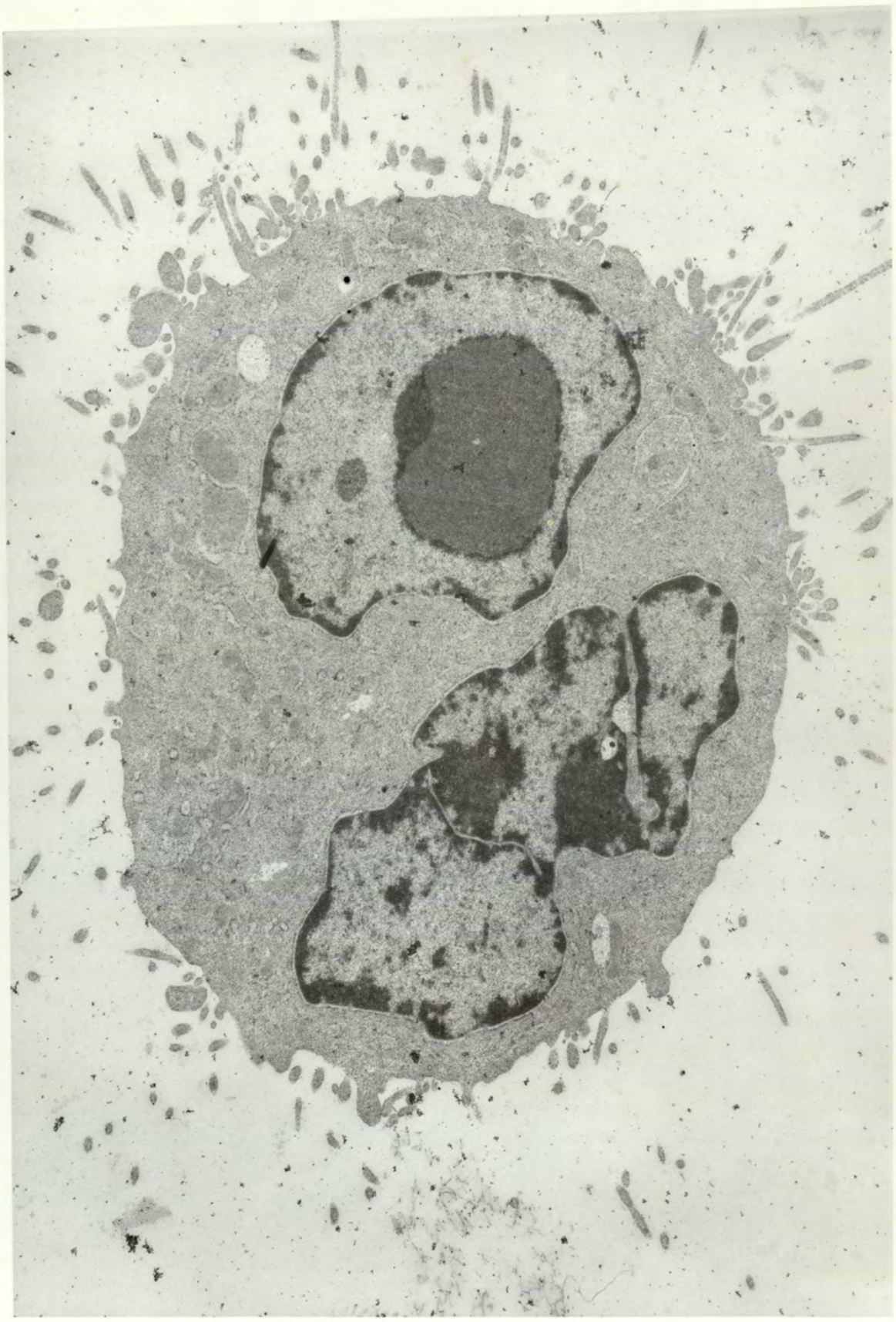

FRONT PLATE

A PC6 CELL AS VIEWED BY A TRANSMISSION ELECTRON MICROSCOPE

To Susan and my Parents

ACKNOWLEDGEMENTS

I would like to thank Dr.J.A.Hickman for his invaluable assistance during this project. I would also like to thank all the members of staff and postgraduates in the Departments of Pharmacy and Biological Sciences whose help and patience is greatly appreciated.

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THE EFFECT OF ALKYLATING AGENTS ON TUMOUR CELL ATPASE ENZYMES

BY

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A THESIS PRESENTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE

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March 1981

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Proposals for the mechanism of action of antitumour nitrogen mustards are reviewed including those which suggest that targets other than DNA may be important for cytotoxicity. The cell membrane as a target for the cytotoxic action of antitumour agents is reviewed. The properties of the cell membrane enzyme sodium potassium dependent magnesium adenosine triphosphatase (Na^+K^+ATPase), which is considered to be synonymous with the "sodium pump", are described and the role of the enzyme and that of particular ions (Na^+,K^+,Mg^{2+} and Ca^{2+}) in cellular metabolism and the regulation of cell replication is reviewed.

The characterisation of enzymes in a crude cell membrane preparation from a plasmacytoma cell line (ADJ/PC6) sensitive to nitrogen mustards *in vivo*, which utilise adenosine triphosphate as a substrate, is described and their activity is compared with those from other sources investigated. Na^+ K^+ATPase, magnesium ATPase, p-nitrophenolphosphatase and external Ca^{2+} ATPase activities are described. Nitrogen mustard (bis (β-chloroethyl) methylamine) and other alkylating agents are shown to be primarily inhibitors of Na^+ K^+ ATPase activity of the crude cell membrane preparation. Nitrogen mustard inhibits the enzyme in a time dependent manner with 100% inhibition at 10^{-10}M after 30 minutes. A monofunctional analogue of nitrogen mustard (N-N-dimethylchloroethylamine) which has no antitumour activity was less potent: 100% inhibition at 10^{-5}M after 30 minutes. The uptake of rubidium (Rb^+) into intact PC6 cells was measured and shown to be ouabain sensitive which suggests it is *via* the sodium pump. Nitrogen mustard is shown to inhibit Rb^+ uptake in a time dependent manner: 10^{-5}M reduced uptake by 45% after 4 hours. However, 10^{-6}M nitrogen mustard, a concentration shown to kill 90% of PC6 cells in an *in vitro* - *in vivo* assay, had no effect on Rb^+ uptake even after 6 hours.

The results are discussed with regard to a hypothesis that inhibition of tumour cell Na^+ K^+ATPase nitrogen mustard may be a cytotoxic lesion.

KEY WORDS: ALKYLATING AGENT, NaKATPASE, CYTOTOXICITY, CELL MEMBRANE.

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INTRODUCTION

1. ANTITUMOUR ALKYLATING AGENTS AND CHEMOTHERAPY:

1.1 HISTORY:

At the 1945 American Association for the Advancement of Science - Gibson Island Research Conference on Tumour Chemotherapy a general review of Tumour Chemotherapy was presented by Woglam (1947) in which he drew the following conclusion, "If we have no cure today, surely it is not from the lack of trying... Those who have not been trained in chemistry or medicine may not realise how difficult the problem of treatment really is. It is almost - not quite but almost - as hard as finding some agent that will dissolve away the left ear, say, yet leave the right ear unharmed: so slight is the difference between the cancer cell and its normal ancestor".

The following year the publication of the first clinical trial of nitrogen mustard (Gilman and Philips 1946) marked the first steps forward towards development of selective cancer chemotherapeutic agents.

Since the biological effects of alkylating agents were first published in 1887 (Meyer 1887) it had been their vesicant properties which attracted most attention. It was noted, however, that the alkylating agent also had a systemic effect, characterised by leucopenia and it was for this reason that it was first suggested that the agents might influence the growth of lymphoid tumours (Gilman and Philips 1946).

The demonstration by Gilman and Philips (1946) that these agents represented a significant advance in the management of lymphoid tumours was followed by further clinical studies on lymphomas, leukaemia and allied diseases (Karnofsky et al 1947, Goodman et al 1947, Wintrobe et al 1947, and Spurr et al 1947).

Since 1946 over a thousand congeners have been synthesized and screened (Ochoa and Hirschberg 1967 p.2) and several alkylating agents have proved clinically useful against tumours in man (Krakoff 1977) (Figure 1).

FIGURE 1

NEOPLASTIC DISEASES THAT RESPOND TO ALKYLATING AGENT CHEMOTHERAPY (TAKEN FROM KRAKOFF 1977)

PROGNOSIS:	TYPE OF CANCER:	AGENT:	% RESPONSE RATE	% SURVIVAL RESPONSES:
Prolonged survival or Cure:	Burketts Lymphoma	Cyclophosphamide	50	Cured
	Seminoma	Cyclophosphamide	90 - 95	50 - 60 cured
	Neuroblastoma	Cyclophosphamide	50	5 - 80 long term survival depending on stage.
	Hodgkins Disease Stages 11B, 111B, & IV	HN2 *	70	40 beyond 5 years
Palliation and Prolongation of life:	Breast Carcinoma	Alkylating Agents**	60 - 80	Increase
	Chronic Lymphocytic Leukaemia	Alkylating Agents	50	Probable increase.
	Lymphosarcoma (adult)	Alkylating Agents	50	Probable increase.
Palliation with uncertain Prolongation of Life:	Chronic Granulocyctic Leukaemia	Alkylating Agents	90	
	Multiple Myeloma	Alkylating Agents	60	
	Ovary	Alkylating Agents	30 - 40	
Uncertain Palliation	Lung	Alkylating Agents	30 - 40	Brief responses
	Head and neck	Alkylating Agents	20 - 30	Brief responses
	Cervix	Alkylating Agents	20	
	Melanoma	Alkylating Agents	20	

* COMBINATION CHEMOTHERAPY SHOWN TO BE EFFECTIVE

** ADJUVANT CHEMOTHERAPY SHOWN TO BE EFFECTIVE

1.2: MECHANISMS OF ALKYLATION:

The alkylation may be defined as the conversion

$$H - Y + R - X \longrightarrow R - Y + H^{+} + X^{-}$$

where R represents an alkyl or substituted alkyl group.

In the biological context, the substrate Y is usually a nucleic acid or protein molecule, which is alkylated on nitrogen, sulphur or oxygen.

The transfer of the carbonium ion R, takes place by two main mechanisms of nucleophilic substitution, SN1 (monomolecular) and SN2 (bimolecular).

SN1:

$$\underset{\text{alkylating agent}}{RX} \xrightarrow{\text{slow}} \underset{\text{carbonium ion}}{R^{+}} + Y^{-}$$

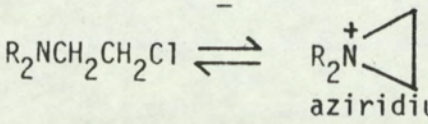
In this type of reaction the rate of formation of the carbonium ion is slow in comparison to the rate of reaction of the carbonium ion with the nucleophile. Therefore, in SN1 reactions the rate limiting step is the ionisation of the alkylating agent in the solvent, a process which occurs more readily in aqueous solution than in less polar solvents. Because of this, any increase in the concentration of the nucleophile Y will not effect the overall rate of reaction.

SN2:

$$\underset{\text{Alkylating agent}}{RX} + \underset{\text{Nucleophile}}{Y^{-}} \longrightarrow \underset{\text{transition state}}{\left[X^{\delta-} \ldots R \ldots Y^{\delta-}\right]} \rightarrow \underset{\text{Alkylated product}}{RY} + X^{-}$$

In this type of nucleophilic substitution, substitution occurs via a transition state which rapidly dissociates to form the alkylated product. The rate of reaction is dependent on the rate of formation of the transition state, and this is influenced by the concentration of nucleophilic centres.

The nitrogen of aliphatic nitrogen mustards may be sufficiently basic to form a cyclic aziridium ion which is more reactive than the typical carbonium ion due to the strained ring system:-

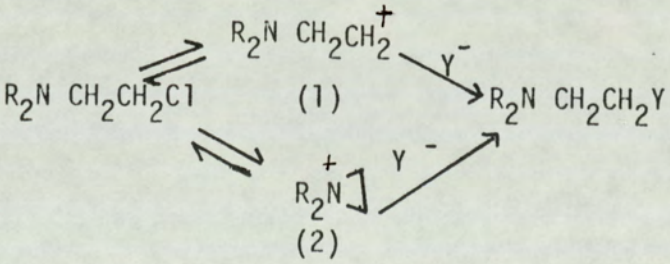


aziridium ion.

The SN1 conversion to the aziridium ion is relatively fast and alkylation of the nucleophile then proceeds in most cases by an SN2 mechanism. In acidic solution the alkylating ability is suppressed.

In the case of the less basic aromatic nitrogen mustards and the sulphur mustards there has been some question concerning their mechanism of alkylation. With these compounds, the lower basicity of the nitrogen or sulphur atom does not allow the formation of a stable cyclic immonium ion analogous to the aliphatic nitrogen mustards. Ross (1962) proposed that these alkylating agents reacted through a carbonium ion formed by unimolecular loss of a chloride ion (Pathway 1 Figure 2) rather than by formation of a cyclic intermediate analogous to the aliphatic nitrogen mustards (Pathway 2 Figure 2).

Figure 2



Although this explanation has been questioned (Triggle 1964) it is now probably accepted that their mechanism of alkylation superficially resembles an SN1 nucleophilic substitution, with the possibility that a cyclic ethyleneimmonium may be involved in the rate determining step (Owen and Stewart 1979).

2: THE MECHANISM OF ACTION OF THE NITROGEN MUSTARDS:

2:1: INTRODUCTION:

Mandel (1959) has stated several rules concerning the studies of the primary mechanism of action of anticancer agents. He stated, "One should not confuse the action of a drug with its mechanism of action. Any carcinostatic drug may produce a variety of biochemical effects, but these may result from, rather than lead to, growth inhibition. Furthermore, many of these effects may not be related to the mechanism by which the drug produces its inhibitory effects ..."

It would seem that unless all of the following criteria were definitely established, conclusions as to mechanism of action are premature.

1) A drug must produce a specific alteration in a biochemical reaction pattern of tissue...

2) The effect must take place at all doses which are tumour inhibitory...

3) In vivo systems should be able to duplicate the in vitro effect in an effort to delineate the specific biochemical step involved...

4) If a particular biochemical reaction is inhibited by a drug, the reversal of all the biological responses by the administration of the product whose synthesis or utilisation is antagonised should be expected...

5) In comparing systems susceptible or resistant to therapy by a drug, a significant quantitative difference in the drug induced alteration should be apparent.

6) The possibility of more than one drug action should be kept in mind, although probably one such effect is the most important.

The nitrogen mustard alkylating agents form very reactive electrophiles that bind covalently and irreversibly to a large number of

nucleophilic centres. It is possible that alkylation at any one of a large number of sites may cause cytotoxicity, but it is more probable, in view of the similar and predictable properties of a wide chemical range of alkylating agents, that toxicity at minimum dose levels is a result of a reaction at one highly sensitive and vital site.

Any hypothesis for a basic mode of action of the alkylating agents must be able to explain why the agents are primarily cytotoxic to cycling cells and produce a late S / G_2 block (Roberts 1975). It must explain why the most cytotoxic alkylating agents and the most potent inhibitors of tumour growth possess at least two alkylating groups per molecule (Connors 1971 p465, Loveless and Ross 1950).

Finally it should be able to account why the alkylating agents exhibit some degree of selective cytotoxicity (Ross 1962 p181-184).

2:2: REACTIONS WITH ENZYMES AND CO-ENZYMES

The early mechanistic studies of alkylating agent cytotoxicity *in vitro* fostered the theory that the primary mechanism of action of the agents was the inactivation of essential cellular enzymes (Peters 1936). Philips (1950) reviewed the "essential enzyme inactivation" theory and stated that a major objection to it was that the sensitivity of most enzymes *in vitro* was not sufficient to implicate their inactivation as a primary factor in cell death. Enzymes that were inhibited by the alkylating agents, for example, nucleotidyltransferase (Wheeler and Alexander 1969),were not appreciably affected at concentrations below 10^{-4}M. Median lethal doses of nitrogen mustard in animals, which were considerably above those required to produce many radiomimetic effects, were only 10^{-5}M if uniform distribution was assumed (Ross 1962 p74).

More quantitative studies suggested that the target sites must react readily with the agents and also be few in number. Most normal cellular enzyme concentrations are such that even if they were inactivated by a stoichiometric reaction with alkylating agents, there would still be an excess of noninactivated enzyme molecules.

Thus Connors (1975, p25) suggested that although enzyme activity was undoubtedly depressed after administration of alkylating agents, this was possibly a secondary effect of alkylation of other sites. Ross (1962 p92) did not rule out the possibility that the primary site of action could be the inactivation of a peculiarly sensitive enzyme system involved in the control of replication.

Respiration and glycolysis are two vitally important biochemical pathways for the cell which have been shown to be sensitive to a variety of alkylating agents in a wide variety of circumstances (Ochoa and Hirschberg 1967 p74).

Tumour cells, which were known to use anaerobic glycolysis as a major source of ATP production, had NAD^+ levels which were readily

depressed by alkylating agents. This resulted in an interference with glycolysis at the stage of the NAD^+ catalysed conversion of 3 - phosphoglyceraldehyde to 1,3 -diphosphoglyceric acid.

There is conflicting evidence concerning alteration of NAD^+ levels after treatment with alkylating agents. Thus, while Wheeler (1962) and Dold et al (1962) showed that alkylating agents reduced the level of this co-enzyme whilst having little effect on other susceptible biochemical pathways, Liss and Palme (1964), using almost identical methods reported the simultaneous inhibitions of DNA synthesis and a reduction in NAD^+ levels.

Recently Smulson et al (1979)have shown a reduction in NAD^+ levels in HeLa cells after treatment with methyl nitrosourea. This was associated with an increase in the activity of the enzyme poly ADP - ribose polymerase. This enzyme is tightly associated with eukaryotic chromatin and is concerned with interaction of nuclear proteins with the nucleosomal substructure of chromatin. Smulson et al (1977) tentatively concluded that the observed decrease in NAD^+ levels after alkylating agent treatment was related in part to enhanced cellular poly ADP ribosylation by the polymerase enzyme which required NAD^+ for the modification. It is thought that one function for this nucleoprotein modification might be related to an alteration in chromatin structure at sites of DNA alkylation damage to allow access for DNA repair enzymes (Smulson 1979 p334).

Thus, the reduction in NAD^+ levels after alkylating agent treatment may be an indirect effect due to enhanced DNA repair induced by the damage to the DNA by the agents. Connors (1975 p25) has suggested that it is unlikely that the cytotoxic effects of the alkylating agent are due primarily to effects on NAD^+ levels and glycolysis although they may be important contributory factors.

2:3 REACTION WITH NUCLEIC ACIDS:

In the original attempts to relate the chemical reactions of the alkylating agents with their biological effects, the fact that at least two alkylating groups per molecule were required for antitumour activity was ascribed to their ability to cross link fibrous macromolecules, in particular those involved in the duplication of the chromosomes (Goldacre et al 1949). Auerbach and Robson (1946), while working on the mutagenic activity of mustard gas, suggested that DNA was an important site for biological alkylation. Elmore et al (1948) proposed that a reaction occurred between DNA and sulphur mustard.

Brookes and Lawley (1961) reported that the N -7 of guanine was preferentially attacked by alkylating agents, because of the arrangement of the purine ring systems in the double helical structure. Price et al (1969) demonstrated that bifunctional alkylating agents could cross link DNA, either by the linkage of one strand in adjacent areas (intra strand cross-linkage), or by the joining together of opposite strands of the helix (interstrand cross linkage).

Drug induced DNA damage has been postulated to interfere with DNA function in two general, although not mutually exclusive ways (Kohn 1979). First, chemical alterations in DNA may effect the direct interactions of the damaged sites with enzymes or other molecules. Typical of this category is DNA base damage. In the second category, are changes that affect topological behaviours of the DNA strands. This may be designated macromolecular damage and is exemplified by chain breaks, interstrand cross links and DNA-protein cross-links.

