro (results 3.5). This result would suggest that death induced by HN2 was not apoptotic. However, 5ug/ml cycloheximide inhibited protein synthesis by only 72% in murine leukaemic L1210 cells (results 3.5) and not the 80% that Lieberman et al (1970) reported as being necessary to protect gut epithelia from the toxic effects of HN2. Lieberman et al (1970) also noted that cycloheximide did not protect the lymphoid tissues of Wistar rats from the toxic effects of HN2. Therefore, the origin and type of tissue employed is also of importance in determining the pattern of cell death.

The concept that cell death can be easily defined into a limited number of categories may be too simplistic, and that in fact it is a very complex process dependent on a large variety of factors. Some of the possible factors have been illustrated above with examples from this thesis. These have included the type of tissue employed; the dose of the toxic insult and the possible existence of two different patterns of cell death within a population of moribund cells.

Little is known of the cellular events which occur after exposure to a toxic insult and how they committ a cell to death. Berger (1986) emphasised that a greater knowledge of the processes and mechanisms of cell death could lead to advances in the treatment of malignant disease. However, until recently, cell death attracted little attention from workers in the field of cancer chemotherapy. Changes in the activity of the $Na^+-K^+-Cl^-$ cotransporter have been implicated in cell division (Panet 1985) and cell death (Wilcock et al 1988). However, inhibition of the $Na^+-K^+-Cl^-$ cotransporter is not a lethal lesion in itself but changes in its activity may be indicative of a committment to death by the cell. Thus, the possible role and importance of ion transporters, and especially the $Na^+-K^+-Cl^-$ cotransporter, in the process of cell death is an area that merits further investigation.

5. REFERENCES.

Alexander, P. Comparision of the mode of action by which some alkylating agents and ionizing radiations kill mammalian cells. Ann. N.Y. Acad. Sci. 163: 652-675 (1969).

Alexander, P. and Mikulski, Z.B. Differences in the response of leukaemia cells in tissue culture to nitrogen mustard and to dimethyl myleran. Biochem. Pharmacol. 5: 275-282 (1961).

Allen, T.D. Ultrastructural aspects of cell death. In: Perspectives on mammalian cell death (Ed. Potten, C.S.) pp 39-66. Oxford University Press, Oxford, New York, Tokyo. (1987).

Andrews, G.K., Harding, M.A., Calvet, J.P. and Adamson, E.D. The heat shock response in HeLa is accompanied by elevated expression of the c-fos proto-oncogene. Mol. Cell. Biol. 7:3452-3458 (1987).

Ankel, E.G., Ring, B.J., Lai, C.-S, and Holcenbrg. The lack of effects of alkylating agents on mammalian cell membranes. Int. J. Tiss. Reac. 8: 347-354 (1986).

Amsler, K., Donahue, J.J., Slayman, C.A., and Adelberg, E.A. Stimulation of bumetanide-sensitive potassium transport in Swiss 3T3 fibroblasts and mitogenic hormones. J. Cell. Physiol. 123: 257-263 (1985).

Auerbach, F. and Robson, J.H. Chemical production of mutations. Nature 157: 302 (1946).

Baserga, R. Growth in size and cell DNA replication. Exp. Cell Res. 151: 1-5.

Baum, B.J., Johnson, L.S., Franzblau, C. and Troxler, R.F. Incorporation of L-azetidine-2-carboxylic acid into hemoglobin in rabbit reticulocytes in vitro. J. Biol. Chem. 250: 1464-1471 (1975).

Baxter, M.A., Chawala, S.B., Hickman, J.A. and Spurgin, G.E. Effects of nitrogen-mustard on the activities of plasma membrane of PCA6 plasmacytoma cells. Biochem. Pharmacol. 31: 1773-1778 (1982).

Ben-Ishay, Z. and Farber, E. Protective effects of an inhibitor of protein synthesis, cycloheximide, on bone marrow damage induced by cytosine araboniside or nitrogen mustard. Lab. Invest. 33:478-490 (1975).

Berger, N.A. Cancer chemotherapy: new strategies for success. J. Clin. Invest. 78: 1131-1135 (1986).

Berman, H.M., McGandy, E.L., Burgner, J.W., VanEtten, R.L. The crystal and molecular structure of L-azetidine-2-carboxylic acid. A naturally occurring homolog of proline. J. Am. Chem. Soc. 91: 6177-6182 (1969).

Berridge, M.J. and Irvine, R.F. Inositol phosphates and cell signalling. Nature 341: 197-205 (1989)

Bhuyan, B.K., Loughman, B.E., Fraser, T.J. and Day, K.J. Comparision of different methods to determine cell viability after exposure to cytotoxic compounds. Exp. Cell Res. 97: 275-280 (1976).

Birnbaumer, L., Codina, J., Mattera, R., Yatani, A., Scherer, N., Toro, M-J and Brown, A.M. Signal transduction by G proteins. Kidney Int. 32: S14-37 (1987).

Blaustein, M.P. and Nelson, M.T. Sodium-calcium exchange: its role in the regulation of cell calcium. In:Membrane transport of calcium p217-236 (Ed. Carafoli, E.) Academic Press, London (1982).

Bocciarelli, D.S. and Violante, A. An experimental leukaemia studied with the electron microscope. Rend. Ist. Super. Sanita 22: 1068-1074 (1960).

Bonner, W.M. and Laskey, R.A. A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83-88 (1974).

Boobis, A.R., Fawthrop, D.J. and Davies, D.S. Mechanisms of cell death. TiPS 10: 275-280 (1989).