Single strand breaks with nitrogen mustard have not been observed, but it is thought possible that they are obscured by cross-links (Kohn 1979 p210). Double strand breaks are of probably greater significance to cytotoxicity than single strand breaks. Although their frequency is less than single strand breaks, their potency in inactivating the biological activity of

DNA is much greater.

The damage caused by both monofunctional and difunctional alkylating agents can be repaired by an excision repair mechanism (Ludlam 1975 p 11-14), although at present there is conflicting evidence on whether or not double strand breaks are repaired in mammalian cells (Kohn 1979 p 212). Thus under certain conditions, for example, in Go of the cell cycle, the cell might be able to repair alkylation damage and then proceed normally through the cell cycle. The time required to undertake repair may explain the extension of the intermitotic period demonstrated by Roberts et al (1968).

Interstrand cross-links have been suspected of being of importance to cytotoxicity. It is thought that they might prevent the DNA strand separation that must occur during normal DNA replication and transcription. A further type of cross-linkage has been demonstrated, that between DNA and protein (Klatt et al 1969, Thomas et al 1978). This type of cross-linkage has been shown to occur at low concentration levels of alkylating agents and has been suggested to alter primer activity (Stozier and Nyhan 1962). These DNA - protein cross-links are repaired in mammalian cells and are currently thought not to be necessarily cytotoxic (Ewig and Kohn 1977).

Apart from observations that nitrogen mustards prevented mitosis, caused deep lesions in the nucleus, enlargement of the nucleoli, chromosomal aberrations and multinucleation in both normal and tumour cells (Wheeler 1962), there is further evidence to implicate a reaction with nucleic acids as an early event in the mode of action of the alkylating agents.

Inhibition of DNA synthesis, as measured by the incorporation of ^{3}H thymidine, at dose levels that have no detectable effect on the synthesis or function of other macromolecules has been demonstrated *in vitro* and *in vivo* (Brewer et al 1961, Crathorn and Roberts 1966, Wheeler 1967, Connors 1975 p26, Roberts 1975). Early evidence for an interaction of alkylating agents with DNA included alterations in the temperature of thermal denaturation,

the sedimentation constant, the diffusion constant, and the electrophoretic mobility of preparations of DNA (Conway et al 1950 , Butler et al 1951 , Butler and James 1951, Wheeler 1962). Kohn et al (1966) demonstrated that nitrogen mustard at a concentration as low as 5×10^{-7}M, which corresponded to an alkylation of approximately 0.005% of the bases, converted an appreciable fraction of bacterial DNA to a form that did not denature after exposure to dilute sodium hydroxide. This suggested that a DNA molecule resistant to denaturation could be produced by the reaction of a single molecule of nitrogen mustard with DNA.

This information was obtained after the treatment of isolated DNA in vitro. Treatment with alkylating agents in vivo prior to the isolation of the DNA, originally failed to detect any alteration in the properties of the DNA (Golder et al 1964,Wheeler and Stephens 1965). This was suggested to indicate that DNA was not a primary target for the alkylating agents (Wheeler 1967), although the limitation in techniques was pointed out (Ochoa and Hirschberg 1967 p91).

More recently Kohn (1976) demonstrated that with the DNA alkaline elution technique it was possible to detect interstrand cross-links and DNA - protein cross-links after alkylating agent treatment in vitro and in vivo. With this technique DNA damage measurements have been obtained at the dose levels that inhibit approximately 90% of colony formation in a clonogenic assay. Kohn (1978) demonstrated that DNA - protein cross-links were selectively removed by the incubation of the cell lysate with proteinase K prior to the alkaline elution, thereby providing an estimate of the relative magnitude of the two types of cross-links.

One criticism of an interaction of alkylating agents with nucleic acids as the primary mode of action of the agents has been the difficulty in explaining the resistance of certain cell lines.

It has been shown for many tumours, that lines resistant to alkylating agents take up less drug than the sensitive tumour. However, the difference in drug concentration between the two tumours was usually about two fold,whilst the degree in resistance in some cases was a

hundred fold or more (Connors 1975 p28). Wheeler and Stephens (1965) and Ball and Roberts (1970) demonstrated that there was no apparent difference between the sensitive and resistant DNA's after treatment with alkylating agents. Ball and Roberts (1970) also found no difference in the level of alkylation between the two DNA's, the rate of excision of alkylated groups, or the ability of the two cell lines to carry out non-semi conservative DNA synthesis after treatment with mustard gas.

More recently, Erickson et al (1978) using the DNA alkaline elution technique has demonstrated a difference in sensitive and resistant human colon carcinoma cells to treatment with the alkylating agent 1 - 2 (chloroethyl) - 3 - (4 trans-methylcyclohexyl) - 1 - nitrosurea (methyl CCNU). A difference between the two relative elution rates was found both *in vitro* and *in vivo* although it was suggested that resistance *in vitro* was associated with the repair of DNA cross-links, whilst *in vivo*, resistance was associated with a lower extent of interstrand cross-link formation.

Zwelling et al (1979) on studying the mechanisms of action of *cis* and *trans* Pt II $(NH_3)_2$ Cl_2 found that it appeared that both isomers produced DNA - protein cross-links in L1210 cells, but that interstrand cross-links were more prominent in the case of the *cis* isomer, which is the active antitumour agent. Cell survival was similar for the two compounds at concentrations that yielded similar extents of interstrand cross-linkage. In the case of concentrations that yielded similar levels of total cross-links, the *cis* isomer was more cytotoxic.

Thus Erickson et al (Kohn 1979 p228) suggested that interstrand cross-links were a more important determinant of cell killing than DNA - protein cross-links.

A further difference between the action of alkylating agents on the chromatin of sensitive and resistant Yoshida cells has been found by Riches et al (1977). They demonstrated that chlorambucil was able to induce nuclear protein phosphorylation in sensitive cells, but not in resistant cells. The nuclear protein phosphorylation was correlated with a loss of condensed chromatin and with subsequent cell death (Riches and Harrap 1973). It was suggested that alkylating agents induce increased nuclear protein phosphorylation which is associated

with enhanced drug binding and subsequent disruption of chromatin integrity. Thus the repair capacity of the cell is inhibited by the chromatin - drug interaction and DNA damage cannot be removed (Wilkinson et al 1979).

This model may provide, in addition, an explanation of why certain corticosteroids potentiate the antitumour efficacy of chlorambucil in a resistant Yoshida tumour (Harrap et al 1977). Corticosteroids have been shown to modify chromatin structure (Whitfield et al 1968). Wilkinson et al (1979) have shown that prednisolone administered 4 hours after chlorambucil treatment produced an increase in DNA cross-linkage in resistant cells to a level similar to that achieved by the alkylating agent alone in sensitive cells. They postulated that agents which modify the transcriptional activity, which is associated with the phosphorylation of non-histone proteins, may also potentiate alkylating agent toxicity.

Thus this model provides a possible mechanism of resistance to alkylating agents. That is, a failure to induce nuclear protein phosphorylation coupled with cross-linkage of DNA which is reversible and apparently repaired and a loss of condensed chromatin (Wilkinson et al 1979). Further, it offers an explanation for the potentiation of the antitumour efficacy of alkylating agents by certain corticosteroids.

Small concentrations of alkylating agents have been shown to readily inhibit the incorporation of radiolabelled precursors into nucleic acids, particularly into DNA (Brewer et al 1961, Crathorn and Roberts 1966, Wheeler 1962, Connors 1975 p26, Roberts 1975). This has often been used as a presumptive measure of the inhibition of nucleic acid synthesis. However, Grunicke et al (1975) demonstrated that whilst the alkylating agent Trenimon caused a rapid decrease in the incorporation of labelled thymidine into the DNA of Yoshida and Ehrlich ascites tumour cells, there was no effect on DNA synthesis as measured by an isotope dilution assay. The reduced incorporation of thymidine into DNA was explained by a decreased transport of the nucleoside into the cells, due to the alkylation of sulphydryl

groups involved in the reactions responsible for transport of the nucleoside. This could explain the paradoxical results of Roberts (1975) and Riches and Harrap (1973). Roberts (1975) showed that in HeLa cells treated with chlorambucil, DNA synthesis decreased in a dose dependent manner, but that total DNA content increased and approached premitotic levels. Riches and Harrap (1973) found that chlorambucil inhibited thymidine incorporation into DNA, whilst there was no effect on DNA synthesis. Further, McCann et al (1971) have shown that radioactive precursor incorporation may not be a reliable measurement of macromolecular synthesis, at least in chick embryos. They demonstrated that the alkylating antibiotic, mitomycin C, had no effect on the incorporation of ^{14}C - leucine into proteins for up to 48 hours, but that net protein synthesis, as judged by total protein per embryo, was inhibited before this. They suggested that the rate of precursor incorporation depended on pool size, on accessibility of the precursor to the cell and on the permeability of the cell. Kay and Handmaker (1970) reached a similar conclusion with regards to uridine incorporation and RNA synthesis in lymphocytes. They found that the incorporation of uridine was probably limited by the phosphorylation of uridine to UMP, and concluded that the incorporation of ^{3}H uridine into RNA was not a valid measure of the rate of RNA synthesis. Thus, the use of the incorporation of radiolabelled precursors, as a measurement for inhibition of macromolecular synthesis may be suspect.

The interaction of alkylating agents with DNA as the primary cytotoxic event makes it difficult to account for the selectivity of the agents. Bis-methane sulphonates have been shown to be more specific inhibitors of the growth of myeloid tumours than the Bis - 2 - chloroethylamines (Elson 1958) whereas the latter compounds are more specific for lymphatic tissue (Elson et al 1958). Table 1 illustrates the selectivity of some Bis - 2 - chloroethylamines against certain experimental animal tumours.

TABLE 1:

SENSITIVITY OF CERTAIN TUMOURS TO BIS - 2 - CHLOROETHYLAMINES (ROSS 1962 p182 - 183)

AGENT	TUMOUR						
	EHRLICH	5180	755	E0771	Yoshida	Walker	537
CHLORAMBUCIL	-	±	-	-	n.d.	+	+
NITROGEN MUSTARD	+	+	-	±	+	+	+
NITROMIN (1)	++	++	n.d.	-	++	++	n.d.
MERPHALAN (2)	+	+	n.d.	n.d.	n.d.	+	n.d.

n.d. not determined

1. Methylbis - (2 chloroethyl) amine oxide.
2. 3 - (Bis - (2 chloroethyl) amino (phenyl) alanine (p-sarcolysin)

Further, Ord and Danielli (1956) and Ord (1956) in a series of experiments on nuclear transfer in amoeba demonstrated that on exposure to nitrogen mustard, damage to the nucleus interfered less with subsequent division than did damage to the cytoplasm. Despite the fact that the nucleus was ten times more sensitive to nitrogen mustard than the cytoplasm, nuclear damage by a given concentration of nitrogen mustard took longer to kill amoeba than did cytoplasmic damage. They also found that amoeba with a lethally damaged cytoplasm usually did not divide, but amoeba with normal cytoplasm and a transferred damaged nucleus were likely to divide several times before death. Thus they concluded that nuclear and cytoplasmic injuries induced by nitrogen mustard were quite distinct and independent phenomena.

In conclusion there is evidence for a reaction of alkylating agents with DNA both *in vitro* and *in vivo*. Two possible mechanisms of resistance have been postulated, and in addition, the requirement for bi or polyfunctional alkylating groups on the molecule has been

explained.

Recent results by Brox et al (1980) have shown that alkylating agents cause a G_2 block. If DNA is cross-linked by the difunctional agents, it is difficult to explain how DNA synthesis is completed and a full premitotic complement obtained. However, they also demonstrated that low levels of agent induced a temporary G_2 block, and that removal of this block was accompanied by a repair of DNA cross-links.

Hence at the present time it is not known whether the interaction with nucleic acids is the only, or primary, mode of cytotoxic action of the alkylating agents in vivo.

2.4: THE INHIBITION OF CYCLIC 3', 5' - NUCLEOTIDE PHOSPHODIESTERASE:

Tisdale and Phillips in a series of papers (Tisdale and Phillips 1975a, 1975b, 1975c, 1976a, 1976b, Tisdale 1974, 1975) have attempted to relate the cytotoxic action of the agents to the inhibition of the subunit enzyme, cyclic 3', 5' - nucleotide phosphodiesterase and to the subsequent rise in cyclic adenosine 3' 5' monophosphate (cyclic AMP). Cyclic AMP has been considered as a possible intracellular mediator by which eukaryotic cells control replication and differentiation (Otten et al 1971, Pastan et al 1975). Cyclic nucleotide phosphodiesterase (adenosine 3', 5'-monophosphate phosphohydrolase EC 3.1.5.17) converts cyclic AMP to 5' AMP, and inhibitors of this enzyme have been shown to induce morphological differentiation of mouse neuroblastoma cells in culture (Prasad and Sheppard 1972). The phosphodiesterase inhibitor theophylline retarded the rate of intradermal growth of Rauscher Leukaemia virus induced tumour in Balb/C mice (Webb et al 1972), and suppressed the growth of subcutaneously inoculated Walker rat carcinoma cells (Keller 1972).

Tisdale and Phillips (1975b) showed that the cyclic AMP level of alkylating agent sensitive tumour cells increased exponentially with increased dosage of chlorambucil. It was shown that the monofunctional N-ethyl analogue of chlorambucil had no effect on cyclic AMP levels 8 hours after treatment. They postulated that the mechanism of elevation of cyclic AMP by the alkylating agents was the inhibition, by cross-linkage, of the low Km form of the enzyme cyclic 3', 5'-nucleotide phosphodiesterase. Resistance was suggested to occur through a reduction in the activity of the low Km form of the enzyme compared to the activity found in sensitive cells. Tisdale and Phillips (1975c) demonstrated that high concentrations of chlorambucil (5 µg/ml) caused an immediate rise in intracellular AMP levels in Walker cells, which reached a maximum within 1 hour. This preceded the effect of the drug on thymidine incorporation into DNA and suggested that cyclic AMP might mediate an effect of chlorambucil on DNA synthesis (Robinson et al 1971).

Thus, this hypothesis accounted for the toxicity of the di and polyfunctional alkylating agents by the cross-linkage of the subunit phosphodiesterase enzyme. It accounted for the natural resistance of cell lines (Tisdale and Phillips 1975a, Tisdale 1975), and the induced resistance of certain cell lines (Tisdale and Phillips 1975b).

Further, the elevation of intracellular cyclic AMP produced may explain the suppressing effect of alkylating agents on antibody formation (Berenbaum 1962), and the delayed hyperglycaemia observed after in vivo administration of alkylating agents (Elson 1963).

Tisdale and Roberts (1978) have also reported that caffeine, a phosphodiesterase inhibitor, potentiated the lethal action of alkylating agents in L cell and Chinese hamster V79 cells, although it has been thought that the role of caffeine might be via cGMP (Wells et al 1975).

However Brox et al (1980) using RPMI 6410 cells, Roberts (1975) using HeLa cells, Wheeler et al (1970) using H.Ep.No.2 cells and Brewer et al (1961) using mouse fibroblasts demonstrated that the bis - 2 - chloroethylamines produced what appeared to be a late S/G_2 block in the cell cycle. Tisdale and Phillips (1975b) suggested that a similarity existed between the antimitotic effects of bis - 2 - chloroethylamines and cyclic AMP since both agents were quoted as causing inhibition during the S phase of the cell cycle. Table 2 demonstrates the conflicting data found by Pastan et al (1975) on the phase in the cell cycle when an elevation in cyclic AMP is thought to act. Whereas there is only evidence for a single late S/G_2 block with bis - 2 - chloroethylamines (Wheeler 1970, Roberts 1975, Brox et al (1980), there is evidence for both a G_1 and G_2 block with cyclic AMP. Thus if the primary mode of action of the alkylating agents is thought to be an elevation in intracellular cyclic AMP levels, in addition to a late S/G_2 block, a possible G_1 block should also have been reported in treated cells. Further Caffino et al (1975) found that an elevation of cyclic AMP produced a G_1 block in S 49 mouse lymphoma cells. They suggested that periodic fluctuations in the levels of cyclic nucleotides were not required, or determined progression through

TABLE 2:

SITE OF GROWTH INHIBITION BY CYCLIC AMP:

(MODIFIED FROM PASTAN 1975)

CELL TYPE	AUTHORS	BLOCK IN G_1	BLOCK IN G_2
3T3	Willingham et al (1972)	+	+
3T3	Kram, Mamont & Tonkins	+	+
SV3T3	Smets	o	n
Human skin fibroblast	Froehlich and Rachmeler	+	n
HeLa	Zeilig	o	+
CHO	Remington and Kievecz (1973)	(+)	+
Lymphoma 549	Bourne et al	+	o
Lymphocytic Leukaemia	Smets	+	o
Lymphocytic Leukaemia	Willis et al	n	+
HeLa	Dipasquale et al (1977)	o	+
Lymphoma 549	Coffino et al (1975)	+	o
Rat fibroblast	Frank	+	n

1. + Inhibition found; o no inhibition; n not examined.

the cell cycle since mutant cells which lacked the cAMP dependent protein Kinase were not affected by dibutryl cyclic AMP and had normal cycles in the presence of elevated cyclic AMP. Further, Tisdale and Phillips (1975b) found that the ID_{50} for chlorambucil against a sensitive line of Walker cells in culture was 0.45 ug/ml. This concentration produced an increase in cyclic AMP levels of approximately 15% after eight hours, and had no significant effect on levels after 24 hours. At a concentration that corresponded to 100% inhibition of growth, the increase in cyclic AMP levels was approximately two fold.

Thus, concentrations of chlorambucil that were found to inhibit cell growth elevated cyclic AMP levels by less than 15% after 8 hours and had no effect on levels after 24 hours. Such small changes may be insufficient to prevent cell replication and are not compatible with the G_2 block (Brox et al 1980) caused by the treatment with an alkylating agent.

2.5: OTHER POSSIBLE SITES OF ACTION

Although the general concensus of opinion is that DNA is the primary target for alkylating agents, it is also thought that other sites might play an important role in the cytotoxic action (Wheeler et al 1970, Pushendorf et al 1971, Grunicke et al 1973).

Evidence has accumulated for an intimate correlation between plasma membrane functions and the regulation of cell division (Pardee 1974, Holley 1972, see 5). The observation by Grunicke et al (1975) that growth inhibiting concentrations of alkylating agents caused alterations of the plasma membrane pointed to the possibility that the antitumour effect of alkylating agents might be due to an interaction with the cell membrane (see 3:5).

3. THE CELL MEMBRANE AS A POSSIBLE TARGET FOR ANTITUMOUR DRUG ACTION:

Rothstein (1959) in his work on the cellular toxicity of heavy metal ions, has brought attention to the fact that the cell membrane is the outer boundary of the cell and hence is exposed to the full extracellular concentration of a drug before the rest of the cell. Since that observation was made little attention has been given to the possibility that the cell membrane might be a pharmacologically important target for the alkylating antitumour drugs.

As discussed earlier (2:3) the reaction of alkylating agents with DNA may not alone be able to account for their cytotoxicity, and reactions with other cell constituents may play a role (Wheeler 1967, Fussganger et al 1967, Grunicke et al 1979).

Evidence has accumulated for a role of the plasma membrane in the regulation of cell function, and it appears that nutrient uptake (Pardee 1974) and ion concentration (Sanui and Rubin 1978, Dornand et al 1978, see section 5) are involved. Ionic dependent nutrient uptake and the intracellular concentration of sodium, potassium, and possibly also calcium and magnesium, are linked by the cell membrane enzyme NaKATPase (EC 3.6.1.3), which is considered synonymous with the sodium pump (Lane et al 1979) (See sections 4 and 5 for further details of ion flux across the cell membrane, and the role of monovalent and divalent ions in cell homeostasis and replication).

Thus the cell membrane, on which much work has been performed with respect to the mechanism of action of antitumour hormone drugs, has come under scrutiny by researchers as a possible target for other antitumour drugs (Murphree et al 1976 , Trittan et al 1977, Juliano and Stamp 1979, Grunicke et al 1979).

3:1 VINBLASTINE AND COLCHICINE

These agents have been suggested to alter various membrane related phenomena through actions other than or in addition to those mediated by microtubule disruption (Furcht and Scott 1975).

Juliano and Stamp (1979) have shown that vinblastine influenced the structure of artificial lipid. They suggested that this effect in vivo could alter the structure of the cell surfaces and hence an alteration in membrane related cellular functions.

3:2 Anthracyclines

The cytotoxicity of anthracyclines, such as the antitumour drug adriamycin, is thought to be due to the intercalation of DNA which leads to an inhibition of DNA and/or RNA synthesis (Di Marco 1975). However, significant mitotic inhibition by adriamycin and daunorubicum has been reported under conditions in which ^{3}H thymidine incorporation has been significantly elevated above that of the control. (Silverstrini et al 1970,1973). Complete inhibition of growth of Sarcoma 180 cells by adriamycin in culture with no decrease in the rate of incorporation of ^{3}H thymidine or ^{3}H uridine into nucleic acids has also been reported (Murphree et al 1976).

A cogener of adriamycin, N-trifluoroacetyl adriamycin - 14 - 14 - valerate (AD - 32) has been reported to be an effective antineoplastic agent. This agent apparently does not enter the cell nucleus (Kristan et al 1976), does not bind to isolated DNA (Sengupta et al 1976), and does not have any effect on DNAase activity (Facchinetti et al 1977). Thus the nucleus may not be an important site of action for AD - 32, and possibly other anthracyclines.

It has been suggested that an action of adriamycin at the membrane level may occur, which could affect cell viability through a change in membrane function, such as a movement of membrane molecules. Adriamycin reacts well with phospholipids in the cell membrane (Anghileri 1977,Goldman et al 1978, Schwartz and Kanter 1979) and possibly with glycoproteins which would allow access of adriamycin into the cell (Schwartz and Kanter 1979).

It has been suggested that phospholipid composition and distribution in cellular membranes might play an important role in the selectivity of anthracycline antibiotics (Schwartz 1976, Tritton et al 1978). The antitumour specificity of adriamycin might be related to the cardiolipin content of membranes (Tritton et al 1978). This lipid, which is normally restricted to mitochondrial membranes, has been found in all cellular membranes after malignant transformation in

liver cells (Bergelson et al 1970,1974). Tritton et al (1978) found that adriamycin reduced the fluidity of artificial membranes only when this lipid was present. They suggested that a differential behaviour on the fluidity of membranes with and without cardiolipin would provide an explanation for some of the agent's selectivity. Moreover, the concept might explain, at least in part, the observed cardiac toxicity of adriamycin due to the high content of cardiolipin rich respiring mitochondria in heart cells (Tritton et al 1978). However, Tritton et al (1978) stressed that this hypothesis did not require the ultimate cellular target for adriamycin to reside at the level of the surface membrane, only that the drug must interact with this membrane, at some time during its encounter with a cell. Thus even if the plasma membrane, is not the primary target for the anthracycline antibiotics, it might still be an important determinant of therapeutic efficacy.

Dasdia et al (1979) have shown that after adriamycin treatment of HeLa cells there was an increase in the intracellular pool of calcium. It was suggested that this might be associated with an action on the sodium pump which could trigger Ca^{2+} release from the bound pool and so modify all the functions related to this action (see 5:6). Interaction with the membrane enzyme NaKATPase has also been linked to the cardiac toxicity of anthracycline antibiotics (Gosalvez et al 1979). It has been shown that adriamycin is a potent inhibitor of NaKATPase *in vitro* (Gosalvez et al 1979) and this effect was suggested to provide a basis for the digitalis type cardiotoxicity of adriamycin (Ghione 1978).

3:3 Antimetabolites:

3:3:1 6-Thioguanylate, a major metabolite of 6-Thioguanine in normal tissue, has been shown to inhibit the *de novo* pathway of purine nucleotide synthesis and purine nucleotide interconversions (reviewed by Paterson and Tidd 1975a). However, there is reasonable doubt that the cytotoxic effects of thioguanine are related. 6-Thioguanine decreased the incorporation of mannose into glycoproteins and it was suggested that this could lead to major alterations in the primary structure of complex sialoglycoproteins (Lazo et al 1979).

Thioguanine may therefore create multiple lesions including a membrane effect as well as the inhibition of purine nucleotide synthesis and the incorporation of 6-thioguanine phosphate into DNA. The multiple lesions created might lessen the probability of cellular repair and ultimately lead to cell death (Lazo et al 1979).

3:3:2 The intracellular presence of the 6-Mercaptopurine anabolites, 6-Methylthioinosinate and 6-Thioinosinate has been shown to inhibit purine nucleotide synthesis interconversions (reviewed by Paterson and Tidd 1975b). In spite of these observations a causal relationship between the inhibition of purine nucleotide interconversions and 6-Mercaptopurine cytotoxicity has not been established. Further, it has not been established that temporary constriction of the production of purine nucleotides is necessarily lethal in the cell. Thus, at present there is not sufficient evidence to attribute the lethal effects of 6-Mercaptopurine exposure to the enzymatic inhibitions discussed above (Paterson and Tidd 1975b p 393).

Patzelt-Wenczler et al (1975) reported that the riboside triphosphate of 6-Mercaptopurine inhibited the cell membrane enzyme NaKATPase by the reaction of the sulphydryl group of the agent with a sulphydryl group at the ATP binding site of the enzyme (see 4:1:1). This agent was used as a biochemical probe and no attempt was made to relate this action with the antitumour action of 6-Mercaptopurine.

3:3:3:The experimental antitumour agent S-trityl-L-cysteine (NSC 83625), shown to be active against L1210 leukaemia *in vivo*, was found to cause membrane perturbation and to interfere with the incorporation of precursors into nucleic acid and proteins in culture (Kessel et al 1976). Alkylation of macromolecules by the trityl component of NSC 83625 has been suggested as a possible mode of action (Zee-Cheng and Cheng 1970) but recent data does not support this (Kessel et al 1976). At present it is not known whether the cell surface changes are directly related to the mode of action of the drug.

3:3:4:Two modes of action have been identified for 5-Fluorourocil *in vivo*. The major mode of cytotoxicity involves drug induced inhibition of the enzyme thymidylate synthetase which lease to a blockade in the normal pathway of nucleic acid synthesis (reviewed

by Heidelberger 1975). Studies on the growth of LS178Y cells revealed that toxicity of 5-Fluorouracil was abolished by the addition of thymidine, provided that the latter was added at a concentration that itself was not growth inhibitory (Margolis et al 1971).

Incorporation of fluorouracil into ribonucleotides has also shown to interfere with the processing and function of RNA (Kessel 1980) . Walliser and Redmann (1978) found that low concentrations (10^{-7}-10^{-8}M) of 5-Fluorouracil caused hyper-polarisation of the cell membranes in HeLa cells after a long (24-96 hours) incubation. Membrane depolarisation was enhanced at higher concentrations and with increased exposure to the agent. The surface change of the cells was altered to a lesser extent than the transmembrane potential. When thymidine, which by itself had no effect on membrane potential, was added along with 5-Fluorouracil, the depolarising action of the antimetabolite was abolished. The results suggested that the effect of 5-Fluorouracil on the cell membrane was related to the influence of the antimetabolite on cell growth. Moreover, Walliser and Redmann suggested that this membrane effect was a possible primary cause for the cytostatic action.

Kessel (1980) found that although exposure of L1210 cells to graded doses of 5-Fluorouracil for 24 hours led to a progressive increase in cell surface hydrophobicity, inhibition of cell division and an increase in cell volume, short term treatment with the drug only altered levels of thymidylate synthetase. It was suggested that cell surfaces were possibly related to long term effects of 5-Fluorouracil associated with impaired synthesis of membrane glycoprotein. No conclusion was drawn as to a possible primary site of cytotoxic action, but Kessel did state that the role of cell surface alterations induced by 5-Fluorouracil remained to be established.

3:4: Experimental Antineoplastic Agents

3:4:1: The diacridines, an experimental class of antitumour agent have been reported to inhibit the growth of leukaemic P-388 cells and L1210 cells, as well as HeLa cells in culture (Canellakis and Chen 1979). Their primary site of inhibition is the synthesis of RNA, whereas the inhibition of the synthesis

of DNA and protein appear to be secondary sites (Canellakis and Bellantone 1976, Canellakis et al 1976). The results with diacridines indicated that the major biochemical site of action was related to their ability to intercalate with nucleic acids and to the metabolic consequences of intercalation. However, the results did not prove that there was a necessary correlation between these major biochemical activities and the antitumour effects of the diacridines. Fico et al (1977), failed to relate the percentage increase in life span (% ILS) to the ability of the diacridines to inhibit DNA synthesis, RNA synthesis and the uptake of the agents by P 388 cells in culture.

It was shown that a highly significant inverse correlation existed between % ILS and the ability of the diacridines to enhance the rate of agglutination of S-180 cells (Fico et al 1977). From these results and data concerned with the lipophilicity of the various diacridines used, it was concluded that the antitumour effectiveness of the diacridines was associated with a reaction with very specific membrane sites that were possibly a prime characteristic of tumour cells (Fico et al 1977).

3:4:2: The alkaloid acronycine (NSC 403169) has been shown to have antitumour activity in several experimental systems (Svoboda et al 1968). Kessel (1977) reported that acronycine inhibited nucleoside transport across the membranes of L1210 and LS178Y cells. The rapidity with which acronycine caused these cell surface alterations led Kessel to suggest that they were primary effects rather than secondary results of drug induced inhibition of macromolecular biosynthesis.

3:4:3: The poylene antibiotic lymphosarcin (NSC 208642) has a wide spectrum of activity against experimental tumours such as P 388 lymphocytic leukaemia, B16 melanoma, colon 38 and Danny Martin mammary carcinoma. Kessel (1978) suggested that at low drug concentrations an alteration in the cell surface occurred which resulted in increased exposure of electronegative groups to the environment. It was thought that the unmasking of sialic acid or anionic phospholipid moeities was possibly involved, and that a higher drug concentration loss of such components or an unmasking of cationic groups might occur (Kessel

1978). In addition, transport of a model amino acid (cycloleucine) was inhibited as were both a non metabolised and metabolised nucleoside (5-deoxyadenosine and uridine). Kessel concluded that it remained to be established whether the selective toxicity towards neoplastic cell types was related to the surface active properties of the drug.

3:4:4: Photoactivated porphyrins for example hematoporphyrin (NSC 59265), have been shown to be active against experimental animal tumours (Kessel 1977b). Significant effects of photo-activated porphyrins on cell surface properties were found at drug concentrations which markedly reduced cell viability but which had no effect on the action of intracellular kinases or incorporation of precursors into nucleic acids. Deuteroporphyrin XI (NSC 19663) was found to inhibit nucleoside and amino acid transport in addition to the observed membrane perturbations. It was postulated that the porphyrins were bound to the hydrophobic region of the cell membrane and that photoactivation led to cross-linkage of membrane proteins, which cause some or all of the observed effects (Kessel 1977b).

3:5: Alkylating Agents

3:5:1: Prior to the theory of the alkylation of DNA, Peters (1947) considered that an attack on cell surfaces by sulphur mustard gas, with consequent modification of permeability was a likely mode of action of this agent.

3:5:2: Chlorambucil was shown to react with the cell membrane of human erythrocytes and Ehrlich ascites tumour cells (Linford et al 1963). It was suggested that the chlorine atoms were replaced by small nucleophilic molecules or anions from the cell surface which weakened the membrane and resulted in cell lysis. Linford et al suggested that chlorambucil was possibly an atypical alkylating agent in that its primary action might be the removal of a simple chemical component from the cell surface.

3:5:3: Trenimon (2,3,5, tris-ethyleneiminobenzoquinane) was reported to inhibit the carrier mediated uptake of thymidine by an impairment of a membrane constituent which catalysed the facilitated diffusion of the nucleotide (Grunicke et al 1975). The observation that a growth inhibitory concentration of alkylating agent caused alterations in the

plasma membrane suggested that the antitumour effects of alkylating agents might be due to an interaction with the cell membrane. Grunicke et al (1979) have since shown that in addition to the impairment of thymidine transport 10^{-6}M per Kg Trenimon inhibited the uptake of aminoisobutyric acid and 3-O-methylglucose after an incubation of four hours.

Grunicke et al showed that on incubation of Ehrlich ascites tumour cells under the same conditions as above reduced $^{86}Rb^{+}$ uptake by approximately 60% in comparison to controls. They suggested that the inhibition of $^{86}Rb^{+}$ uptake within 30 seconds of treatment supported the assumption of a direct effect on the cell membrane, although it must be noted that to achieve that result in such a short time interval, a concentration of 10^{-4}M Trenimon was required. The inhibition of $^{86}Rb^{+}$ uptake is a presumptive measure of the inhibition of NaKATPase. An attempt was made to study the effect of alkylation under conditions in which only the cell surface was alkylated by the use of polyethyleneimines of various chain lengths to which chlorambucil was covalently bound. The pure polymer had no cytotoxic effect, whilst the alkylating polymer exhibited significant antitumour activity. Although it was considered unlikely that the bound molecule was taken up into the cell, this has not been proven. Furthermore, studies are still in progress to check whether the alkylating groups remained bound to the carrier or were hydrolysed off.

Grunicke et al (1979) concluded that their present data did not permit a decision whether the uncoupling of DNA and histone synthesis or the alkylation of the plasma membrane was responsible for the antitumour effect.

3:5:4: The trifunctional nitrogen mustard HN3 (Tris(2 Chloroethyl)-amine HCl) has been shown to react with the membrane proteins of human erythrocytes (Wildenauer and Weger 1979). With the use of ^{14}C radiolabelled HN3 23.5% of the total radioactivity taken up by the cells was incorporated into the membranes. The 76.5% remaining in the haemolysate represented the amount of unreacted or bound HN3 or its hydrolysis products in the cytoplasm. A comparison of the specific activity of total HN3 labelled membrane with the specific activity of HN3 labelled cytoplasmic haemoglobin suggested that the membrane was

primary target for the alkylation of human erthrocytes by HN3.

Spectrin, which together with actin, is thought to form a network responsible for cell shape (Nicolson 1976) and is considered to be involved in the linkage and control of outer surface plasma membrane components (Nicolson 1976) was shown by gel electrophoresis to be one of the targets of the alkylating agent. In addition, glycophorin (PAS 1), an antigenic protein which spans the membrane and is the receptor for certain lectins (Marchesi and Furthmayr 1976) was also alkylated. Wildenauer and Weger concluded that the information that an alkylating agent reacted with membrane proteins and modified proteins such as spectrin, which are important in cytoskeleton and transmembrane control, might contribute to the understanding of the mode of action of the alkylating agents.

4: THE CELL MEMBRANE AND MONOVALENT ION TRANSPORT:

4:1: The Sodium Pump

Animal cells maintain their cytoplasmic concentrations of Na^+ below and K^+ above those in the extracellular fluid, by the active transport of the ions against an electrochemical gradient. This is a process dependent upon metabolic energy, and the cell expends about one third of its total energy metabolism in the maintenance of this gradient (Segal et al 1978).

The first report of the active transport of monovalent ions was by Schatzmann (1953). He also showed that the cardiac glycoside, ouabain, inhibited pump mediated fluxes but not the passive leakage of Na^+ and K^+ down their respective electrochemical gradients. Skou (1957) reported the isolation of a membrane enzyme that hydrolysed ATP and required in excess of 50mM Na^+ and 20mM K^+ for maximal activity. He suggested that this enzyme was the molecular basis for the vectorial transport of Na^+ and K^+ across the cell membrane. Later, he showed that this enzyme could be specifically inhibited by ouabain (Skou 1960) and the unique sensitivity of this enzyme to the cardiac glycoside was offered as evidence that this enzyme was in fact related to the active transport of Na^+ and K^+ across the plasma membrane (Glynn 1964).

The term Na^+ K^+ activated (NaKATPase) was suggested by Bonting et al (1961). Previously the enzyme was known as either membrane ATPase, pump ATPase, or Na stimulated ATPase. "NaKATPase" was used to distinguish the enzyme from other ATPases and this term highlighted its presumed role in cation transport without implying that it was definitely the pump enzyme or that it was located in the plasma membrane.

4:1:1: Structure

The enzyme has now been shown to span the plasma membrane and consist of two types of chains (reviewed by Schwartz et al 1975).

The α chain carries the ouabain binding site, is phosphorylated by ATP (Forbush and Hoffman 1979) and is considered to be the catalytic subunit. The β chain is a sialoglycoprotein. Molecular weight determination of the and chains give values of 106,000 and 37,000 respectively (Hastings and Reynolds 1979). Due to differences in the value for the molecular weight of the enzyme there are conflicting reports on the molar ratios of the two chains. Early results suggested a molecular weight of 140,000 - 200,000 giving rise to ratios of $\alpha\beta$, $\alpha_2\beta$ and $\alpha\beta_2$ (Schwartz et al 1975). Recently Hastings and Reynolds (1979) have determined the molecular weight of 380,000 $\pm$ 10,500 giving a subunit ratio of $\alpha_2\beta_4$. Using a molecular weight of 276,000 - 307,000 Esmann et al (1979) give a subunit ratio of $\alpha_2\beta_2$. They point out that α : β weight ratio found by gel scanning suggests $\alpha_2\beta_3$ but this could be due to proteolysis of some α chains giving rise to fragments staining in the β chain region. At the moment $\alpha_2\beta_2$ is the most generally accepted structure (Stein 1974, Huang and Askari 1979).

The proposed $\alpha_2\beta_2$ structure consist of two $\alpha\beta$ dimers and the cross-linkage of the two dimers by Cu, Cu phenanthroline (Huang and Askari 1979) or 3, 3 dithiobispropionimidate (De Pont 1979) renders the enzyme inactive. This suggests that a free mobility of the subunits relative to each other is essential for proper functioning of the NaKATPase (De Pont 1979).

Since the enzyme spans the cell membrane the role of the membrane lipids in the structure have been investigated. Besides forming a matrix for the enzyme it would appear that lipids are involved in the modulation of the enzyme conformation. In this role phosphatidylserine may be a requirement, or at least, is more effective than other lipids in stimulating catalysis (Fenster and Copenhaver 1967).

Work on the tertiary protein structure of NaKATPase has centred

on the sulphydryl groups within the enzyme. Titration of the number of sulphydryl groups after complete solubilisation of the enzyme in detergent yields 36 sulphydryl groups per molecule of NaKATPase. Sodiumdodecylsulphate electro-phoregrams show that 34 sulphydryl groups are located on the catalytic subunit and only 2 on the glycoprotein subunit (Schoot et al 1979). These have been subdivided into three classes by Schoot et al (1978):-

CLASS A - 12 easily accessible sulphydryl groups which react with such sulphydryl reagents as N - ethylmaleimide and 5, 5' dithiobis (2 nitrobenzoic acid). They are all located on the catalytic subunit. At least one of these groups is essential for enzyme activity.

CLASS B - At least 14 sulphydryl groups located in a more lipophilic area, which are less accessible and react only with N - ethylmaleimide. They are also located on the catalytic subunit and include at least one sulphydryl group essential for enzyme activity.

CLASS C - At most 10 sulphydryl groups which do not react with either N - ethylmaleimide or 5,5' dithiobis (2 mitro-benzoic acid). Two of these groups are only modified after solubilisation of the protein in detergent. It is not known whether any of these Class C groups are essential for enzymatic activity (Schoot et al 1978).