Bouchy, M., Donner, M. and Andre, J.C. Evolution of flourescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) during the labelling of living cells. Exp. Cell Res. 133: 39-46 (1981).

Brewer, H.B., Comstock, J.P., and Aronow, L. Effects of nitrogen mustard on protein and nucleic acid synthesis in mouse fibroblasts growing in vitro. Biochem. Pharmacol. 8: 281-287 (1961).

Brock, T.A., Brugnara, C., Canessa, M. and Gimbrone Jr., A. Bradykinin and vasopressin stimulate $Na^+-K^+-Cl^-$ cotransport in cultured endothelial cells. Am. J. Physiol. 250: C888-C895 (1986).

Brookes,, P. and Lawley, P.D. The reaction of mono- and di-functional alkylating agents with nucleic acids. Biochem. J. 80: 496-503 (1961).

Brox,L.W., Gowans, B. and Belch, A. L-phenylalanine mustard (Melphalan) uptake and crosslinking in the RPMI 1640 human lymphoblastoid cell line. Cancer Res. 40: 1169-1172 (1980).

Burdon, R.H. Heat shock and the heat shock proteins. Biochem. J. 240: 313-324 (1986).

Buttyan, R., Zakeri, Z., Lockshin, R. and Wolgemuth, D. Cascade induction of c-fos, c-myc, and heat shock 70K transcripts during regression of the rat ventral prostate gland. Mol. Endo. 2: 650-657 (1988).

Caspersson, T., Farber, S., Foley, G.E. and Killander, D. Cytochemical observations on the nucleolus-ribosome system: effects of actinomycin D and nitrogen mustard. Exp. Cell Res. 32: 529-552 (1963).

Chassande, O. Frelin, C., Farahifar, D., Jean, T. and Lazdunski, M. The $Na^+-K^+-Cl^-$ cotransport in C6 glioma cells. Eur. J. Biochem. 177: 425-433 (1988).

Chahwala, S.B. and Hickman, J.A. Investigations of the action of the antitumour drug adriamycin on tumour cells membrane function. Biochem. Pharmacol. 34: 1501-1505 (1985).

Chipperfield, A.R. The $Na^+-K^+-Cl^-$ cotransport system. Clin. Sci. 71: 456-476 (1986).

Chmielewicz, Z.F., Fiel, R.J., Bardos, T.J. and Ambrus, J.L. Alterations of some macromolecular and biochemical properties of calf thymus DNA caused by "Dual antagonists" and nitrogen mustard. Cancer Res. 27: 1248-1257 (1967).

Cho Chung, Y.S. On the mechanism of cyclic AMP-mediated growth arrest of solid tumours. Advances in Cyclic Nucleotides Research 12: 111-121 (1980).

Chu, M.Y. and Fisher, G.A. Incorporation of cytosine-^{3}H-arabinoside and its effect on the murine leukaemic cell (LY5178Y). Biochem. Pharmacol. 17: 753-767 (1968).

Cobb, J.P. The comparative cytological effects of several alkylating agents on human normal and neoplastic cells in tissue culture. Ann. N.Y. Acad. Sci. 84: 515-542 (1960).

Crathorn, A.R. and Roberts, J.J. Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. Nature 211: 150-153 (1966).

Dean, S.W. and Fox, M. DNA repair, DNA synthesis and cell cycle in human lymphoblastoid cells differentially sensitive to the cytotoxic effects of nitrogen mustard. Mutation Res. 132: 63-72 (1984).

DeCosse, J.J. and Gelfont, S. Effects of nitrogen mustard during the cell cycle of the Ehrlich ascites tumor. Exp. Cell Res. 60: 185-190 (1970).

Doppler, W., Hofman, J., Oberhuber, H., Maly, K. and Grunicke, H. Nitrogen-mustard interference with potassium transport systems in Erlich Ascites cells. Eur. J. Can. Res. Clin. Oncol. 110: 35-41 (1985).

Duhm, J. and Gobel, B.O. Role of the furosemide-sensitive Na^+/K^+ transport system in determining the steady state Na^+ and K^+ content and volume of human erythrocytes in vitro and in vivo. J. Membrane Biol. 77: 243-254 (1984).

Dumont, J.E., Jauniaux, J-C and Roger, P.P. The cyclic AMP-mediated stimulation of cell proliferation. TIBS 14: 67-71 (1989).

Duncan, R.F. and Hershey, J.W.B. Translational repression by chemical inducers of the stress response occurs by different pathways. Arch. Biochem. Biophys. 256: 651-661 (1987).

Edelstein, M.B. and Heilburn L.K. Specificity, schedule and proliferation dependence of infused l-histidinol after 5-flourouracil in mice. Cancer Res. 48: 1470-1475 (1988).

Ellory, A.C. and Stewart, G.W. The human erythrocyte Cl-dependent Na-K cotransport system as a possible model for studying the action of loop diuretics. Br. J. Pharmac. 75: 183-188 (1982).

Ewig, R.A.G. and Kohn, K.W. DNA damage and repair in mouse leukemia cells treated with nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. Cancer Res. 37: 2114-2122 (1977).

Feit, P.W., Hoffman, E.K., Schiodt, M., Kristensen, P., Jessen, F. and Dunham, P.B. Purification of Na/Cl cotransporter proteins from membranes of Ehrlich ascites cells using a bumetanide-Sepharose affinity column. J. Membrane Biol. 103: 135-147 (1988).

Forbush, B. and Palfrey, H.C. [^{3}H] Bumetanide binding to membranes isolated from dog kidney outer medulla. J. Biol. Chem. 258: 11787-11792 (1983).