It has been suggested by Schoot et al (1978) there are at least two,possibly three sulphydryl groups which are essential for NaKATPase catalytic activity. One Class A essential group is involved in the reactions that follow binding of ATP, and may possibly be involved in the binding to the 6 amino group of ATP (Patzelt-Wenczler et al 1975). Another Class A essential group may exist which is involved in the potassium phosphatase activity. This is generally considered to be a partial reaction of NaKATPase activity, although problems have arisen in the identification of the

substrate sites (Swann and Albers 1975) and localisation of the activating K^+ sites (Tashimo 1978). This -SH group is protected from sulphydryl reagents by ATP and the synthetic substrate 4-nitrophenylphosphate (Schoot et al 1978). Alkylation of the Class B essential group inhibits the overall activity and the partial reactions to the same extent (Schoot et al 1978).

In addition to cysteine at least one other amino acid is considered important for enzyme activity. Several arginine residues may exist which have equal importance for enzyme activity. The Class A essential sulphydryl group concerned with ATP binding and the arginine residue are adjacent to each other so that modification of one leads to modification of the other. At pH 7.4 the arginine residue is positively charged and it is thought that this functions by binding the negatively charged triphosphate backbone of ATP. Protection against inactivation by 2,3 butanedione, which modifies arginine residues, is only found when ATP or ADP, but not when AMP is bound to the enzyme. Hence, the essential arginine residue must bind the phosphate group of ATP (Schoot et al 1979).

4:1:2: REACTION SEQUENCE OF NaKATPase

Early studies (Post et al 1965, Fahn et al 1966) showed that not only was the enzyme phosphorylated in the presence of Na^+, Mg^{2+} and ATP but also that the phosphoenzyme existed in two forms:- E_1-P sensitive to dephosphorylation by ADP, and E_2-P, sensitive to dephosphorylation in the presence of K^+. It was considered that E—P differed only in conformational state and for symmetry two conformational states of the dephosphorylated enzyme were proposed, so giving rise to the scheme:-

$$E_1 \rightarrow E_1\text{—}P \rightarrow E_2\text{—}P \longrightarrow E_2 \longrightarrow E_1$$

In this scheme E_1—P was generally assumed to precede E_2-P (Fahn et al 1966), but this assumption has been questioned (Tonomura and Fukushima 1974).

Siegal et al (1967) have proposed a model for ATP hydrolysis based on the scheme (Figure 3). Part (1) of the reaction is classified as the sodium dependent phosphorylation which is dependent on both

Na^+ and Mg^{2+}. Part (2) is an Mg^{2+} dependent conformational charge involved in ion transport and part (3) is K^+ dephosphorylation of the enzyme. This latter reaction was also thought to reflect the non specific hydrolysis of the various phosphoric acid anhydrides, for example p-nitrophenylphosphate, umbelliferone phosphate and acetyl phosphate. This has been termed K^+ dependent phosphatase activity but problems have arisen in the identification of the subtrate sites (Swann and Albers 1975) and localisation of the activating K^+ sites (Tashima 1978). The final part of the sequence (4) is again a conformational change linked to ion transport.

Figure 3: REACTION SCHEME PROPOSED FOR ATP HYDROLYSIS (SIEGAL ET AL 1967):

$$ATP + E \xrightleftharpoons{Na^+ \quad Mg^{2+}} E_1 \sim P + ADP \qquad (1)$$

$$E_1 \sim P \xrightleftharpoons{Mg^{2+}} E_2 - P \qquad (2)$$

$$E_2P + H_2O \xrightleftharpoons{K^+} E_2 + P \qquad (3)$$

$$E_2 \rightleftharpoons E_1 \qquad (4)$$

4:1:3 Transport Modes of the Pump

Glynn and Karlish (1975) have distinguished four discrete modes of action of the sodium pump in red blood cells, at least three of which are macroscopically reversible:-

a) Coupled Na^+/K^+ exchange
b) Uncoupled Na^+ efflux
c) Na^+/Na^+ exchange
d) K^+/K^+ exchange.

Detailed reviewsof the role and mechanism of NaKATPase in ion transport have been given by Glynn (1964), Skou (1973, 1979), Askari (1974), Schwartz (1975), Kaplan (1979) and Robinson (1979).

4:2 THE ASSOCIATION INDUCTION HYPOTHESIS:

Although the role of the coupled sodium-potassium pump in the movement of ions across the cell membrane is accepted by a majority of workers, there has been some criticism of it (Ling 1965, 1966, 1978).

According to the coupled pump hypothesis, the bulk of intracellular K^+ and Na^+ ions exist in a free state. The steady levels of these ions are maintained by a pump in the cell membrane which actively transports K^+ ions into and Na^+ ions out of the cell in a coupled cyclic manner (see 4:1).

According to the association induction hypothesis, the intracellular ions (and other solutes) fall into two categories: interstitial and absorbed. Interstitial ions are found in the cell water in the free state, and as a rule are at lower concentrations than in the external medium. This is thought to be due to the existence of cell water polarised into multilayers by macromolecules. Negendank and Shaller (1979a) defined this structured water as neither "bound" nor "ice-like", and no part of it was truly "non solvent". This structured cell water is sufficiently orientated to exclude solutes on both an entropic and enthalpic basis, to varying degrees dependent on a) their molecular size, b) their hydrogen bonding and c) their own tendency to polarise water. This is felt to be the primary mechanism of Na^+ exclusion from the cell (Ling 1965, 1966, 1978).

Sodium and potassium ions are assumed to be absorbed on to monovalent anionic sites such as the β and γ carboxyl group of cell proteins. These anionic sites also help to maintain cell shape and volume by the formation of salt linkages with oppositvely charged groups such as imidazole, ε- amino and guanidyl groups on neighbouring proteins within the cells. Theoretical calculations, made on the basis of fundamental physical constants, show that when the electron density of the anionic group changes, the preference of the site may change from a preponderent preference for K^+ or Na^+ to another where this preference is diminished or reversed. Due to the fact that the electron density favours K^+ and also that there is a co-operative interaction between sites, 99% of the intracellular

K^+ is considered to be in a bound state, whilst only 50% of the Na^+ is bound (Ling 1966). Thus K^+ is concentrated in the cell because of its selective binding, and Na^+ concentration is low due to its exclusion by tightly structured water.

This theory is further supported by Negendank and Shaller (1979a) who argue, along with Ling, that agents such as ouabain and ATP exert their effects by the modulation of a large number of sites which results in a lower equilibrium constant for K^+ and Na^+.

This theory questions the role of NaKATPase in ion transport and cellular metabolism control, but although not widely held, Ling (1978) and Negendank and Shaller (1979b) have good evidence for the absorption of monovalent ions onto proteins, and this has been suggested to play an important role in the functioning of the cell (Kellermayer and Hazlewood 1979).

5: THE ROLE OF MONOVALENT AND DIVALENT CATIONS IN THE REGULATION OF CELLULAR METABOLISM AND REPLICATION:

5:1: INTRODUCTION:

The fact that the cell maintains an intracellular environment vastly different from the extracellular environment, and expends about one third of its total energy metabolism in doing so (Segal et al 1978) suggests that ions may play an important role in cellular metabolism.

Kaplan (1979) stated that changes in rates of cation movement had been implicated in the initiation and maintenance of the transformed state of lymphocytes (Quastel and Kaplan 1968). Inhibition of cell proliferation under conditions that led to a marked decrease in intracellular K^+ concentrations has been demonstrated in Sarcoma 180 cells (Lubin 1967), Girardi heart cells (Lamb and McCall 1972), BHK cells (McDonald et al 1972), L cells (Quissel and Suttie 1973), mouse lymphoblasts (Shank and Smith 1976) and human fibroblasts (Ledbetter and Lubin 1977). The enzyme responsible for the active transport of monovalent cations across the cell membrane, NaKATPase, has been shown to have both increased or decreased activity in transformed cells (Kasarov and Friedman 1974, Kimelburg and Mayhew 1975, Spaggiare et al 1976, Banerjee and Bosmann 1976, Elligsen et al 1974). Quastel and Kaplan (1968) reported that ouabain, the cardiac glycoside which is a specific inhibitor of NaKATPase, inhibited the incorporation of radiolabelled precursors into DNA, RNA and protein of stimulated human lymphocytes as well as inhibiting the blast transformation process itself. This inhibition was reversible upon either washing out the ouabain, or upon addition of excess K^+, which inhibits ouabain binding by competing for the same site on the ATPase enzyme.

These and other findings suggest that monovalent ions not only play an important role in the regulation of cellular metabolism but also might be important in the initiation and maintenance of cell replication.

However, Hammarström and Smith (1979) pointed out that the effects of ouabain on NaKATPase may simply constitute a model for the effect of this agent on other enzyme systems which may be the ones responsible for inhibition of mitogenesis.

5:2: THE ROLE OF Na^+ IN THE REGULATION OF CELLULAR METABOLISM AND REPLICATION:

5:2:1 METABOLISM:

The transport system for neutral amino acids has been broadly classified into two groups. The first has been denoted System A and is dependent on an Na^+ gradient across the cell membrane. The second has been denoted System L and is independent of an Na^+ gradient. Both systems are thought to have a wide specificity for neutral amino acids and essentially all the neutral amino acids are to some degree transported by both of these systems, although in different proportions. System A is strongly concentrative, System L more weakly so. It has been shown that steady states are set up in which net uptake for various amino acids takes place by System A, and net exodus by System L (Christensen 1977). Amino acids with weak reactivity with System A and much stronger affinity for System L maintain only moderate gradients across the membrane, whereas those with the opposite pattern of preference maintain rather high cellular levels relative to the extracellular fluid (Christensen 1977).

Another Na^+ dependent system almost completely specific to glycine has been described for nucleated and reticulated red blood cells (Christensen 1977), and it may occur in a variant form with broader specificity in the intestine and kidney (Christensen 1977). A third Na^+ dependent system denoted ASC has an intermediate range of specificity embracing 3 to 5 carbon straight chain amino acids and their hydroxy and sulphydryl derivatives, also asparagine, glutamine and the prolines. This system is less sensitive to H^+ and will not accept Li^+ as a substitute for Na^+. The receptor site of system ASC binds the alkali ion at a closely specified point in juxtaposition to the hydroxyl group of 4 hydroxyproline (Thomas and Christensen 1970), whereas System A binds Na^+ or L^+ at a different, less precisely localised point rather nearer the β carbon atom of the bound acid substrate (Christensen 1977).

Shank and Smith (1976) have suggested that cellular amino acid transport is maintained at a critical level such that small

changes in transport significantly influence cell metabolism.

Thus the Na^+ dependent uptake of certain amino acids can be explained by a transport system that combines with both Na^+ and the amino acid and causes translocation of both solutes across the membranes. The continued operation of such a system requires that Na^+ be extruded from the cell by NaKATPase in order to maintain the Na^+ gradient (reviewed by Schultz and Curran 1970).

5:2:2: REPLICATION

Cone (1974) proposed that electrical transmembrane potential and associated ionic concentration differences across the cell membrane were functionally involved in the control of mitogenesis in somatic cells.

The results obtained by Cone (1974) for CHO and 3T3 cells revealed that at lower density levels the cells maintained log-phase growth and a low electrical transmembrane potential (E_M). As the cell density increased, and substantial direct cell-to-cell surface contact began to develop, mitotic activity decreased, with a corresponding rise in the E_M. Thus Cone suggested a direct, functional involvement of the intracellular ionic environment, particularly the Na^+ concentration, in mitogenesis control.

Figure 4 is a schematic diagram of the hypothetical chain of conditions involved in mitogenesis control in somatic cells proposed by Cone.

This work was confirmed by Congote et al (1973) who showed that high intracellular Na^+ levels stimulated RNA synthesis and Stillwell et al (1973) who suggested that Na^+ was a mitogen. Cone (1974) predicted that intracellular Na^+ concentrations are elevated in rapidly dividing normal cells and tumour cells and this has been confirmed using energy-dispersive X-Ray microanalysis (Cameron et al 1980). The theory also predicts that ouabain or any agent that

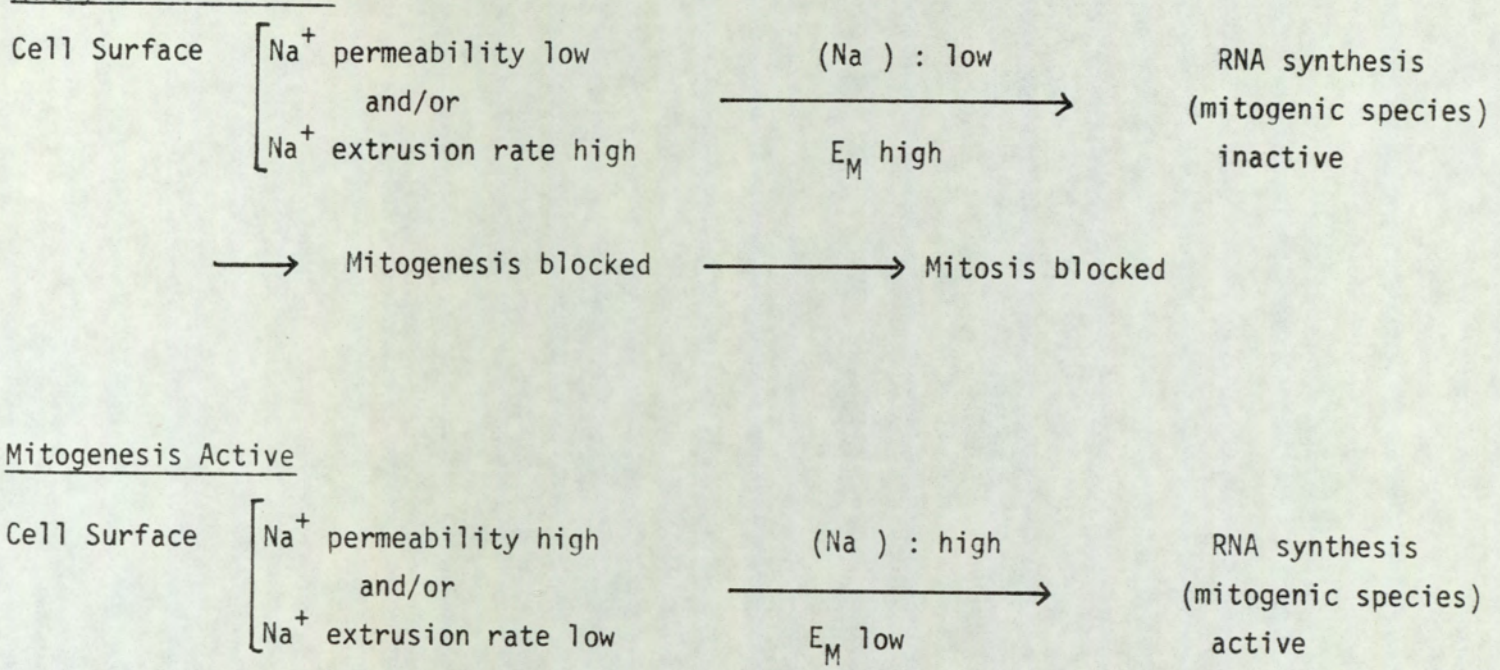
blocks Na^+ extrusion should be a mitogen by alteration of the transmembrane potential. Cone and Cone (1978) showed that an ouabain induced depolarisation of spinal cord neurones led to activation of DNA synthesis and nuclear division. The fact that many cells did not complete cytokinesis was considered to be due to the assay conditions, and not necessarily a direct effect of ouabain.

The mitogenic effect of ouabain found by Cone and Cone is contrary to the majority of findings where ouabain blocks mitogenesis (Lamb and McCall 1972 Cuff and Lichtman 1975a). Further, Shen et al (1978) found that although the transmembrane potentials of tumour cells were more Na^+ dependent than normal or preneoplastic cells, the transmembrane potential values obtained did not always show values expected by Coné's theory. This was also observed by Starnbrook et al (1975) who showed that through the cell cycle, the transmembrane potential of isolated V79 Chinese hamster cells in a synchronised colony varied in a way opposite to that predicted by Cone. The periodic increase in transmembrane potential did not appear to play a causal role in the regulation of cells through the cycle.

Thus, although there is some evidence that alterations in trans-membrane potential play a role in the activation of eukaryotic cells, possibly via a change in the intracellular content of Na^+, the results at present do not prove or disprove Cone's theory.

In addition to the role of Na^+ in the determination of transmembrane potential, Na^+ has also been shown to effect the transcription activity of genes. Kroeger (1963) showed that Na^+ was capable of reverting the pattern of gene transcription activities of mature differentiated cells to those characteristic of more immature stages of cell maturation. Recently Kellermayer and Hazlewood (1979) have suggested that the desorption of sodium and/or potassium ions from proteins (see 4:2) may play an important role in the reversible structural changes in DNA during mitosis.

FIGURE 4 The hypothetical chain of conditions involved in mitogenesis control in somatic cells (Cone 1974)



Isoleucine, an amino acid with a Na^+ dependent component of uptake has been considered to have a direct regulatory effect on DNA synthesis (Tobey and Let 1971). Oxender et al (1977) showed that the content of cellular amino acids increased two to three fold in quiescent cells possibly as a consequence of reduced protein synthesis.

It was therefore concluded by these authors that it was unlikely that the levels of intracellular amino acids regulated replication. Pardee et al (1978) in their review on the animal cell cycle concluded that decreased transport activity associated with quiescence was only a feedback type adaption to a reduced requirement for nutrients and not a major control mechanism for the regulation of cell replication.

5:3 THE ROLE OF K^+ IN THE CONTROL OF CELLULAR METABOLISM AND REPLICATION

5:3:1 METABOLISM

Potassium ion flux has been thought to be critical to cellular metabolism particularly because of the importance of the intracellular concentration of the ion in protein synthesis. The regulation of protein, RNA and DNA synthesis in cultured human fibroblasts by changes in the intracellular concentration of K^+ has been suggested to be possible by the regulation of peptide bond synthesis (Ledbetter and Lubin 1977). Naslund and Hutton (1971) have shown that the internal structure of both ribosomal subunits was destabilised by K^+ deficiency. They also showed that as a result of K^+ deficiency monoribosomes lost their ability *in vitro* to use poly (U) as an articiail messenger for phenylalanine incorporation and the ribosomes dissociated in their respective subunits. This effect was irreversible, even after addition of K^+.

A coupling between the potassium transport across reticulocyte membrane and protein synthesis within the cells has been suggested. It was shown that two potassium ionophores, valinomycin and dicyclohexyl - 18 - crown - 6 inhibited protein synthesis in reticulocytes. This inhibition was not due to direct changes in the intracellular potassium concentration. However, it was shown that both agents, the former at low concentrations only, acted reversibly and the inhibitory effect could be released by washing the reticulocytes. No effect was detected by the addition of the drugs to a reticulocyte cell free system, which suggested that the inhibition was likely to be membrane mediated (Herzberg et al 1974). Panet and Atlan (1979) expanded this work and found that under conditions where the two K^+ ionophores inhibited protein synthesis, there was a reduction in the ouabain resistant K^+ through the reticulocyte membrane. Moreover, evidence was given for a possible intermediate step in the coupling in that both ionophores caused a subtle interference with ATP metabolism. Thus they suggested that a change in K^+ efflux caused a reduction in the ATP/ADP ratio, which in turn inhibited protein synthesis. The mechanism of this coupling remains to be elucidated.

5:3:2: REPLICATION

The regulation of cell proliferation by K^+ has been suggested to a number of workers (Lubin 1967, Quastel and Kaplan 1970, Lamb and McCall 1972, McDonald et al 1972, Quissel and Suttie 1973, Cuff and Lichtman 1975a, Shank and Smith 1976, Ledbetter and Lubin 1977, Adam et al 1979).

Quastel and Kaplan (1970) reported that the transformation of human lymphocytes by phytohaemogglutinin (PHA) caused a rapid increase in the K^+ in flux. This data was consistent with the hypothesis that one of the essential early changes induced by the stimulation of resting lymphocytes was an increase in the number of sites for the transport of monovalent cations at the cell surface, either by exposure of preformed but previously cryptic pump sites, or by the aggregation of inactive subunits to active oligomers on the membrane. The increase in the number of functional NaKATPase sites would result in increased internal K^+ concentration above a threshold concentration required for all the train of events that link a stimulus at the membrane to the synthesis of DNA and to the mitosis that ultimately ensues (Kaplan et al 1976). This work was extended by Averdunk (1972) who demonstrated an increase in K^+ uptake within a minute of the addition of PHA to the medium in lymphocytes.

The hypothesis that an increase in intracellular K^+ concentration above some threshold level might play a part in the initiation mechanism of PHA treatment of lymphocytes has been questioned (Kaplan 1979). Results from Kay and Negendank (reviewed by Kaplan 1979) showed that intracellular K^+ concentrations fell after stimulation. Some of these paradoxical findings have been explained by Segal et al (1975). They demonstrated that PHA produced a rapid alteration in lymphocyte plasma membranes which allowed abnormal K^+ exchange. They therefore suggested that the change in K^+ content measured by other workers was possibly an indirect effect due to ion exchange during the preparative washes. These washes were performed to remove the agents, for example PHA and ouabain, prior to the determination of ionic content.

Segal et al (1976a) thus found no alteration in K^+ content after PHA treatment, from which they concluded that it was improbable that a significant increment in cell K^+ content was an early requirement for the initiation of blastogenesis and proliferation.

Homeostasis within the cell after PHA treatment was found to be maintained by an increase in K^+ efflux (Segal et al 1976b). This was confirmed by Iverson (1976), and therefore the observed increase in K^+ uptake after PHA treatment would appear to be a reflection of increased total flux of K^+ with an increased efflux to compensate for the enhanced uptake of K^+ (Segal et al 1976b). It has been speculated that the physiological benefit of this increased Na KATPase activity might be the enhanced uptake of the amino acids prior to blast transformation (Kaplan 1979, see 5:2:1). Another suggestion is that some critical step early in the train of events of stimulation requires that the cyclic AMP level of the cell will be reduced (see 2:4). If both adenyl cyclase and Na KATPase compete for a common pool of ATP the adoptive significance of the increase in pump activity might be the reduction in substrate available for cyclic AMP synthesis. It has been demonstrated that in cells derived from the MOPC 173 murine plasmocytoma, inhibitory concentrations of ouabain on cell growth caused an increase in intracellular cAMP concentration (Lelievre et al 1977).

Recently Holien et al (1979) have shown evidence for an immediate transient decrease in lymphocyte intracellular water volume in response to PHA. This contrasted with the result of Segal et al (1976a). Holien et al (1979) also showed that during the volume decrease there was no measurable change in the absolute intracellular content of K^+, but a transient increase in intracellular K^+ concentration. The decrease in lymphocyte water volume induced by PHA was also observed for concanavalin A which also stimulates lymphocyte proliferation, but not for wheat germ lectin, an agglutinating agent which is not mitogenic. Thus it was suggested that volume regulation, which would result in an alteration in intracellular K^+ concentration, may be closely associated with the mitogenicity of these compounds.

Cuff and Lichtman in a series of papers (1975a, 1975b, 1975c). showed that ouabain produced a concentration dependent decrease in K^+ content and growth rate in lymphoblasts (1975a).Continued exposure of the cells resulted in adaptation, that is, the K^+ content and growth rates returned to control values (1975b). A three hour lag occurred between the time of ouabain addition and the inhibition of proliferation (1975c). This finding resembles that of Quastel and Kaplan (1970)who showed that at least one hour of incubation with ouabain was required before inhibition of protein synthesis in lymphocytes become detectable, and two hours before incorporation of radiolabelled precursors into DNA and RNA was noticably affected. Cuff and Lichtman (1975c) showed that the inhibition of cell proliferation by ouabain was probably due to a block in late S phase, although all stages of the cycle seemed to be sensitive to some extent. Their data also suggested that the restoration of potassium and/or sodium to normal concentrations during late S phase was essential to the progress of mouse lymphoblasts through the cell cycle (1975c).

Mayhew and Levinson (1968) showed that ouabain inhibited the proliferation of Ehrlich ascites tumour cells and induced a change in K^+ content from 1.45 moles/10^7 cells to 1.05 moles/10^7 cells within one hour. This result resembles those of Adam et al (1979) who showed that protein synthesis was severely inhibited when intracellular potassium content fell below 60-80% of normal, although RNA synthesis was hardly affected.

In comparison to this, Andersson (1979) agreed that although an elevation of intracellular K^+ might increase protein synthesis it was unlikely that the absolute level of intracellular K^+ was directly related to the mechanism of stimulation since DNA synthesis was capable of being maintained at low K^+ levels. He proposed that transmembrane potential (E_m) and ionic events which co-ordinated DNA synthesis, karyokinesis and cytokinesis were local permeability changes by self-electrophoresis aggregation of charged membrane constituents in the plane of the plasma membrane.

5:4: THE ROLE OF TOTAL MONOVALENT CATION CONTENT AND/OR THE SODIUM:POTASSIUM RATIO IN THE CONTROL OF CELLULAR METABOLISM AND REPLICATION:

In addition to the effects based on a single monovalent ion outlined above, it has been suggested that total monovalent cation content and/or the Na^+ : K^+ ratio is important in the regulation of cellular metabolism (Kimelburg and Mayhew 1975, Fossel and Soloman 1979).

Cell free experiments have indicated that specific mRNA's are preferentially translated depending upon the Na^+ and K^+ concentration (Carrasco and Smith 1976). It has also been shown that changes in the intracellular levels of Na^+ and K^+ effect anabolism and catabolism of proteins during cataract formation,(Shinohara and Piatigorsky 1977, Piatigorsky et al 1978). However, Piatigorsky et al (1978) stated that the observed effects might be a secondary consequence of other changes. Protein degradation has been observed to be caused by the activity of a neutral endopeptidase which is associated with α - crystallin and apparently activated by hydration as Na^+ enters the lens.

Moscatelli et al (1979) have shown that decreasing the K^+ concentration of the extracellular medium from 5mM to 0.59 mM decreased the intracellular K^+ content of chick embryo fibroblasts to 22% of control values and increased the Na^+content to 82% of control values. The alteration of monovalent cation content occurred within two hours, but had no effect on the rate of ^3H thymidine incorporation for at least 16 hours. On decreasing the extracellular Na^+ concentration, a 50% reduction in intracellular $Na^+$was obtained, with no effect on ^3H thymidine incorporation. It was concluded that as these changes in cellular Na^+ and K^+ were much longer than any known to occur under physiological conditions and since they had no effect on ^3H thymidine incorporation, Na^+ and K^+ did not play a critical role in the determination of replication rate.

5:5 THE ROLE OF Mg^{2+} IN THE CONTROL OF CELLULAR METABOLISM AND REPLICATION

Much of the Mg^{2+} present in cells is bound to membranes, and it has been shown that Mg^{2+} is bound exchangeably to the same sites as Ca^{2+} (Sanui and Pace 1967). Of the small fraction of non membrane bound Mg^{2+}, a part is free or bound to adenine nucleotides (Rubin 1975a).

Many biochemical pathways controlled by magnesium in the cell are required to accelerate the progress of cells through the G1 phase into the S phase of the cell cycle (Rubin and Fodge 1974). In addition, another group of reactions which are independent of one another, and are not required to stimulate DNA synthesis, are known to be accelerated by a variety of unrelated growth stimulators (Rubin and Koide 1975, Rubin 1975b). These reactions include the transport of hexoses and uridine (Sefton and Rubin 1971, Weber and Rubin 1971), the steps of glycolysis catalysed by phosphofructokinase (Fodge and Rubin 1975), hexokinase and pyruvate kinase (Singhet et al 1974), the conversion of amino acids to lactate (Fodge and Rubin 1975) and the synthesis of hyaluronic acid and collagen (Moscatelli and Rubin 1977).

Rubin (1975b) proposed that, since Mg^{2+} was the only cellular constituent known to provide positive control in such a diversity of reactions, the availability of Mg^{2+} within the cell was the central mediator of the co-ordinate control of metabolism and replication of Ca^{2+} is modulated by an indirect effect on the intracellular free Mg^{2+}.

A reduction in free Mg^{2+} leads to an inhibition of thymidine incorporation into DNA, uridine into RNA and proline into protein. In the case of uridine, the uptake of this compound has been shown to be limited by the rate of its phosphorylation rather than its transport (Rozengurt et al 1977, Martin and Tashjian 1978, Plagemann et al 1978). The rate of uridine phosphorylation has been shown *in vitro* to vary directly with the intracellular free Mg^{2+} concentration but remained unaffected by Ca^{2+} (Bowen-Pope et al 1979). This was suggested to show that the availability of Mg^{2+} modulated the uptake of uridine, and the

authors concluded that serious consideration should be given to the mediation of Mg^{2+} in the other reactions of the co-ordinate response.

It has been suggested by Rubin (1977) that since the total intracellular Mg^{2+} content is less than is required for maximal activity of regulating enzymes, such as those which carry out the transphosphorylation of glycolysis (Mansour and Ahlfohrs 1968), small changes in the concentration of available Mg^{2+} would exert a considerable degree of control on general cell metabolism.

The inhibition of DNA synthesis which followed the chelation of Ca^{2+} by EGTA has been proposed to be due to changes in Mg^{2+} levels caused by the chelation of Ca^{2+}. Chelation of Ca^{2+} by EGTA is also believed to lead to the removal of bound ions from the membrane and their replacement of Mg^{2+}, thus lowering the free Mg^{2+} available to adenine nucleotides which in turn causes a reduction in DNA synthesis. Conversely, the stimulation of cell growth by excess Ca^{2+} is thought to be due to a displacement of Mg^{2+} from its membrane sites, which is considered to increase free Mg^{2+} available to adenine nucleotides and so leads to a stimulation of DNA synthesis (Rubin 1975a).

Sanui and Rubin (1978) concluded that although there was evidence for a role of Na^{+} and K^{+} in the regulation of cell metabolism and replication, they considered that there was a primary control role of intracellular free Mg^{2+} in the regulation of lymphocyte replication rate (Brennan and Lichtmann 1973) and in the co-ordinate control of metabolism and replication in animal cells in general (Rubin 1975a).

5:6 THE ROLE OF Ca^{2+} IN THE REGULATION OF CELLULAR METABOLISM AND REPLICATION

In animal cells the concentration of Ca^{2+} in the cytoplasm is in the range of 10^{-5} M - 10^{-8} M (Hodgkin and Keynes 1957 , Naninga 1961). In contrast, the concentration in mammalian extracellular fluid is close to 10^{-3} M. Thus the concentration ratio across the cell membrane is 10^{2} - 10^{5}, but the predicted ratio based on the Nernst equation is approximately 10^{-2}. Thus Ca^{2+} ions are not distributed according to their electrochemical potential across the membrane. Addition of radiolabelled calcium to the cell results in uptake followed by loss, that is, calcium exchange takes place across the cell membrane. It was suggested that Ca^{2+} entered the cell by a passive process, some of which was possibly dependent on the intracellular Na^{+} concentration, and was pumped out by an active process or "calcium pump" (Rasmussen 1970).

Smith and Vernon (1979) have shown extracellular Ca^{2+} exerted two effects on Na^{+} and K^{+} transport. First, Ca^{2+} caused a reduction in membrane permeability to K^{+}. Second, Ca^{2+} altered the coupling of the Na^{+}/K^{+} active transport mechanism which led to an electrogenic hyperpolarisation of the membrane. Conversely, alterations in the intracellular Na^{+} concentration have been shown to alter the influx of Ca^{2+} ions into the cell (Baker et al 1969). Glitsh et al (1970) have shown that a rise in intracellular Na^{+} led to an influx of Ca^{2+} ions and they proposed that even if the rise in Na^{+} was only slight, there would be an appreciable Ca^{2+} influx.

Ca^{2+} has been shown to have profound effects on cellular and enzyme function (Izquierdo and Izquierdo 1967, Paul and Ristow 1979). In addition calcium has been shown to be a key component of the plasma membrane (Manery 1966), with changes in Ca^{2+} binding altering many of the physical properties of the membrane. For example, changes in the permability to water, other ions and solutes occur as well as deformability of the membrane structure (Nanninga 1961, Manery 1966, Rasmussen 1970).

The amount of bound Ca^{2+} has been shown to be dependent on the

concentration of the free ion (Rasmussen 1970). The level of free Ca^{2+} in the cell has been proposed as the intracellular regulator of the proliferation rate in normal, but not transformed cells (Balk 1971). Boynton et al (1977) maintained that extracellular Ca^{2+} was a regulatory factor that was only needed briefly in the cell cycle. After phytohaemogglutinin treatment of lymphocytes an influx of Ca^{2+} was observed by Maino et al (1974), although it was stated that it was not clear if this was the only, or even the main consequence of phytohaemogglutinin binding. They also stated that at least three intracellular processes were controlled by the concentration of Ca^{2+} prior to DNA synthesis in stimulated lymphocytes -

a) Activation of phosphorylase kinase was required for the initiation of glycolysis for the provision of energy.

b) The microfilament - microtubule system was required for endocytosis and blockade by cytochalasin B stopped transformation by phytohaemogglutinin.

c) The level of the cyclic nucleotide cGMP, which is thought by some workers to be the nucleotide which initiates events culminating in mitosis (Hadden et al 1972) has been shown to be Ca^{2+} dependent in some cells (Schultz et al 1973).

Apart from its role in proliferation, Ca^{2+} has been shown to be involved in the regulation of glycogenolysis in tumour ascites cells via the ionic control of pyruvate kinase. Bygrave (1967) demonstrated that this enzyme was activated by Mg^{2+} and K^{+} ions and inhibited by Ca^{2+} and Na^{+} ions, so illustrating the close inter-relationship between the monovalent and divalent cations in cellular metabolism.

Thus, although Ca^{2+} is required as an enzymatic co-factor in relatively few intracellular reactions, it has been proposed as an intracellular regulator of replication. Paul and Ristow (1979) reported that 3T3 and SV3T3 has a Ca^{2+} sensitive restriction point at which cells reversibly arrest in G_1, which they suggested meant

that Ca^{2+} ions were required at an early point after growth stimulation of quiescent cells by serum. SV3T3 cells had a decreased requirement for Ca^{2+}. Since this decrease paralleled relaxation of growth control mechanisms it was suggested that the transformed cells had lost an important element of the original set of mechanisms that control proliferation, and the progressive loss of such mechanisms accompanied transformation (Paul and Ristow 1979).

WORKING HYPOTHESIS

The basis of the present investigation concerned the hypothesis that nitrogen mustard may inhibit the cell membrane enzyme, NaKATPase. It was postulated that since this enzyme was known to be sensitive to alkylating agents such as iodoacetic acid, inhibition of this enzyme by the nitrogen mustards might affect the intracellular cationic content of cells and thus effect consequent cellular metabolism and replication processes known to be controlled by these cations. Hence, the NaKATPase enzyme might be a vital target which may explain the cytotoxicity of the nitrogen mustards.

The cell line chosen in the study was the ADJ/PC6A plasmacytoma, a line which has previously been shown to be highly sensitive to nitrogen mustard type alkylating agents (e.g. Connors and Hare 1975). In addition to the studies using a sensitive cell line, it was proposed that the formation of a PC6A cell line resistant to alkylating agents might allow a comparison to be made of the enzyme activity on alkylating agent sensitive and resistant cell lines.

The assay systems used were designed to study the ability of the simplest of the antitumour β chloroethylamine alkylating agents, nitrogen mustard, to inhibit the enzyme NaKATPase *in vitro* in both a broken cell preparation and in whole cells. Prior to the study of the effects of nitrogen mustard on a crude cell membrane system from broken PC6A cells, the properties of the enzyme were compared with the known properties of NaKATPase from other sources.

6. MATERIALS AND METHODS:

6:1 GENERAL

6:1:1 CHEMICALS AND REAGENTS

All the chemicals used were obtained from British Drug Houses Ltd., Poole, Dorset (B.D.H.), unless otherwise stated. The disodium salt of adenosine triphosphate (Na_2ATP) obtained was free of the vanadate ion (vanadium in the 5^+ oxidation state) (Josephson and Cantley 1977) (personal communication B.D.H.). Iodoacetic acid and N - ethyl maleimide were obtained from the Boehringer Corporation London. p - Chloromercuribenzoic acid was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. Ouabain octahydrate was obtained from Sigma Chemicals Ltd., Poole, Dorset. Chlorambucil was obtained from the Wellcome Foundation, London. Nitrogen mustard was a kind gift from the Boots Co. Ltd., Beeston, Nottingham. Monofunctional chlorambucil was a kind gift from Dr.M.Tisdale, Department of Biochemistry, St.Thomas' Hospital, London and N,N dimethyl - 2 chloroethylamine was a kind gift from Dr.A.Creighton, Imperial Cancer Research Fund Laboratories, Lincolns Inn Fields, London. *Cis* diammine dichloro platinum and its *trans* isomer were a kind gift from Dr.M.Claire, Johnson Matthey Ltd. (Reading).

6:2: BUFFER SOLUTIONS Molarities shown are at final concentration in the assay.

6:2:1: a) 30mM Tris/HCL buffer pH 7.4

Tris (hydroxymethyl) amino methane base		3.63g
Water	to	1 litre

Adjusted to pH 7.4 by the dropwise addition of concentrated hydrochloric acid prior to making up to volume.

6:2:2: b) 100 mM Na^+/20mM K^+/5mM Mg^{2+} 30mM Tris buffer pH 7.4

Sodium Chloride		9.000g
Potassium Chloride		2.292g
Magnesium Chloride		1.561g
Tris (hydroxymethyl) amine methane base		3.63 g
Water	to	1 litre

Adjusted to pH 7.4 as before.

6:2:3: c) 5mM Mg^{2+} 30mM Tris buffer pH 7.4

Magnesium Chloride		1.561g
Tris (hydroxymethyl)amino methane base		3.63 g
Water	to	1 litre

Adjusted to pH 7.4 as before.

6:2:4: d) 1M Tris/HCl buffer pH 9.5

Tris (hydroxymethyl) amino methane base		131.0g
Water	to	1 litre

Adjusted to pH 9.5 by the dropwise addition of concentrated hydrochloric acid prior to making up to volume.

6:2:5: e) 8mM Mg/20mM K^+ 1M Tris pH 9.5

Magnesium Chloride		2.498g
Potassium Chloride		2.292g
Tris (hydroxmethyl) amino methane base		121.0g
Water	to	1 litre

Adjusted to pH 9.5 as before

6:2:6 f) 100 mM Na^+/20mM K^+/5mM Mg/0.2mM Ca^{2+} 30mM Tris buffer pH 7.4

Sodium chloride	9.00g
Potassium chloride	2.292g
Magnesium chloride	1.561g
Calcium chloride	0.067g
Tris (hydroxmethyl)amino methane base	3.63g
Water to	1 litre

Adjusted to pH 7.4 as before

6:2:7 g) 5mM Mg/0.2mM Ca^{2+} 30mM Tris buffer pH 7.4

Magnesium chloride	1.561g
Calcium chloride	0.067g
Tris (hydroxymethyl) amino methane base	3.63g
Water to	1 litre

Adjusted to pH 7.4 as before.

6:2:8 h) Dulbecco's Phosphate Buffered Saline (Dulbecco and Vogt 1954)

Calcium chloride	0.133g
Potassium chloride	0.200g
Di-potassium dihydrogen phosphate	0.200g
Sodium chloride	0.800g
Di-sodium hydrogen phosphate	1.150g
Water to	1 litre

DRUG SOLUTIONS

Drugs were dissolved in the solvent specified in the text and diluted to the required concentration with Tris buffer (6:2:1).