Fornace, A.J., Alamo, I., Hollander, M.C. and Lamoreaux, E. Induction of heat shock protein transcripts and B2 transcripts by various stresses in Chinese Hamster cells. Exp. Cell. Res. $\underline{182}$: 61-74 (1989).

Frelin, C., Chassander, O. and Lazdunski, M. Biochemical characterisation of the $Na^+-K^+-Cl^-$ co-transport in chick cardiac cells. Biochem. Biophys. Res. Commun. $\underline{134}$: 326-331 (1986).

Friedman, M.O. and Boger, E. Colorimetric estimation of nitrogen mustards in aqueous media. Anal. Chem. $\underline{33}$: 906-910 (1961).

Futscher, B.W. and Erickson, L.C. Changes in c-myc and c-fos expression in a human tumour cell line following exposure to bifunctional alkylating agents. Cancer Res. $\underline{50}$: 62-66 (1990).

Garay, P.R. Inhibition of the $2Na^+/K^+$ cotransport system by cAMP and intracellular Ca^{2+} in human red cells. Biochim. Biophys. Acta $\underline{688}$: 786-792 (1982).

Gardos, G. The function of calcium in the potassium permeability of human erythrocytes. Biochem. Biophys. Acta $\underline{30}$: 653-654 (91958).

Ganz, M.B., Boyarsky, GT., Sterzel, R.B. and Boron, W.F. Arginine vasopressin enhances pH_i regulation in the presence of HCO_3^- by stimulating three acid-base transport systems. Nature $\underline{337}$: 648-650 (1989).

Geck, P., Pietrzyk, C., Burckhardt, B-C., Pfeiffer, B. and Heinz, E. Electrically silent cotransport of Na^+,K^+ and Cl^- in Ehrlich Ascites cells. Biochim. Biophys. Acta $\underline{600}$: 432-447 (1980).

Geck, P., Heinz, E. and Pfeiffer, B. Influence of high ceiling diuretics on ion fluxes and cell volume of Ehrlich ascites tumor cells. Scand. Audiol. Suppl. $\underline{14}$: 25-37 (1981).

Geck, P. and Pfeiffer, B. $Na^+-K^+-Cl^+$ cotransport in animal cells and its role in volume regulation. Ann. N.Y. Acad. Sci. USA $\underline{456}$: 166-182 (1985).

Geck, P. and Heinz, E. The $Na^+-K^+-Cl^-$ cotransport system. A topical review. J. Membrane Biol. $\underline{91}$: 97-105 (1986).

Geiduschek, E.P. "Reversible" DNA. Proc. Nat. Acad. Sci. $\underline{47}$: 950-955 (1961).

Goldacre, R.J., Loveless, A. and Ross, W.C.J. Mode of production of chromosome abnormalities by the nitrogen mustards. Nature 163: 667-669 (1949).

Goldenberg, G.J. Repair of sub-lethal damage of L5178Y lymphoblasts in vitro treated with dimethyl myleran and nitrogen mustard. Biochem. Pharmacol. 17: 820-824 (1966).

Goldenberg, G.J. and Alexander, P. The effects of nitrogen mustard and dimethyl myleran in murine leukaemic cell lines of different radiosensitivity in vitro. Cancer res. 25: 1401-1409 (1965).

Grinstein, S., Rothstein, A., Sarkardi, B. and Gelfand, E.W. Responses of lymphocytes to anisotonic media: volume-regulatory behaviour. Am. J. Physiol. 246: C204-215 (1984).

Grinstein, S., Rotin, D. and Mason, M.J. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. Biochim. Biophys. Acta 988: 73-97 (1989).

Grunicke, H., Hirsch, F., Wolf, H., Bauer, U. and Kiefer, G. Selective inhibition of thymidine transport at low doses of alkylating agent triethyleneiminobenzoquione (Trenimon). Exp. Cell. Res. 90: 357-364 (1975).

Grunicke, H., Gantner, G., Holzweber, M., Ihlenfeldt, M. and Puschendorf, B. New concepts on the interference of alkylating antitumour agents with the regulation of cell division. Adv. Enz. Regul. 17: 291-305 (1979).

Grunicke, H., Putzer, H., Scheidll, F., Wolff-Schreiner, E. and Grunewald, K. Inhibition of tumour growth by alkylation of the plasma membrane. Bioscience Reports 2: 601-604 (1982).

Grunicke, H., Grunewald, W., Helliger, W., Scheidl, F. Wolff-Schreiner, E. and Puschendorf, B. Inhibition of tumour growth by an alkylation of the plasma membrane. Adv. Enz. Regul. 21: 21-30 (1983).

Grunicke, H., Doppler, W., Finch, S.A.E., Greinert, R., Grunewald, K., Hofmann, J., Maly, K., Stier, A., Scheidl, F. and Thomas, J.K. Effects of nitrogen-mustard on potassium transport systems and membrane structure of Ehrlich Ascites tumour cells. Adv. Enz. Regul. 23: 227-290 (1985a).

Grunicke, H., Doppler, W., Hofmann, J., Linder, H., Maly, K., Oberhauer, H., Ringsdorf, H. and Roberts, J.J. The plasma membrane as a target of alkylating agents. Adv. Enz. Regul. 24: 247-261 (1985b).

Haas, M. and McManus, T.J. Bumetanide inhibits (Na+K+2Cl) co-transport at a chloride site. Am. J. Phsiol. 245: C235-C240 (1983).

Haas, M. and Forbush III, B. Photolabeling of a 150 kDa (Na+K+Cl) cotransport protein from dog kidney with a bumetanide analogue. Am. J. Phsiol. 253: C243-C250 (1987).

Hahn, G.M. and Li, G.C. Thermotolerance and heat shock proteins in mammalian cells. Radiat. Res. 72: 452-457 (1982).

Hendil, K.B. and Hoffmann, E.K. Cell volume regulation in Ehrlich ascites tumour cells. J. Cell. Physiol. 84: 115-126 (1974).

Hendry, J.H. and Scott, D. Loss of reproductive integrity of irradiated cells, and its importance in tissues. In: Perspectives on mammalian cell death (Ed. Potten, C.S.) pp160-183. Oxford University Press, New York, Toyko. (1987).

Henle, R.J. and Leeper, D.B. Interaction of hyperthermia and radiation in CHO cells: recovery kinetics. Radiat. Res. 90: 339-347 (1976).

Hesselwood, I.P. DNA strand breaks in resistant and sensitive murine lymphoma cells detected by hydroxyapatite chromatographic technique. Intern. J. Radiation Biol. 34: 461-469 (1978).

Hoffmann, E.K. Role of seperate K^+ and Cl^- channels and of Na^+/Cl^- cotransport in volume regulation in Ehrlich cells. Fed. Proc. 44: 2513-2519 (1985).

Hollander, M.C. and Fornace, Jr. A.J. Induction of fos RNA by DNA-damaging agents. Cancer Res. 49: 1687-1692 (1989).

Hume, W.J. A pathologist's view of cell death. In: Perspectives of mammalian cell death (Ed. Potten, C.S.) pp 66-92. Oxford University Press, Oxford, New York, Toyko (1987).

Ihlenfeldt, M., Ganter, G., Harer, M., Puschendorf B., Putzer, H. and Grunicke, H. Interaction of the alkylating antitumour agent 2,3,5-Tris(ethyleneimino)-benzoquinone with the plasma membrane of Ehrlich Ascites cells. Cancer Res. 41: 289-293 (1981).

Jewell, S.A., Bellomo, G., Thor, H. and Orrenius,S. Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. Science 217: 1257-1258 (1982).

Jorgensen, P. Petersen, J. and Rees, W.D. Identification of the Na^+-K^+-Cl^- cotransporter protein of Mr 34,000 from the kidney by photolabelling with [^{3}H]bumetanide. Biochim. Biophys. Acta 775: 105-110 (1984).

Kaji, D. Volume sensitive K transport in human erythrocytes. J. Gen. Physiol. 88: 719-738 (1986).

Kaufmann, S.C. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukaemia cells by etoposide, campotothecin and other cytotoxic anticancer drugs: a cautionary note. Cancer Res. 49: 5870-5878 (1989).

Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26: 239-257 (1972).

Kerr, J.F.R., Searle, J., Harmon, B.V. and Bishop, C.J. Apoptosis. In: Perspectives on mammalian cell death (Ed. Potten, C.S.) pp93-129. Oxford University Press, Oxford, New York, Tokyo. (1987).

Keyes, S.R., Hickman, J.A. and Sartorelli, A.C. The effects of adriamycin on intracellular calcium concentrations of L1210 murine leukaemic cells. Eur. J. Cancer Clin. Oncol. 23: 295-302 (1987).

Kim, H.D., Tsai, Y-S, Franklin, C.C. and Turner, J.T. Characterisation of Na^+/K^+/Cl^- cotransport in cultured HT29 human colonic adenocarcinoma cells. Biochem. Biophys. Acta 946: 397-404 (1988).

Koetles, G.J., Kubasova, T. and Varga, L. ^{3}H-concanavalin A binding of X-irradiated human fibroblasts. Nature 259:507-508 (1979).

Kohn, K.W., Spears, C.L. and Doty, P. Inter-strand crosslinking of DNA by nitrogen mustard. J. Mol. Biol. 19: 266-288 (1966).

Kohn. K.W. and Green, D.M. Transforming activity of nitrogen mustard-crosslinked DNA. J. Mol. Biol. 19: 289-296 (1966).

Koller, P.C. Comparative effects of alkylating agents on cellular morphology. Ann. N.Y. Acad. sci. 68: 783-801 (1957).

Kracke, G.C., Anatra, M.A. and Dunham, P.B. Asymmetry of Na$^+$-K$^+$-Cl$^-$ cotransport in human erythrocytes. Am. J. Physiol. <u>254</u>: C243-250 (1988).

Kregnow, F.M. Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. Ann. Rev. Physiol. <u>43</u>: 493-505 (1981).

Lauf, P.K. K$^+$:Cl$^-$ cotransport: sulfhydryls, divalent cations, and the mechanism of volume activation in a red cell. J. Membrane Biol. <u>88</u>: 1-13 (1985).

Ledbetter, M.L. and Lubin, M. Control of protein synthesis in human fibroblats by intracellular potassium. Exp. Cell. Res. <u>105</u>: 223-226 (1975).

Leffert, H.L. Growth regulation by ion fluxes. Ann. N.Y. Acad. Sci. <u>339</u>: (1980).

Lemasters, J.J., DiGuiseppi, J., Nieminen, A-L and Herman, B. Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. Nature <u>325</u>: 78-81 (1987).

Levinson, C. Volume regulatory activity of the Ehrlich ascites tumor cell and its relationship to ion transport. J. Membrane Biol. <u>100</u>: 183-191 (1987).

Levis, A.G., Danieli, G.A. and Piccinni, E. Nucleic acid synthesis and the mitotic cycle in mammalian cells treated with nitrogen mustard in culture. Nature <u>207</u>: 608-610 (1965).