6:3: ANIMALS

Female Balb/C mice from the Department of Pharmacy, University of Aston part outbred colony were used throughout, unless otherwise stated.

6:4: TUMOUR TRANSPLANTATION

(PC6 and PC6A are taken to be synonymous).

The ADJ/PC6A ascites tumour, which has been shown to be sensitive to alkylating agents (Connors and Hare 1975) was grown in the peritoneal cavity of 20g female Balb/C mice and maintained by routine passaging every 7 days.

Ascites cells were removed from the donor animal and 0.5ml of the ascites suspension was injected intraperitoneally (i.p.) into each recipient mouse.

6:5: IN VITRO - IN VIVO ASSAY ON SOLID PC6A TUMOUR

1.0ml of PC6A ascites tumour was suspended in 8.9ml of media (RPMI 1640 + Horse Serum (Gibco Europe, Glasgow) 6:4). 0.1 ml of drug solution or 30mM Tris buffer pH 7.4 (6:2:1) was added and the suspension incubated at 37^{o} for 2 hours. 0.1 ml aliquots were then injected subcutaneously into each recipient 20g female mouse.

After 28 days the animals were killed by cervical dislocation and the solid tumours dissected out and weighed.

6:6: CELL COUNTING

A Coulter particle counter, model ZB1 was used. Dilutions were made with Isoton (Modified Eagle's medium from Coulter Electronics, Harpenden, Herts.) so that a final cell count of between 10,000 and 60,000 was obtained. The count was then corrected for coincidence using the chart supplied by Coulter Electronics.

The settings used for counting PC6A cells were:-

Amplitude 8 Threshold - Lower 18 Upper 85 Aperture Current 0.5 matching 20K.

The settings for amplitude and aperture current were determined by trial and error to give the optimum picture on the Coulter oscilloscope according to the Coulter manual. The threshold setting was determined by the use of pollen grains of a known diameter (13.31 microns).

6:7 WASHED WHOLE ASCITES CELLS

3.0 ml of ascites cells were removed from the animal and re-suspended in RMPI 1640 media at 37°. The suspension was centrifuged at approximately 500g (speed 5) on an MSE Minor bench centrifuge for 60 seconds and then the pellet resuspended in RPMI 1640 media. A sample was counted on the Coulter counter, corrected for coincidence, and the cells resuspended in RPMI 1640 media at 10^7/ml unless otherwise stated.

6:8 IN VITRO-IN VIVO ASSAY OF CYTOTOXICITY TO AN ASCITES PC6A TUMOUR.

Washed ADJ/PC6A ascites cells in RPMI 1640 media contained in sterile polypropylene tubes (16 x 100 mm round bottom/screw cap, Sterilin Ltd., Middlesex) at a concentration of 2 x 10^7 ml, plus drug or 30 mM Tris buffer (6:2:1), were incubated for 2 hours (unless otherwise stated) at 37° in a revolving frame. 0.5ml aliquots were then injected i.p. into each recipient 20g female mouse.

After 7 days the animals were killed by cervical dislocation, the tumour cells removed and counted as before. The peritoneum was washed with 1.0 ml of sterile saline and this washing added to the removed cells.

To prevent cell clumping prior to counting the cells were suspended in approximately 70ml of Isoton.

6:9: CELL HOMOGENATE PREPARATION FROM PC6A CELLS.

The ascites cells were removed from the animal seven days after transplantation. 3.0 ml of a neat cell suspension was centrifuged at approximately 300 g (speed 3) on an MSE Minor bench centrifuge for two minutes and then resuspended in 7.7 mM Mg^{2+} 30mM Tris/HCl buffer pH 7.4 to a volume of 15ml.

Different methods of cell disruption were used to determine any difference in the total ATPase, total NaKATPase and ouabain sensitive NaKATPase activities obtained.

6:9:1: SONICATION

Sonication was performed on an Ultrasonic A350G sonicator (Ultrasonics Ltd.,Shipley, Yorks.) with a 3mm titanium probe. Cell disruption was achieved using four 5 second bursts of sonication at mark 6, after fine tuning. The suspension was cooled in ice for 30 seconds after each sonication.

6:9:2: POTTER HOMOGENISER/FREEZE AND THAW

The cell suspension was rapidly frozen in crushed dry ice, thawed quickly at 37^{o} and homogenised with five strokes using a 23mm Teflon Potter homogeniser. The cell suspension was then frozen again and the process repeated eight times.

6:9:3: HYPOTONIC SOLUTION

The cell suspension was centrifuged at approximately 300g (speed 3) on an MSE Minor bench centrifuge for two minutes and then the pellet was resuspended in 10 micromoldr sodium bicarbonate solution and left for 30 minutes.

The cell suspensions were then treated as below.

After each process the disruption was checked using a Wild Binocular light microscope (Wild Microinstruments Ltd.) at a magnification of x400. In addition negative stained slides were prepared and viewed with an EM 6B AEI electron microscope. This demonstrated that few (<1%) whole cells remained after disruption.

6:9:4:

After disruption was obtained, the suspension was centrifuged at 800g on an MSE18 centrifuge for 10 minutes at 4^{o}, and the pellets obtained resuspended in 30mM Tris buffer pH 7.4 to a volume of 30ml. The preparation obtained consisted of broken cells, nuclei and about 1% whole cells. The broken cell preparations were used either immediately or stored overnight at 4^{o}c. This was due to the stability of the preparation (see 7:4 results).

6:10: CELL HOMOGENATE PREPARATION FROM OTHER TISSUES.

6:10:1: GUT MUCOSAL CELLS

The intestinal mucosa was scraped from the everted intestine of female Balb/C mice into cold 7.7mM Mg^{2+} 30mM Tris buffer (6:2:3) and the cell homogenate prepared by the Potter homogeniser/freeze and thaw technique. (6:9:2).

6:10:2: HEART CELLS

The heart was removed from a freshly killed animal into cold 7.7mM Mg^{2+} 30mM Tris buffer (6:2:3), and chopped into approximately 1mm cubes. The cell homogenate was prepared by the Potter homogeniser/freeze and thaw technique (6:9:2).

6:10:3: TLX5 CELLS

TLX5 cells were removed from a male CBA/Lac mouse, which had been injected i.p. seven days earlier with approximately 10^{6} TLX5 cells, into cold 7.7mM Mg^{2+} 30mM Tris buffer (6:2:3). The cell homogenate was prepared by sonication as before (6:9:1).

In all cases the broken cell preparations were obtained as before (6:9:4).

6:11: ASSAY PROCEDURES:

6:11:1: INORGANIC PHOSPHATE ASSAY

The procedure was based on the method of Baginski and Zak (1960).

The reactions were stopped with ice cold 10% Trichloroacetic acid and the solutions were centrifuged at approximately 8000g (speed 8) for 5 minutes on an MSE Minor bench centrifuge. 1.0ml aliquots of the supernatent were removed and the inorganic phosphate estimated by the addition in turn of 0.2mL 10% ascorbic acid, 0.5ml of molybdate reagent and 1.0 ml of arsenite - citrate reagent. The blue colour was allowed to develop for 15 minutes, then the absorbance measured at 600mm using a Beckmann Acta V spectrophotometer.

Standards were prepared according to Baginski and Zak (1960) but the standard curve was modified to test 1.0 ml instead of the 0.1ml stated.

The reason for the modification was the low levels of release of phosphate obtained in the ATPase assays. To obtain absorbance in the region of 0.15 - 0.35 units, the test volume was increased from 0.1ml to 1.0 ml with the corresponding alterations to the standard curve.

A standard curve was prepared for every assay performed.

6:11:2: ATPASE ASSAY

The total ATPase, MgATPase and NaKATPase activities of the broken cell preparations were determined by a procedure based on the method of Taylor (1967) and are described below:

6:11:3: ASSAY OF TOTAL ATPASE AND Mg ATPASE ACTIVITY

Unless otherwise stated buffer solution B (6:2:2) (1.3ml) was incubated with 100 µl of the broken cell preparation prepared as under (6:9:1), and 100 µl of 30mM Tris pH 7.4 (6:2:1) for 15 minutes at 37°. The reaction was started by the addition of 0.5 ml of 10mM Na_2 ATP in Tris buffer at pH 7.4 and allowed to proceed for 10 minutes as in the method of Taylor (1967).

The MgATPase activity was determined in the same manner using buffer solution C (6:2:3).

6:11:4 Na K ATPASE ACTIVITY

NaKATPase activity was determined and defined in two ways:-

a) 100µl of 3mM ouabain was added to the Total ATPase assay 15 minutes prior to the addition of ATP. The difference in activity obtained with and without the presence of ouabain was defined was ouabain sensitive NaKATPase activity.

b) By the subtraction of the MgATPase activity from the Total ATPase activity. This activity was defined as the total NaKATPase activity.

In all cases correction was made for the spontaneous non enzymic breakdown of ATP, measured as inorganic phosphate, and the phosphate content of the broken cell preparation and buffer solution.

Protein content, which was approximately 40 - 60µg/ml final concentration, was measured by the method of Lowry et al (1951) using bovine serum albumin as standard. Results of ATPase assays were expressed as µMPhosphate/mg protein/hour.

6:11:5 EFFECT OF SULPHYDRYL AND CYTOTOXIC REAGENTS ON OUABAIN SENSITIVE NaKATPase ACTIVITY AND TOTAL ATPASE ACTIVITY.

Assays were set up as in (6:11:3). In addition, 100µl of the sulphydryl reagents solution was added to the drug treated assays. After 15 minutes incubation at 37°, ouabain was added to one of the control assays and one of the drug treated assays. After a further incubation of 15 minutes ATP was added to all the assays and the reaction allowed to proceed for 10 minutes. The reactions were terminated with 2.0 ml of ice cold 10% Trichloroacetic acid and tested for phosphate as before (6:11:1). The effect of the sulphydryl reagents on the ATPase activities were defined as follows:-

a) Control activity - Control activity plus ouabain = Ouabain sensitive NaKATPase activity (6:11:4).

b) Activity of the drug treated preparation with sulphydryl reagent alone - activity of the drug treated preparation with sulphydryl reagent plus ouabain = Effect of ouabain on ATPase activity after drug treatment.

A comparison of the effect of ouabain on ATPase activity after 30 minutes drug treatment (b), against ouabain sensitive NaKATPase activity (a), shows the effect of the sulphydryl reagent on ouabain sensitive NaKATPase activity after a 30 minute preincubation. If (b) equals zero then there is 100% inhibition of ouabain sensitive NaKATPase. If (b) equals the ouabain sensitive NaKATPase activity (a) there is 0% inhibition of ouabain sensitive NaKATPase.

6:11:6: EFFECT OF SULPHYDRYL REAGENTS ON MgATPASE ACTIVITY

MgATPase activity was determined as before (6:11:3), with the modification that the control MgATPase activity was determined after a 30 minute incubation at 37^{o} , and that 100µl of a sulphydryl reagent solution was added at zero time, 30 minutes prior to the addition of ATP.

% Inhibition was determined after a 30 minute preincubation by subtracting the sulphydryl treated MgATPase activity from the control MgATPase activity and comparing this value to the control MgATPase activity.

6:11:7: p-NITROPHENYLPHOSPHATASE ACTIVITY

1.3ml of buffer solution E (6:2:5), plus 0.5ml of broken cell preparation and 0.1ml of solution D (6:2:4) were incubated for 15 minutes at 37^{o} . The reaction was started by the addition of 0.1ml of p-nitrophenylphosphate solution to give a final subtrate concentration of 1mM. The reaction was allowed to proceed for 10 minutes and then terminated by the addition of 2.0ml of ice cold 20% trichloroacetic-acid.

p-Nitrophenylphosphatase activity was defined as the activity obtained in a solution containing 8mM Mg^{2+} plus 20mM K^{+} (Solution E. 6:2:5) minus the activity obtained in a solution without these ions (Solution D. 6:2:4).

Normally the above reaction is stopped with sodium hydroxide and the p-nitrophenol content measured after the protein precipitate is removed. Using this technique the absorbence obtained was negligible (below 0.05 absorbence units) and so the inorganic phosphate content was determined as before (6:11:1) a method used by Monteil et al (1974). Values were corrected for the release of phosphate from p-nitrophenylphosphate, broken cell preparation and buffer solution. Protein determination was as before (Lowry et al 1951) and the results were expressed as μM phosphate/mg protein/hour.

6:11:8: EXTERNAL CaMgATPase ACTIVITY (RONQUIST AND AGREN 1975)

1.3ml of buffer solution F (6:2:6) was preincubated with 10^6 intact washed ascites cells and 100μl of 30mM Tris pH 7.4 (6:2:1) for 15 minutes at 37°. The reaction was started by the addition of 0.5ml of 10mM Na_2ATP in Tris buffer at pH 7.4 and allowed to continue for 10 minutes. The reaction was terminated by the addition of 2.0ml of ice cold 10% trichloroacetic acid and the phosphate content determined as before. Results were expressed as μM Phosphate/10^6cells/hour.

6:11:9: ASSAY OF CELL VOLUME MEASUREMENTS IN A HYPOTONIC SOLUTION

The method of Shank and Smith (1976) was used. Washed cells were subjected to a hypotonic shock and the cell numbers at a particular size range determined over a period of time using the Coulter counter. An aliquot of cells were resuspended in buffer solution H (Dulbecco's Phosphate Buffered Saline). This solution was then diluted by 25% by the addition of distilled - deionised water. Cell numbers were repeatedly counted at 37° over a period of 15 minutes in the Coulter counter with a narrow window setting:- Threshold Lower 23 Threshold Upper 28.

6:11:10: EFFECT OF OUABAIN ON THE CELL VOLUME MEASUREMENTS IN A HYPOTONIC SOLUTION

Ten minutes prior to the dilution of the media various concentrations of ouabain were added to the cell cultures.

The hypotonic shock was obtained as before, with the addition that ouabain was present in the water used for the dilution at the same concentration as in the culture. Results were expressed as the ratio of the number of cells counted 15 minutes after hypotonic shock, to the number of cells counted immediately after hypotonic shock.

6:12: STUDY OF THE EFFECT OF ALKYLATING AGENTS AND OUABAIN ON THE UPTAKE OF RADIOLABELLED RUBIDIUM INTO PC6 CELLS

6:12:1: SUMMARY

The technique used to separate the cells from the media after incubation with radiolabelled rubidium involved the rapid centrifugation of an aliquot (200 µl) of the cell suspension after it had been placed on top of an oil layer (100µl) which itself was placed over formic acid (50µl). The density of the oil was adjusted to allow the passage of the cells through it and into the formic acid.

6:12:2: DETERMINATION OF THE OPTIMUM OIL DENSITY

Formic acid (50µl) was pipetted into each polypropylene tube (400µl polypropylene tube, Beckman Ltd., High Wycombe, Bucks.) and rapidly spun down (9000g) in a Beckman microfuge B.
On top of the formic acid was layered 100µl of each of the following oil mixtures:-

Silicon Oil*	+	Corn Oil+
5		0
5		0.5
5		1.0
5		1.5
5		2.0

*Silicon oil DC500 Hopkin and Williams Ltd., Chadwell Heath, Essex.
+Mazola pure corn oil.

The oil layer was allowed to settle by gravity before proceeding. 200µl aliquots of a cell suspension containing 10^7 PC6 cells per ml in RPMI 1640 media were carefully layered on top of the oil phase and the tubes centrifuged for 2 minutes in a Beckman microfuge B. The tubes were then checked visually for the passage of cells through the oil layer and photographed.

6:12:3: UPTAKE OF RADIOLABELLED RUBIDIUM

The procedure used was as above. The optimum oil density was found to be 5 parts Silicon oil plus 1.5 parts Corn oil (results Plate 1). 50µl of labelled rubidium was added at time zero to the PC6 cell suspension and after centrifugation the tubes were rapidly frozen in liquid nitrogen and cut through the oil layer. Both parts of the tube thus produced were placed in polypropylene centrifuge tubes (LP3 tubes, Luckham Ltd.,Burgess Hill, Sussex) and were counted on an AEG gamma counter with settings for rubidium of:-

HV x 2 : 393 Gain 4 Threshold 292 Window 832

6:12:4: STUDIES ON THE EFFECT OF INULIN CARRYOVER

Inulin is a carbohydrate which does not enter mammalian cells. Therefore it is used in studies with radiolabelled compounds to determine the amount of radiolabelled compound trapped between the cells after separation from the media. Using the above separation technique the determination of the amount of radiolabelled compound trapped between the cells and carried over through the oil was performed. The procedure for separating the cells from the media was as above and 50µl of ^3H inulin was added prior to the assay.

After cutting through the oil phase, the upper and lower sections of the tubes were placed in 20ml glass counting vials, allowed to thaw, then counted on a Tri-Carb 2660 beta counter (Packard Instrument Co., Illinois, U.S.A.) with 10.0ml of Picofluor 30 as the scintillant. (Packard Instrument Co., Caversham, Berks.)

The results were determined as disintegrations per minute (d.p.m.) obtained from a quench curve for formic acid and Picofluor 30 on the beta counter used provided by Mr.M.Atkinson, Biological Sciences Department, University of Aston (Figure 5).

6:12:5: DETERMINATION OF THE NUMBER OF CELLS WHICH PASS THROUGH THE OIL LAYER USING RADIOLABELLED CHROMIUM

The Chromium ion is absorbed onto the outer surface of cell membranes, and this allows its use as a tracer to determine the total number of cells which pass through the oil layer after centrifugation. The resuspended cells were labelled with chromium and incubated for 60 minutes at 37^{o} in a revolving frame to ensure mixing. The suspension was then washed four times with fresh RPMI 1640 media and aliquots treated as before (6:12:3). The chromium activity of both the supernatent and and pellet were counted on the appropriate mode on the AEG gamma counter.

FIGURE 5

QUENCH CURVE FOR FORMIC ACID IN PICOFLUOR 30

STANDARD		RATIO	EFFICIENCY
FORMIC ACID	PICOFLUOR 30		
1.0ml	10.0ml	0.2285	11.97
0.875ml	10.125ml	0.2809	15.89
0.750ml	10.275ml	0.3814	22.85
0.625ml	10.375ml	0.5303	36.56
0.500ml	10.5ml	0.5602	39.69
0.375ml	10.625ml	0.5864	41.33
0.250ml	10.750ml	0.6008	42.52
0.125ml	10.875ml	0.6522	48.44
0	11.0ml	0.6685	47.67

6:13: DETERMINATION OF AVAILABLE Cl AND HALF LIFE OF NITROGEN MUSTARD (DAVIS AND ROSS 1965)

19.3mg of nitrogen mustard was dissolved in 10ml distilled water at various temperatures and left for either 30 seconds, 1 minute or 5 minutes. 10ml of 0.1M sodium thiosulphate was added and titrated with 0.1M sodium hydroxide to the phenolphthalein end point.

The solution was then warmed for a short time and back titrated with 0.1M iodine (starch indicator).

7: RESULTS

7;1: PHOSPHATE STANDARD CURVE

Figure 6 shows three phosphate standard curves determined according to Baginski and Zak (1960). It can be seen that there is some variation and thus a standard curve was prepared each time an assay was performed.

7:2: EFFECT OF PROTEIN CONCENTRATION ON TOTAL ATPASE ACTIVITY

Figure 7 shows that there was a linear relationship between protein concentration and the rate of ATP hydrolysis as measured by phosphate production (see 6:11:1). Table 3 shows the same data with relation to ATPase activity. Three broken cell preparations were made and the Total ATPase (6:11:3) determined on each. The results show that over the range used the protein content had no effect on the Total ATPase activity observed.