Levy, M. The effect of mustard gases upon the property of nucleated erythrocytes and their ghosts. Cancer Res. <u>25</u>: 752-759 (1965).

Lew, V.L. and Ferreira, H.C. Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. Curret topics in membranes <u>10</u>: 218-273 (1978).

Li, G.C. and Laszlo, A. Amino acid analogs while inducing heat shock proteins sensitize CHO cells to thermal damage. J. Cell. Phys. <u>122</u>: 91-97 (1985).

Lieberman, M.W., Verbin, R.S., Landay,M., Liang, H., Farber, E., Tsun-Nin, L. and Starr, R. A probable role for protein synthesis in intestinal epithelial cell damage induced <u>in</u> <u>vivo</u> by cytosine arabinoside, nitrogen mustard or X-irradiation. Cancer Res. <u>30</u>: 942-951 (1970).

Lindquist, S. The heat-shock response. Ann. Rev. Biochem. <u>55</u>: 1151-1191 (1986).

Lockshin, R.A. and Beaulaton, J. Cell death: questions for histochemists concerning the causes of the various cytological changes. Histochem. J. 13: 659-666 (1981).

McConkey, D.J., Hartzell, P., Duddy, S.K., Hakansson, H. and Orrenius, S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. Science 242: 256-259 (1988).

McConkey, D.J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A.H. and Orrenius, S. Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. Arch. Biochem. Biophys. 269: 365-370 (1989).

McGown, L.T., Poppitt, D.G., Swindell, R. and Fox, B.W. The effect of vinca alkaloids in enhancing the sensitivity of a methotrexate reistant (L1210/R7A) line, studied by flow cytometric and chromosome number analysis. Cancer Chemother. Pharmacol. 13: 47-53.

Mauro, F. and Madoc-Jones, H. Age responses of cultured mammalian cells to cytotoxic drugs. Cancer Res. 30: 1397-1408 (1970).

Meares, P., Foley, T. and Mengual, J.I. Interaction of tracer and non tracer fluxes in membranes. In: Membrane transport processes (Ed. Hoffmann, J.F.) Vol. 1, p1-12. Raven Press, Nerw York (1978).

Medrano, E.E. and Pardee, A.B. Prevalent deficiency in tumour cells of cycloheximide-induced cycle arrest. Proc. Natl. Acad. Sci. USA 77: 4123-4126 (1980).

Melchior, D.L. and Steim, J.M. Thermotropic transitions in membranes Ann. Rev. Biophys. Bioeng. 5: 205-238 (1976).

Mesland, D.A.M., Los, G. and Spiele, H. Cytochalasin B disrupts the assocciation of filamentous web and plasma membrane in hepatocytes. Exp. Cell Res. 135: 431-435 (1981).

Micetich, K., Zwelling, L.A. and Kohn, K.W. quenching of DNA: platinum (II) monoadducts as a possible mechanism of resistance to cis-diamminedichloroplatinum(II) in L1210 cells. Cancer Res. 43: 3609-3613 (1983).

Milarski, K.L. and Morimoto, R.L. Expression of human HSP-70 during the synthetic phase of the cell cycle. Proc. Natl. Acad. Sci. USA 83:9517-9521 (1986).

Mills, D.C.B. and Thomas, d.P. Blood platelet nucleotides in man and other species. Nature 222: 991-992 (1969).

Moolenar, W.H., Tsien, R.Y., van der Sang, P.T. and de Laat, S.W. Na^+/H^+ exchange and cytoplasmic in the action of growth factors in human fibroblasts. Nature $\underline{304}$: 645-648 (1983).

Nicotera, P., Hartzell, P., Baldi, C., Svensson, S-K., Bellomo, G., and Orrenius, S. Cystamine induces toxicity in hepatocytes through the elevation of cytosolic Ca^{2+} and the stimulation of a nonlysomal proteolytic system. J. Biol. Chem. $\underline{261}$: 14628-14635 (1986).

Nieminen, A-L., Gores, G.J., Wray, B.E., Tanaka, Y., Herman, B. and Lemasters, J.J. Calcium dependence of bleb formation and cell death in hepatocytes. Cell Calcium $\underline{9}$: 237-246 (1988).

Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature $\underline{308}$:693-698 (1984).

Nishikura, K. and Murray, J.M. Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells. Mol. Cell. Biol. $\underline{7}$: 639-649 (1987).

O'Brien, T.G. and Krzeminski, K. Phorbolester inhibits furosemide-sensitive potassium transport in BALB/c 3T3 preadipose cells. Proc. Natl. Acad. Sci. USA $\underline{80}$: 4334-4338 (1983).

O'Brien, T.G. and Prettyman, R. Phorbol esters and mitogenesis: comparision of the proliferative response of parental and $Na^+-K^+-Cl^-$ cotransport defective BALB/c 3T3 cells to 12-O-tetradecanoylphorbol-13-acetate. J. Cell. Physiol. $\underline{130}$: 377-381 (1987).

O'Brien, T.G., George, K. and Prettyman, R. Protein kinase C and membrane transport: divergent responses of $Na^+/K^+/Cl^-$ cotransport and sugar transport to exogenous diacylglycerol. Biochim. Biophys. Acta $\underline{945}$: 41-50 (1988).

O'Donnell, M.E. and Owen, N.E. Role of cyclic GMP in atrial natriuretic factor stimulation of $Na^+-K^+-Cl^-$ cotransport in vascular smooth muscle cells. J. Biol. Chem. $\underline{261}$: 15461-15466 (1986a).

O'Donnell, M.E. and Owen, N.E. Atrial natriuretic factor stimulates $Na^+-K^+-Cl^-$ cotransportin vascular smooth muscle cells. Proc. Natl. Acad. Sci. USA. $\underline{83}$: 6132-6136 (1986b).

O'Donnel, M.E., Bush, E.N. Holleman, W. and Owen, N.E. Biologically active atrial natriuretic peptides selectively activate Na/K/Cl cotransport in vascular smooth muscle cells. J. Pharmacol. Expl. Ther. $\underline{243}$: 822-828 (1987).

O'Grady, S.M., Palfrey, H.C. and Field, M. Charateristics and functions of $Na^+-K^+-Cl^-$ cotransport in epithelial tissues. Am. J. Physiol. <u>253</u>: C177-C192 (1987).

O'Neill, W.C. and Mikkelsen, R.B. Furosemide-sensitive Na^+ and K^+ transport and human erythrocyte volume. Biochim. Biophys. Acta <u>896</u>: 196-202 (1987).

Ord, M.J. The site of damage in amoebae exposed to low concentrations of Methyl di(ß-chloroethyl)-amine (a Nitrogen Mustard). Qrt. J. Micros. Sci. <u>97</u>: 39-48 (1958).

Ord, M.J. and Danielli, J.F. The site of damage in amoebae exposed to lethal concentrations of methyl di-(ß-chlorethyl)-amine (a Nitrogen Mustard). Qrt. J. Micros. Sci. <u>97</u>: 17-29 (1958).

Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. Role of Ca^{2+} in toxic cell killing. TiPS <u>10</u>: 281-285 (1989).

Owen, N.E. Regulation of Na/K/Cl cotransport in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. <u>125</u>: 500-508 (1984).

Owen, N.E. and Prastein, M.L. Na/K/Cl cotransport in cultured human fibroblasts. J. Biol. Chem. <u>260</u>: 1445-1451 (1985).

Owen, N.E. Effect of TPA on ion fluxes and DNA synthesis in vascular smooth muscle cells. J. Cell Biol. <u>101</u>: 454-459. (1985).

Owen, N.E. and Ridge, K.M. Mechanism of angiotensin II stimulation of $Na^+-K^+Cl^-$ cotransport of vascular smooth muscle cells. Am. J. Physiol. <u>257</u>: C629-639 (1989).

Palfrey, H.C. and Greengard, P. Hormone-sensitive ion transport systems in erythrocytes as models for epithelial ion pathways. Ann. N.Y. Acad. Sci. USA <u>372</u>: 291-307 (1981).

Palfrey, H.C. and Rao, M.C. $Na^+-K^+-Cl^-$ cotransport and its regulation. J. Exp. Biol. <u>106</u>: 43-54 (1983).

Panet, R. Serum induced net potassium influx performed by diuretic-sensitive transport system in quiescent NIH 3T3 mouse fibroblasts. Biochim. Biophys. Acta <u>813</u>: 141-144 (1985).

Pang, K-Y., Chang, T-L. and Miller, K.W. On the coupling between anesthetic induced membrane fluidization and cation permeability in lipid vesicles. Mol. Pharmacol. 15: 729-738 (1979).

Papahadjopoulos, D., Jacobson, K. and Isac, T. Phase transitions in lipid vesicles: fluorescence polarization and permeability measurements concerning the effects of temperature and cholestrol. Biochim. Biophys. Acta 311: 330-348 (1973).

Paris, S. and Pouyssegur, J. Growth factors activate the bumetanide-sensitive Na^+-K^+-Cl^- cotransport in Hamster fibroblasts. J.Biol. Chem. 261: 6177-6183 (1986).

Paris, S. and Pouyssegur, J. Further evidence for a phospholipase C coupled G-protein in Hasmster fibroblasts. J. Biol. Chem. 262: 1970-1976 (1987).

Paris, S., Chambard, J-C. and Pouyssegur.J. Coupling between phosphoinositide breakdown and early mitogenic events in fibrblasts. J. Biol. Chem. 262: 1977-1983 (1987).

Peters, R.A. Biochemical research at Oxford upon nitrogen-mustard gas. Nature 159: 149-151 (1947).

Pratt, W.B. and Ruddon, R.W. The anticancer drugs. Oxford University Press, Oxford, New York. (1979)

Price, C.C., Gaucher, G.M., Koneru, P., Shibakawa, J.R., Sowa, R., and Yamaguchi, M. Mechanism of action alkylating agents. Ann. N.Y. Acad. Sci. 164: 593-600 (1969).

Rauscher, F.J., Cohen, D.R., Curran, T., Bos, T.J., Vogt, P.K., Bohmann, D., Tijan, T. and Franza, R. Fos-associated protein p39 is the product of the jun proto-oncogene. Science 240:1009-1016 (1988).

Roberts, J.J., Brent, T.P. and Crathorn, A.R. Evidence for the inactivation and repair of the mammalian DNA template after alkylation by mustard gas and half-mustard gas. Europ. J. Cancer 7: 515-524 (1971).

Roper, P.R. and Drewinko, B. Comparision of in vitro methods to determine drug-induced cell lethality. Cancer Res. 36: 2182-2188 (1976).

Ross, W.E., Ewig, R.G. and Kohn, K.W. Differences between the formation and removal melphalan and nitrogen-mustard in of DNA cross-links. Cancer Res. 38: 1502-1506 (1978).

Rossier, B.C., Geering, K. and Kraehenbul, J.P. Regulation of the sodium pump: how and why? TIBS 12: 483-487 (1987).

Ruddon, R.W., Johnson, J.M. The effects of nitrogen mustard on DNA template activity in purified DNA and RNA polymerase systems. Mol. Pharmacol. 4: 258-273 (1968).