7:3: EFFECT ON SOURCE OF ATP ON OUABAIN SENSITIVE NaKATPASE ACTIVITY (SEE METHODS 6:11:4)

Josephson and Cantley (1977) have shown that Na_2ATP (Sigma Chemicals) contains a potent inhibitor of NaKATPase. Table 4 shows the effect of the source of ATP on the NaKATPase activity obtained. It shows that when a simultaneous assay on the ouabain sensitive NaKATPase activity of a broken cell preparation is performed, ouabain sensitive NaKATPase activity was only found when Tris ATP (Sigma Chemicals) was used.

The second part of the table shows that when a broken cell preparation was assayed simultaneously with Tris ATP (Sigma Chemicals) or Na_2ATP (BDH), comparable ouabain sensitive NaKATPase activity was obtained. All further experiments were performed using Na_2ATP(BDH) unless otherwise stated.

FIGURE 6

STANDARD PHOSPHATE CURVES PREPARED ON CONSECUTIVE DAYS USING THE MODIFIED TECHNIQUE OF BAGINSKI AND ZAK

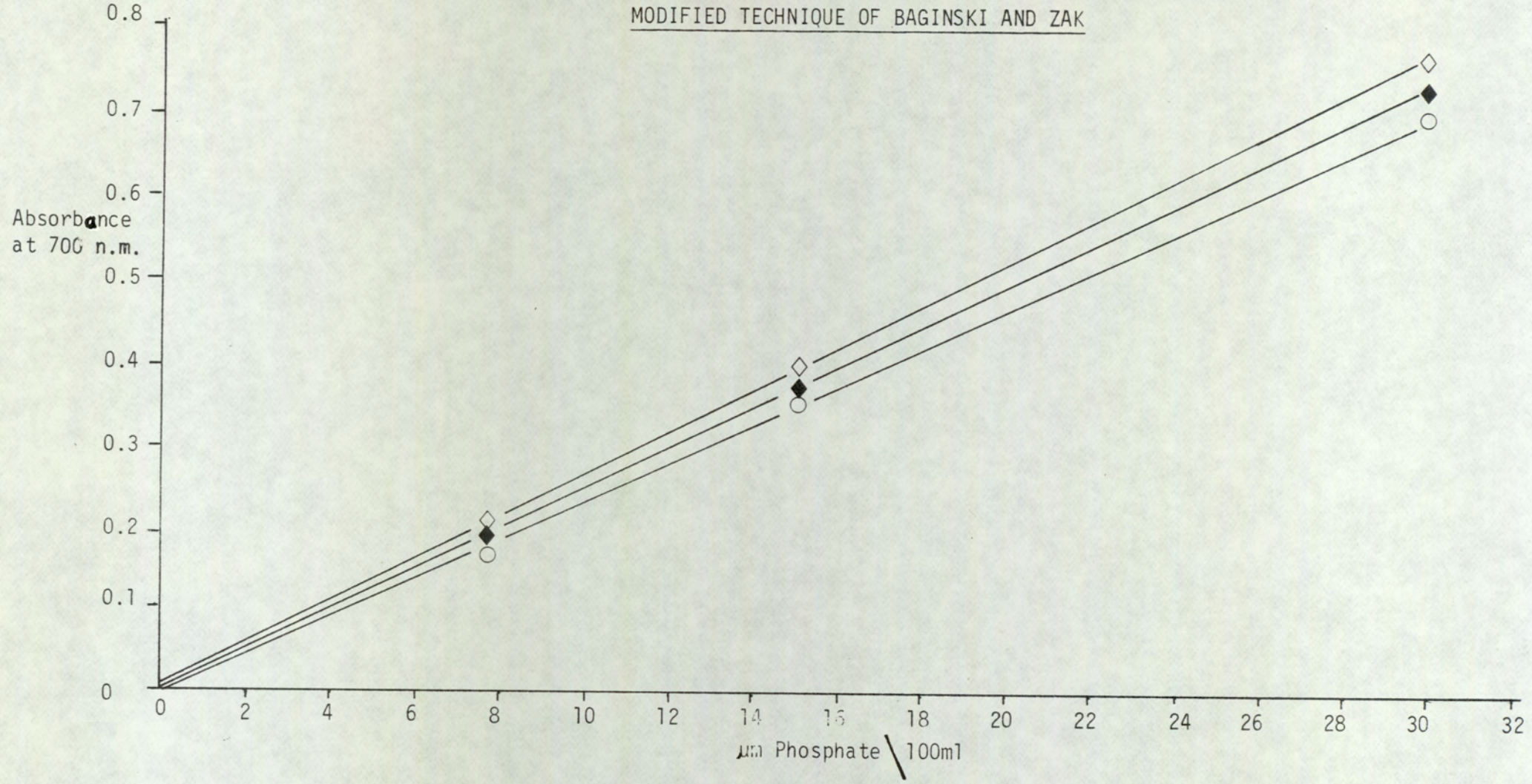


FIGURE 7

EFFECT OF PROTEIN CONCENTRATION ON ATP HYDROLYSIS BY A BROKEN CELL MEMBRANE PREPARATION

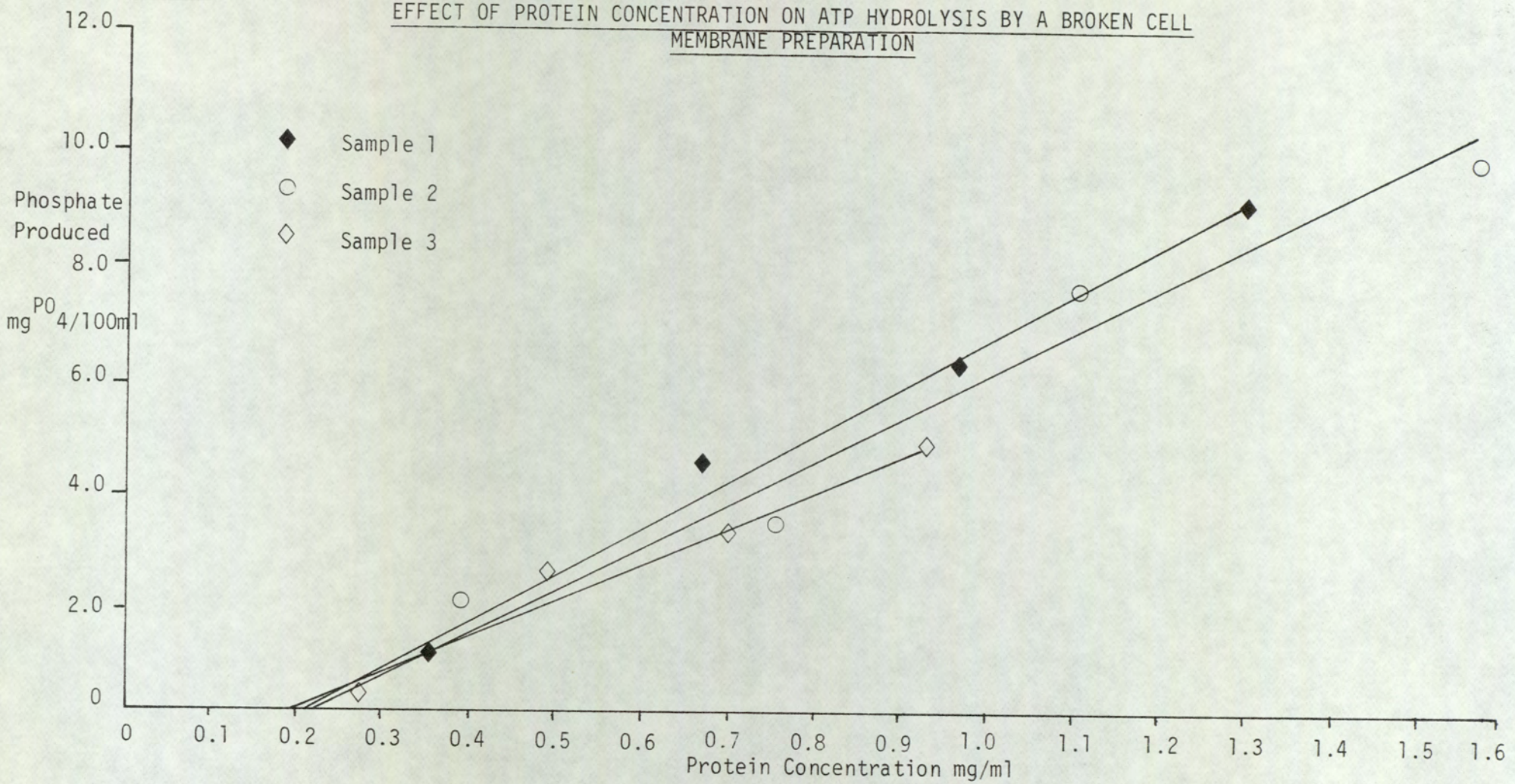


TABLE 3

EFFECT OF PROTEIN CONCENTRATION ON TOTAL ATPASE ACTIVITY

SAMPLE 1	µMPi/mg/hr ACTIVITY	SAMPLE 2	µMPi/mg/hr ACTIVITY	SAMPLE 3	µMPi/mg/hr ACTIVITY
1.31mg/ml (1)	8.51	1.49mg/ml	8.14	0.94mg/ml	7.41
0.98mg/ml	8.69	1.12mg/ml	7.37	0.71mg/ml	8.69
0.66mg/ml	7.04	0.75mg/ml	4.83	0.47mg/ml	8.54
0.33mg/ml	8.78	0.37mg/ml	8.23	0.24mg/ml	4.10

(1) The concentration shown is that of the prepared broken cell preparation, not the final concentration in the assay.

TABLE 4

EFFECT OF SOURCE OF ATP ON OUABAIN SENSITIVE NAKATPASE ACTIVITY

ATP SOURCE	OUABAIN SENSITIVE NaKATPASE ACTIVITY µMPi/mg/hr
Na_2ATP (Sigma) TRIS ATP (Sigma)	No activity 2.33 n = 1
TRIS ATP (Sigma) NaATP (BDH)	3.32 3.29 n = 1

7:4: STABILITY OF BROKEN CELL PREPARATION STORED AT $4^{o}c$

In Table 5 the effect of storage on the ATPase activity of the broken cell preparation at $4^{o}c$ is shown. The preparations were thawed and then incubated for 30 minutes prior to the addition of ATP, ouabain was added after 15 minutes where applicable. The table shows that a preparation stored overnight had activity comparable to that which was used immediately after preparation. There was some loss in Total ATPase activity on storage at $4^{o}c$ for 4 days and a complete loss of ouabain sensitive NaKATPase activity after this time.

7:5: CELL HOMOGENATE PREPARATION (SEE METHODS 6:9)

The techniques used to prepare the cell homogenates were:-

Sonication
Hypotonic solution
Potter homogeniser with freezing and thawing

The results in Table 6 show the ouabain sensitive activity and total NaKATPase activityof the broken cell preparations prepared from the cell homogenates obtained by the three techniques.

Sonication and the Potter homogeniser gave comparable results, but due to the time required to obtain adequate cell lysis with the Potter homogeniser, in all subsequent preparations of the broken cell preparation, the A350G sonicator was used unless otherwise stated.

Hypotonic lysis gave a preparation with low NaKATPase activity, but common to all three methods was the high degree of variation in the Total NaKATPase and ouabain sensitive NaKATPase activities.

TABLE 5

STABILITY OF THE BROKEN CELL PREPARATION STORED AT 4 °C PREPARED BY SONICATION

n = 3

	TOTAL ATPASE µMPi/mg/hr	µMPi/mg/hr OUABAIN SENSITIVE NaKATPASE
USED IMMEDIATELY AFTER PREPARATION	6.22 ± 1.66	2.02 ± 1.16
STORED 18 HOURS @ 4°C	6.98 ± 2.30	2.10 ± 0.40
STORED 4 DAYS @ 4°C	1.22 ± 1.07	No activity

TABLE 6

EFFECT OF PREPARATION ON THE TOTAL ATPASE TOTAL NaKATPASE AND OUABAIN SENSITIVE NaKATPASE ACTIVITY

METHOD OF DISRUPTION	TOTAL ATPASE µMPi/mg/hr	OUABAIN SENSITIVE NaKATPASE µMPi/mg/hr	TOTAL NaKATPASE µMPi/mg/hr
SONICATION	10.22 ± 2.95	2.68 ± 1.41	3.25 ± 2.19
HYPOTONIC SOLUTION	8.58 ± 1.13	1.10 ± 1.41	1.99 ± 1.78
POTTER HOMOGENISER/ FREEZE AND THAW	8.17 ± 0.66	2.55 ± 2.22	3.71 ± 0.83

7:6: EFFECT OF THE CHANGE IN IONIC CONCENTRATION ON ACTIVITY

The activity of the ATPase enzyme has been shown to require the presence of Na^+, K^+ and Mg^{2+} for maximum ATP hydrolysis (Skou 1957). Figure 8 shows the effect of altering the concentration of Na^+ and K^+ on the Total ATPase activity (6:11:3). Maximal activity occurred when the ratio of Na^+ : K^+ was in the order of 5 : 1. This same ratio was also required for maximal total NaKATPase activity (6:11:4). Ouabain inhibition has been shown to be antagonised by high K^+ concentrations (Palmer and Nechay 1964, Nechay et al 1967, Nelson and Nechay 1971 and Nechay et al 1975), therefore in these experiments where high $[K^+]$ was used NaKATPase activity was determined as the total NaKATPase activity and not as ouabain sensitive activity (6:11:4) (Figure 9).

Figure 8 also shows that there was an ATPase activity significantly different ($p=0.01$) from the activity obtained with Tris buffer alone, irrespective of the ionic concentration. It was considered that this was due to MgATPase activity.

In addition figure 8 demonstrates the high degree of variation in the results obtained at high K^+ concentrations. This high variation renders the results with 100mM K^+ non significant from the result with 100mM Na^+ and 20mM K^+ ($p>0.5$).

Figure 9 shows that increased Na^+ concentration at a constant Na:K ratio of 1:1 caused inhibition of NaKATPase activity as the cationic content was increased.

7:7: EFFECT OF IONIC CONTENT ON ACTIVITY

Figure 10 shows that for the hydrolysis of ATP the enzyme requires Mg^{2+} (columns 3 and 4). Addition of 100mM Na^+ to the Mg^{2+} had no significant effect on activity ($p>0.5$), but the addition of K^+ to the Mg^{2+} showed a significant increase in activity ($p=0.2$).

FIGURE 8

THE EFFECT OF VARYING THE MONOVALENT CATIONIC CONCENTRATION ON THE TOTAL ATPASE ACTIVITY OF A BROKEN CELL MEMBRANE PREPARATION OF PC6 CELLS

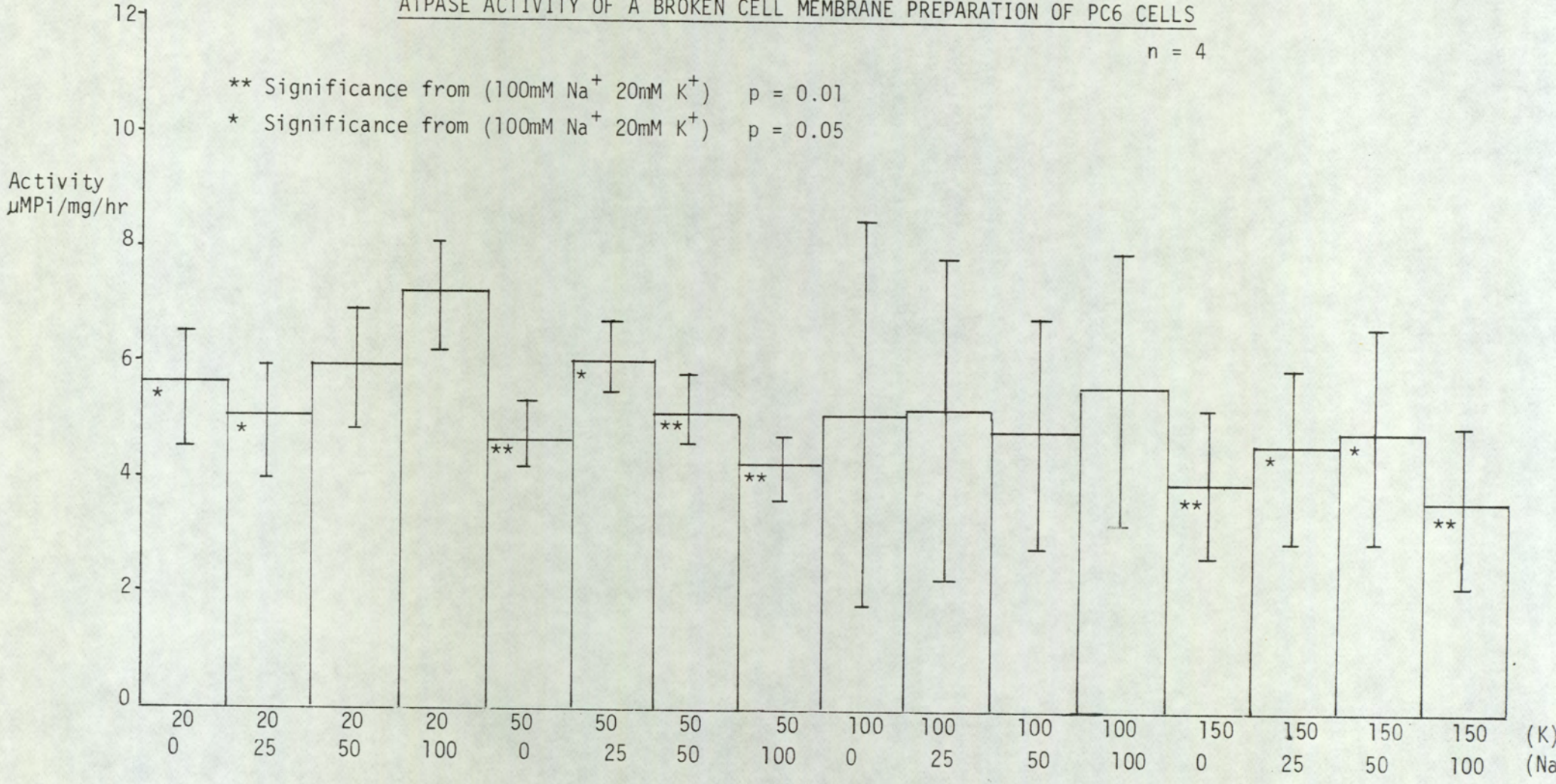


FIGURE 9

THE EFFECT OF VARYING THE MONOVALENT CATION CONCENTRATION ON THE ATPASE ACTIVITY (MINUS MgATPASE)