Saier, M.H. and Boyden, D.A. Mechanism, regulation and physiological significance of the loop diuretic-sensitive NaCl\KCl symport system in animal cells. Mol. Cell. Biochem. 59: 11-32 (1984).

Sakamoto, K. and Elkind, M.M. X-rays and nitrogen mustard: independent action in chinese hamster cells. Biophys. J. 7: 1115-1130 (1969).

Sato, C. and Yonei, S. Membrane changes. In: Perspectives on mammalian cell death (Ed. Potten, C.S.) pp1-18. Oxford University Press, Oxford, New York, Tokyo (1987).

Schaefer, E.L., Morimoto, R.I., Theodorakis, N.G. and Seidenfeld, J. Chemical specificity for induction of stress response genes by DNA-damaging drugs in human adenocarcinoma cells. Carcinogenesis 9: 1733-1738 (1988).

Schanne, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L. Calcium dependence of toxic cell death: a final common pathway. Science 206: 700-703 (1979).

Schatzmann, H.J. and Vincenzi, F.F. Calcium movemets across the membrane of human red cells. J. Physiol. 201: 369-395 (1969).

Searle, J. Lawson, T.A., Abbott, P.J., Harmon, B. and Kerr, J.F.R. An electron-microscope study of the mode of cell death induced by cancer chemotherapeutic agents in populations of proliferating normal and neoplastic cells. J. Path. 116: 129-138 (1975).

Setoyama, C. Frunzio, R., Liau, G., Mudryi, M., De Crombrugghe, B. Transcriptional activation encoded by the vfos gene. Proc. Natl. Acad. Sci. USA 83: 3213-3217 (1986).

Skou, J.C. Enzymatic basis for the active transpoirt of Na^+ and K^+ across the cell membrane. J. Physiol. 45: 596-615 (1965).

Spurgin, G.E. The effect of alkylating agent on tumour cell ATPase enzymes. Ph.D. Thesis, Departemnt of Pharmacy, University of Aston (1981).

Stolfi, R.L., Sawyer, R.C. and Martin, D.S. Failure of l-histidinol to improve the therapeutic efficiency of 5-fluorouracil against murine breast tumours. Cancer Res. 47: 16-20 (1987).

Strandberg, M.C., Bresnick, E. and Eastman, A. The significance of DNA cross-linking to cis-diamminedichloro-platinum (II)-induced cytotoxicity in sensitive and resistant lines of murine leukaemia L1210 cells. Chem. Biol. Inter. $\underline{15}$: 169-180 (1982).

Smith, J.B. and Smith, L. $Na^+-K^+-Cl^-$ cotransport in cultured vascular smooth muscle cells: stimulation by angiotensin II and calcium ionophores , inhibition by cAMP and calmodulin antagonists. J. Membrane Biol. $\underline{99}$: 51-63 (1987).

Sussman, I. and O'Brien, T.G. Characterization of a BALB/c 3T3 preadipose cell mutant with altered $Na^+-K^+-Cl^-$ cotransport activity. J. Cell. Physiol. $\underline{124}$: 153-159 (1985).

Sussman, I., Prettyman, R. and O'Brien, T.G. Phorbol esters and gene expression: the role of rapid changes in K^+ transport in the induction of ornithine decarboxylase by 12-O-teradecanoylphorbol-13-acetate in BALB/c 3T3 cells and a mutant cell line defective in $Na^+-K^+-Cl^-$ cotransport. J. Cell Biol. $\underline{101}$: 2316-2323 (1985).

Tas, P.W.L., Massa, P.T., Kress, H.G. and Koschel, K. Characterisation of an $Na^+-K^+-Cl^-$ co-transport in primary cultures of rat astrocytes. Biochim. Biophys. Acta. $\underline{903}$: 411-416 (1987).

Thomas,G.P. and Mathews, M.B. Alterations of transcription and translation in HeLa cells exposed to amino acid analogs. Mol. Cell. Biol. $\underline{4}$: 1063-1072 (1984).

Thomlinson, R.H. and Gray, L.H. The histilogical structure of some human lung cancers and the possible implications for radiotherapy. Br. J. Cancer $\underline{9}$: 539-548 (1956).

Tisdale, M.J. and Phillips, B.J. Comparative effects of alkyalting agents and other anti-tumour agents on the intracellular level of adenosine 3',5'-monophosphate in Walker carcinoma. Biochem. Pharmacol. $\underline{24}$: 1271-1276 (1975).

Toteson, D.C. and Hoffman, J.F. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J. Gen. Physiol. $\underline{44}$: 169-194 (1960).

Tritton, T.R. and Hickman, J.A. Cell surface membranes as chemotherapeutic targets In Chemotherapy. vol.II (McGuire.W.E., ed.) Marinus Nijhof, Den Hague (1984).

Trump, B.F. and Berezesky, I.K. Ion regulation, cell injury and carcinogenesis. Carcinogenesis $\underline{8}$: 1027-1031 (1987).

Tsien, R.Y, Pozzan, T. and Rink, T.J. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped flourescent indicator. J. Cell Biol. 94: 325-334 (1982).

van Ankeren, S.C. and Wheeler, K.T. Relationship between DNA repair and cell recovery: importance of competing biochemical and metabolic processes. Radiat. Environ. Biophys. 24: 271-280 (1985).

van Putten, L.M. and Lelieveld, P. Factors determining cell killing by chemotherapeutic agents in vivo. Europ. J. Cancer 7: 11-16 (1971).

Velazquez, J.M. and Lindquist, S. hsp70: Nuclear concentration during enviromental stress and cytoplasmic storage during recovery. Cell 36: 655-662 (1984).

Verma, I.M. Proto-oncogene fos: a multifaceted gene. TIG 2: 93-96 (1986).

Warwick, G.P. The mechanism of alkylating agents. Cancer Res. 23: 1315-1333 (1963).

Warrington, R.C. A novel approach for improving the efficacy of experimental cancer chemotherapy using combinations of anticancer drugs and L-histidinol. Anticancer Res. 6: 451-464 (1986).

Warrington, R.C. and Fang, W.D. Proliferative responses to L-histidinol are correlated with the phenotypic status of normal and transformed CHO cells. Cell Biol. Int. Reports 6: 309-317 (1982).

Warrington, R.C., Muzyka, T.G. and Fang, W.D. Histidinol-mediated improvement in the specificity of 1-ß-D-arabinofuranosylcytosine and 5-flourouracil in L1210 leukaemia-bearing mice. Cancer Res. 44: 2929-2935 (1984).

Warrington, R.C., Cheng, I. and Fang, W.D. Effects of 1-histidinol on the susceptibility of P815 mastocytoma cells to selected anticancer drugs in vitro and in DBA/2J mice. J. Natl. Cancer Inst. 78: 1177-1183 (1987).

Warrington, R.C. and Fang,W.D. L-Histidinol improves the selectivity and efficacy of alkylating agents and daunomycin in mice with P388 leukaemia. Br. J. Cancer 60: 652-656 (1989).

Watowich, S.S. and Morimoto R.I. Complex regulation of heat shock and glucose-responsive genes in Human cells. Mol. Cell. Biol. 8: 393-405 (1988).

Weissberg, J.B., Herion, J.C., Walker, R.I. and Palmer, J.G. Effect of cycloheximide on the bone marrow toxicity of nitrogen mustard. Cancer Res. 38: 1523-1527 (1978).

Wheeler, G.P. Studies related to the mechanisms of action of cytotoxic alkylating agents: a review. Cancer Res. 22: 651-688 (1962).

Wiater, L.A. and Dunham, P.B. Passive transport of K^+ and Na^+ in human red blood cells: sulfhydryl binding agents and furosemide. Am. J. Phsiol. 245: C348-C356 (1983).

Wilcock, C. Effects of nitrogen-mustard on the plasma membrane function. pH.D thesis, Aston University (1987).

Wilcock, C., Chahwala, S.B. and Hickman, J.A. Selective inhibition by bis(2-chloroethyl)methylamine (nitrogen mustard) of the $Na^+/K^+/Cl^-$ cotransporter of murine L1210 leukaemia cells. Biochim. Biophys. Acta 946: 368-378 (1988).

Wilcock, C. and Hickman, J.A. Characterisation of $Na^+/K^+/Cl^-$ cotransporter in alkylating agent-sensitive L1210 murine leukemia cells. Biochim. Biophys. Acta. 946: 359-367 (1988).

Wildenauer, D.B. and Weger, N. Reactions of the trifunctional nitrogen mustard tris(2-chloroethyl)amine (HN3) with human erythrocyte membranes in vitro. Biochem. Pharmacol. 28: 2761-2769 (1979).

Wildenauer, D.B., Reuther, H. and Remien, J. Reactions of the alkylating agent Tris(2-chloroethyl)amine with the erthrocyte membrane. Effects on shape changes of human erthrocytes and ghosts. Biochim. Biophys. Acta 603: 101-116 (1980).

Williams, J.R. and Little, J.B. Association of mammalian cell death with a specific endonucleolytic degradation of DNA. Nature 252: 754-755 (1974).

Williamson, C.E., Kirby, J.G., Miller, J.I., Sass, S., Kramer, S.P., Seligman, A.M. and Witten, B. Enzyme-alterable agents. IX The enzymatic transformation of some nitrogen mustards in the presence of carbon dioxide: implications in respiration. Cancer Res. 26: 323-330 (1966).

Wheeler, G.P. Studies related to the mechanisms ofaction of cytotoxic alkylatng agents: A review. Cancer Res. 22: 651-688 (1962).

Wheeler, G.P. Some biochemical effects of alkylating agents. Fed 26: 885-892 (1967).

Wu, B.J. and Morimoto, R.I. Transcription of the human hsp 70 gene is induced by serum stimulation. Proc. Natl. Acad. Sci. USA 82: 6070-6074 (1985).

Wyllie, A.H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555-556 (1980).

Wyllie, A.H. Cell death: a new classification seperating apoptosis from necrosis, pp9-34. In cell death in biology and pathology (eds. Bowen I.D. and Lockshin R.A.), Chapman and Hall, London, New York (1981).

Wyllie, A.H. The biology of cell death in tumours. Anticancer Res. 5: 131-136 (1985).

Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. Cell death: the significance of apoptosis. Int. Rev. Cytol. 68: 251-306 (1980).

Wyllie, A.H., Morris, R.G., Smith, A.L. and Dunlop, D. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. 142: 67-77 (1984).

Yin, L., Chun, E.H.L., Rutman, R.J. A comparision of the effects of alkylation of DNA of sensitive and resistant Lettre-Ehrlich cells following in vivo exposure to nitrogen-mustard. Biochim. Biophys. Acta 324: 472-481 (1973).

Zwelling, L.A., Michaels, S., Schwartz, H., Dobson, P.P. and Kohn, K.W. DNA cross-linking as an indicator of sensitivity and resistance of mouse L1210 leukaemia to cis-diamminedichloroplatinum(II) and L-phenylalanine mustard. Cancer Res. 41: 640-649 (1981).