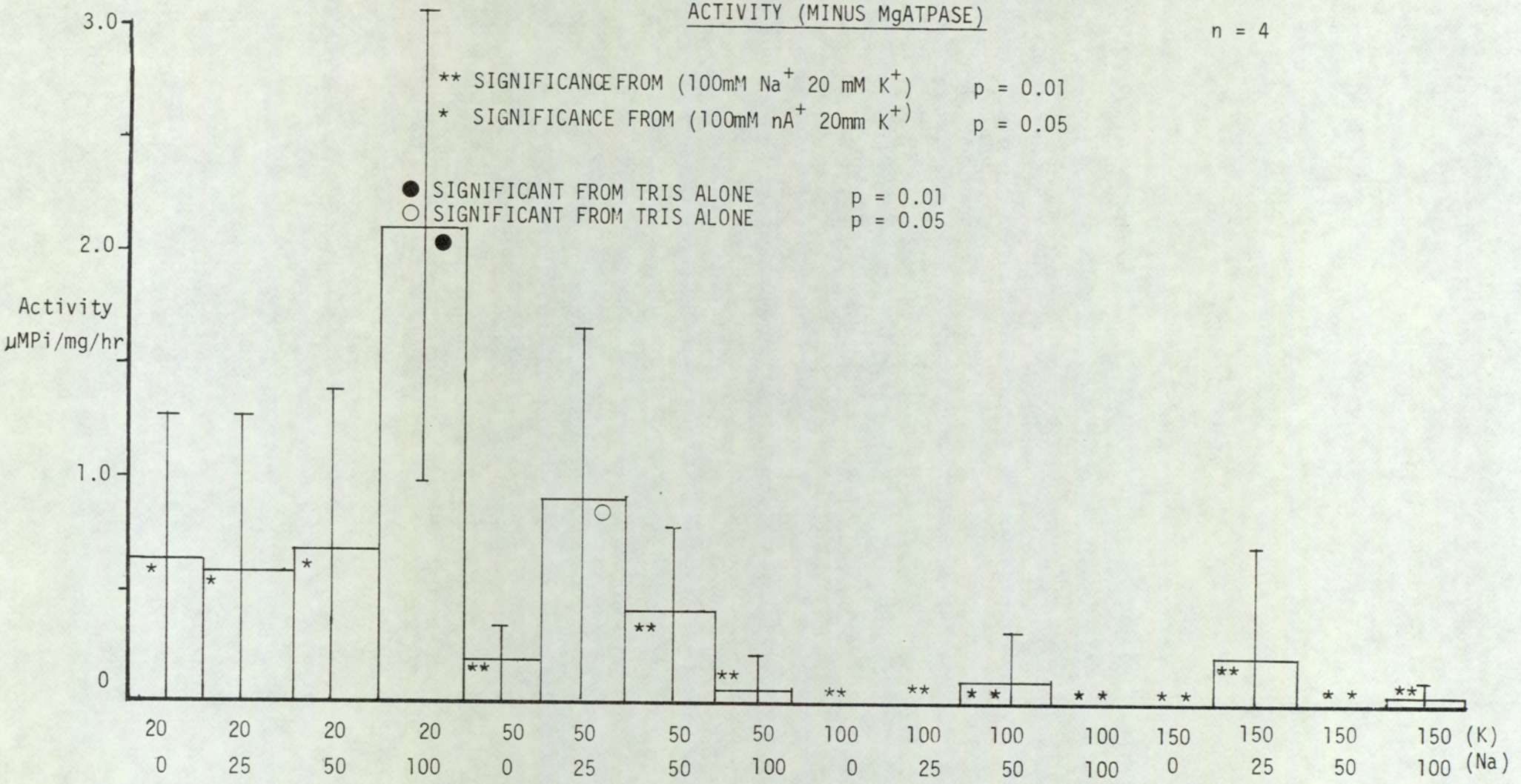
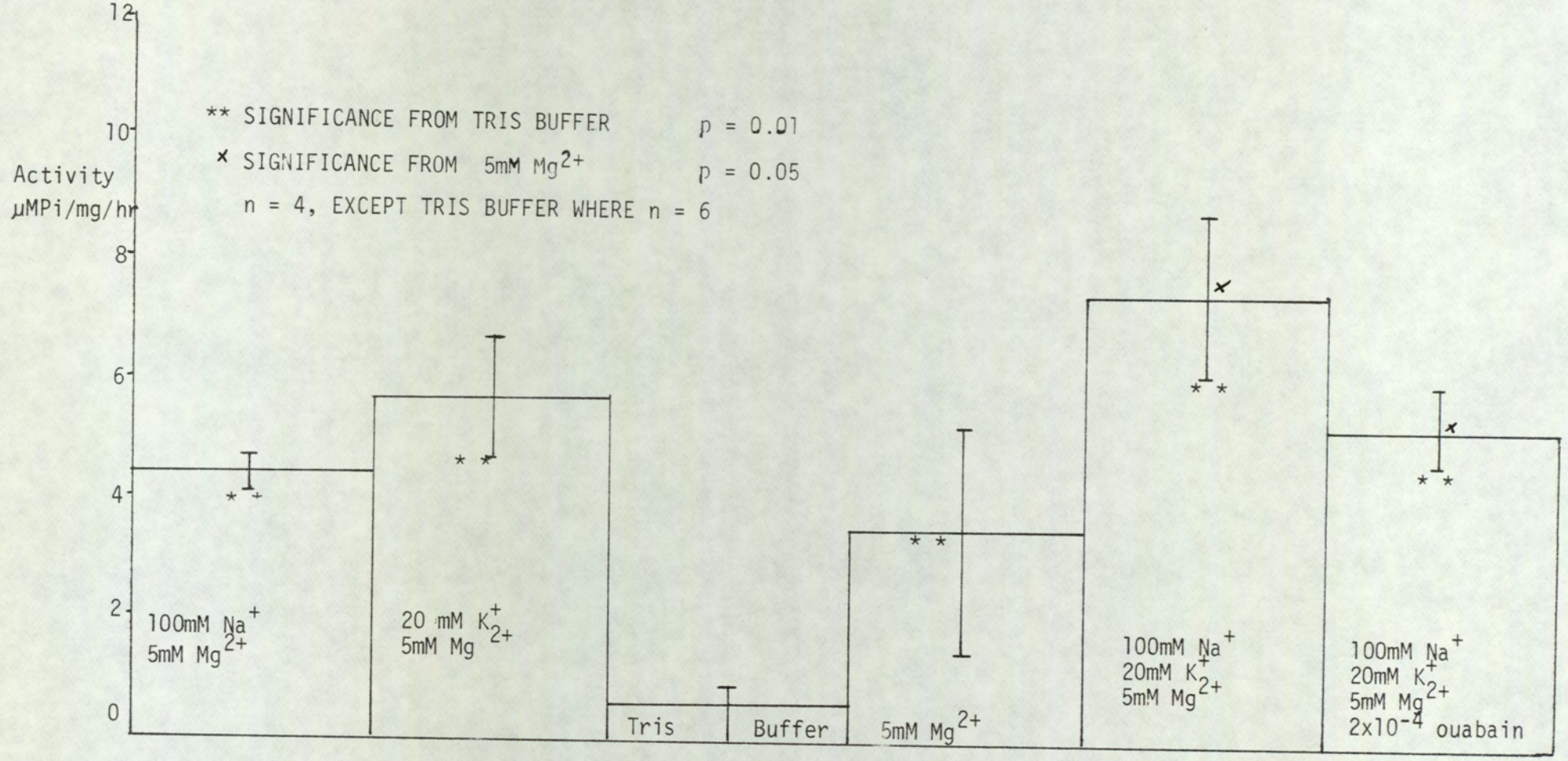


FIGURE 10

THE EFFECT OF IONIC CONTENT ON ATPASE ACTIVITY



When Na^+ and K^+ were added to the Mg^{2+} there was a large and significant increase ($p=0.05$) in activity compared to the mean MgATPase activity, so demonstrating the synergistic effect of Na^+ and K^+ on the ATPase activity. The addition of ouabain (column 6) reduced activity in the presence of Na^+, K^+ and Mg^{2+}, and this reduction was due to ouabain sensitive NaKATPase inhibition. The reduction obtained with ouabain was significantly different ($p=0.02$) from the Total ATPase activity.

Figure 10 demonstrates that the mean of 4 experiments gave a Total ATPase activity as 46% MgATPase and 54% NaKATPase under optimal ionic conditions at the time of these experiments. In Table 5 the total NaKATPase activity was only 32% of the Total ATPase activity. This demonstrates the variation in theMgATPase: NaKATPase ratio obtained with the different sonicated preparations.

7:8: THE DETERMINATION OF Vmax AND Km VALUES FOR ATP AND UTP ON OUABAIN SENSITIVE NaKATPASE ACTIVITY.

Table 7 shows the Km values obtained for ATP and UTP on ouabain sensitive NaKATPase activity. The enzyme has a higher affinity for ATP than UTP. This has also been shown to be the case for ITP and CTP (Skou 1973b, Monteil et al 1974).

The Vmax value obtained with ATP was 9.25mM, but with UTP no Vmax was obtained with the substrate concentrations used.

7:9: OUABAIN SENSITIVE NaKATPASE ACTIVITY FROM GUT MUCOSAL AND HEART CELLS

Table 8 shows the ouabain sensitive NaKATPase activity obtained with the broken cell preparations from gut mucosal and heart cells. The results were compared to the activity obtained from a broken cell preparation of PC6Acells produced by the Potter homogeniser. This comparison was used due to technical difficulties in the production of a heart cell homogenate with the sonicator.

The results show that there was no significant difference

TABLE 7

VMAX AND KM VALUES FOR ATP AND UTP ON OUABAIN SENSITIVE NaKATPASE ACTIVITY		
	KM	VMAX
ATP	2.5mM	9.25mM
UTP	>15mM	n.d.

1. Concentrations above 15mM not possible due to the limited quantity of UTP.

TABLE 8

OUABAIN SENSITIVE NaKATPASE ACTIVITY FROM DIFFERENT TISSUES n = 3	
ENZYME SOURCE[1]	ACTIVITY μMPi/mg/hour
GUT MUCOSAL CELLS	1.50 ± 0.06
HEART CELLS	1.77 ± 0.92
ADJ/PC6 CELLS	2.55 ± 2.22

1. Broken cell preparations (6:9:2) produced by Potter homogeniser with freezing and thawing.

($p > 0.5$) between the gut mucosal and heart cell preparations and the PC6A cell preparation. In addition the results show an increased variation in the PC6A enzyme activity compared to that of the other two broken cell preparations (see discussion).

7:10: OUABAIN SENSITIVE NaKATPASE ACTIVITY FROM TLX5 CELLS

Table 9 shows the ouabain sensitive NaKATPase activity in a broken cell prepatation from TLX5 cells (see methods 6:10:3). The cell homogenate was prepared by sonication and so the TLX5 enzyme activity was compared to the PC6A enzyme activity also prepared by sonication.

The results show that there was no significant difference ($p > 0.5$) between the two enzyme activities.

TABLE 9

OUABAIN SENSITIVE NaKATPASE ACTIVITY
FROM TLX5 CELLS

n = 3

ENZYME SOURCE[1]	ACTIVITY μMPi/mg/hr
TLX5 CELLS	1.95 ± 0.80
ADJ/PC6 CELLS	2.68 ± 1.41

1. Broken cell preparation produced by sonication (6:9:1)

7:11: EFFECT OF TEMPERATURE ON TOTAL ATPASE AND TOTAL NaKATPase ACTIVITY

Figure 11 shows the effect of temperature on the ATPase enzyme activity of both Total and NaKATPase increased with temperature until a maximum was reached at 37^{o}. After the maximum activity was obtained there was a drop in activity at 45^{o}., which in the case of NaKATPase inhibited the enzyme.

In both cases the enzyme system worked below physiological temperature, a result noted before (Russell and Chambers 1976).

7:12: THE EFFECT OF INCUBATION TIME ON TOTAL ATPASE ACTIVITY.

In the previous experiments to determine ouabain sensitive NaKATPase activity the broken cell preparation was incubated for 15 minutes at 37^{o} prior to the addition of ATP. In the study of the effect of sulphydryl reagents on NaKATPase and MgATPase it was necessary to increase this incubation time from 15 minutes to 30 minutes (see methods 6:11:5). Figure 12 shows the effect of incubation at 37^{o} on Total ATPase activity. The results show that up to 30 minutes there was no significant effect ($p>0.5$) on activity but if the incubation time was increased to 60 minutes there was a fall of 56% in activity in comparison to the result at 5 minutes. Therefore to take the effect of incubation into account all subsequent experiments with sulphydryl reagents were compared to a control total ATPase and/or ouabain sensitive NaKATPase activity determined after 30 minutes incubation (see methods 6:11:5).

7:13: EFFECT OF PREINCUBATION TIME WITH OUABAIN ON OUABAIN SENSITIVE NaKATPase ACTIVITY

Figure 13 shows the effect of varying the time ouabain was in contact with the broken cell preparation prior to the addition of ATP. The result shows that there was a stimulation after 5 minutes of Total ATPase activity by the ouabain, but within 15 minutes there was a levelling off of ouabain sensitive NaKATPase activity.

FIGURE 11

THE EFFECT OF TEMPERATURE ON THE TOTAL ATPASE AND TOTAL NaKATPASE ACTIVITY IN A BROKEN CELL MEMBRANE PREPARATION

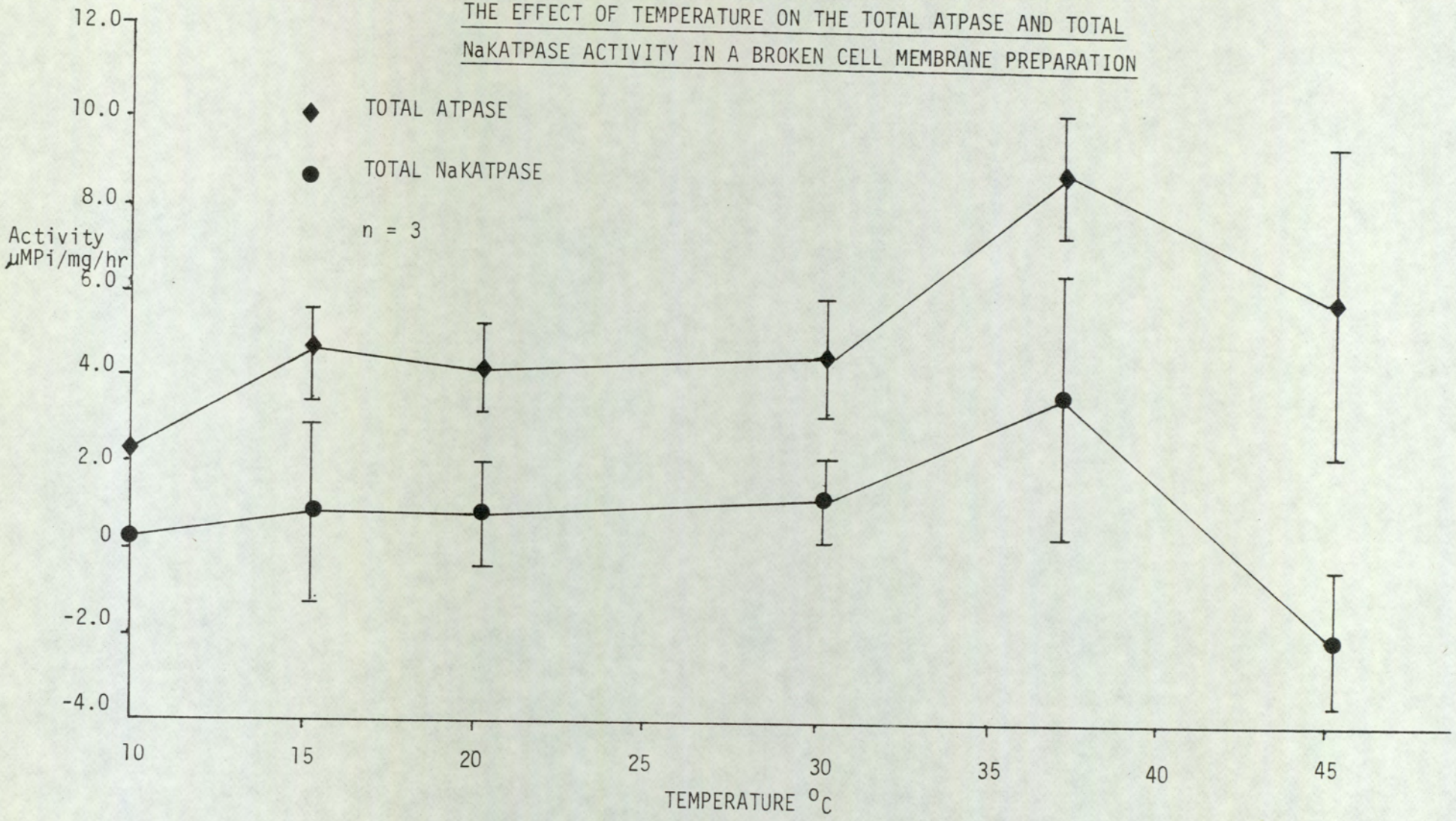


FIGURE 12

THE EFFECT OF PREINCUBATION* TIME ON THE TOTAL ATPASE ACTIVITY OF A BROKEN CELL MEMBRANE PREPARATION

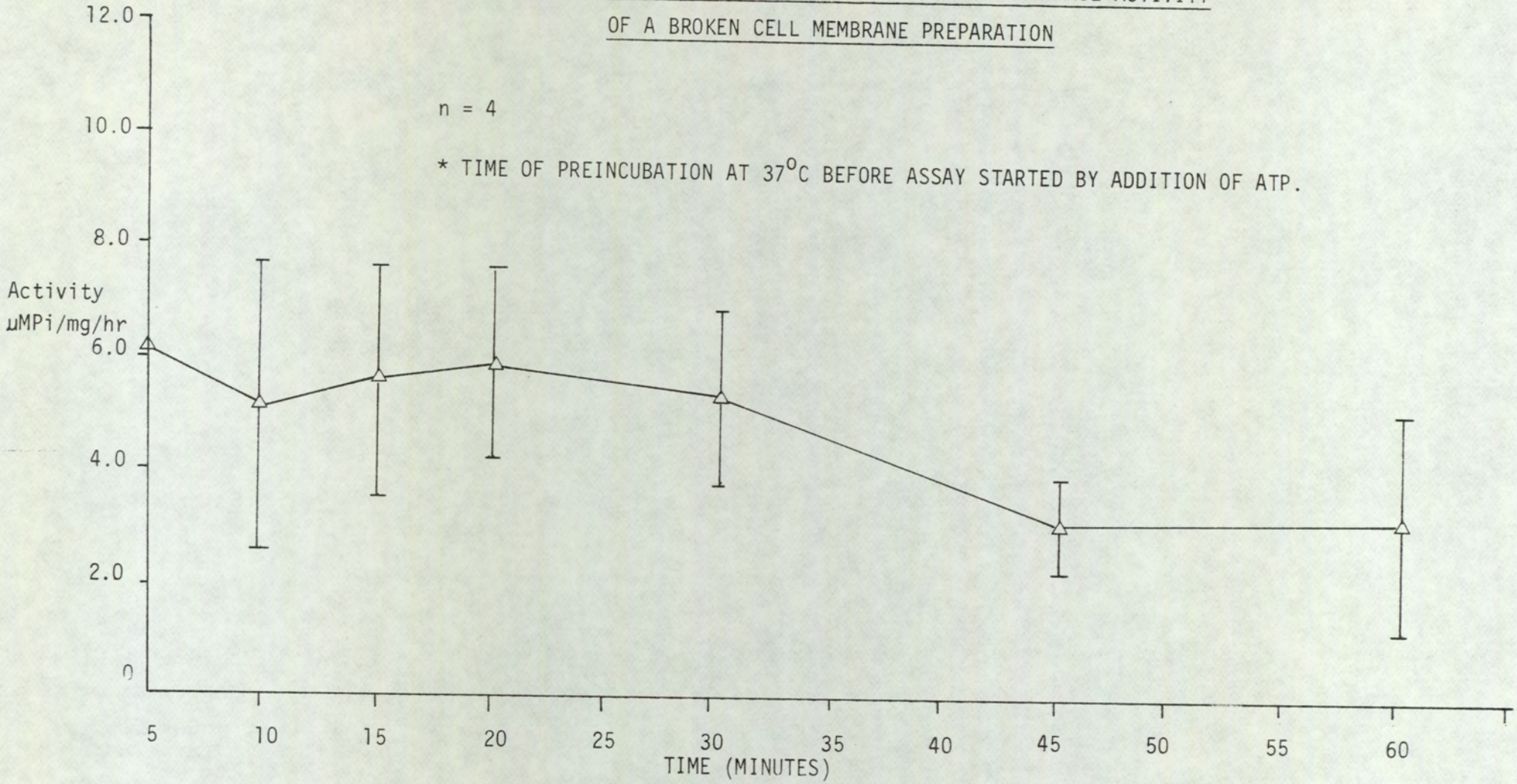
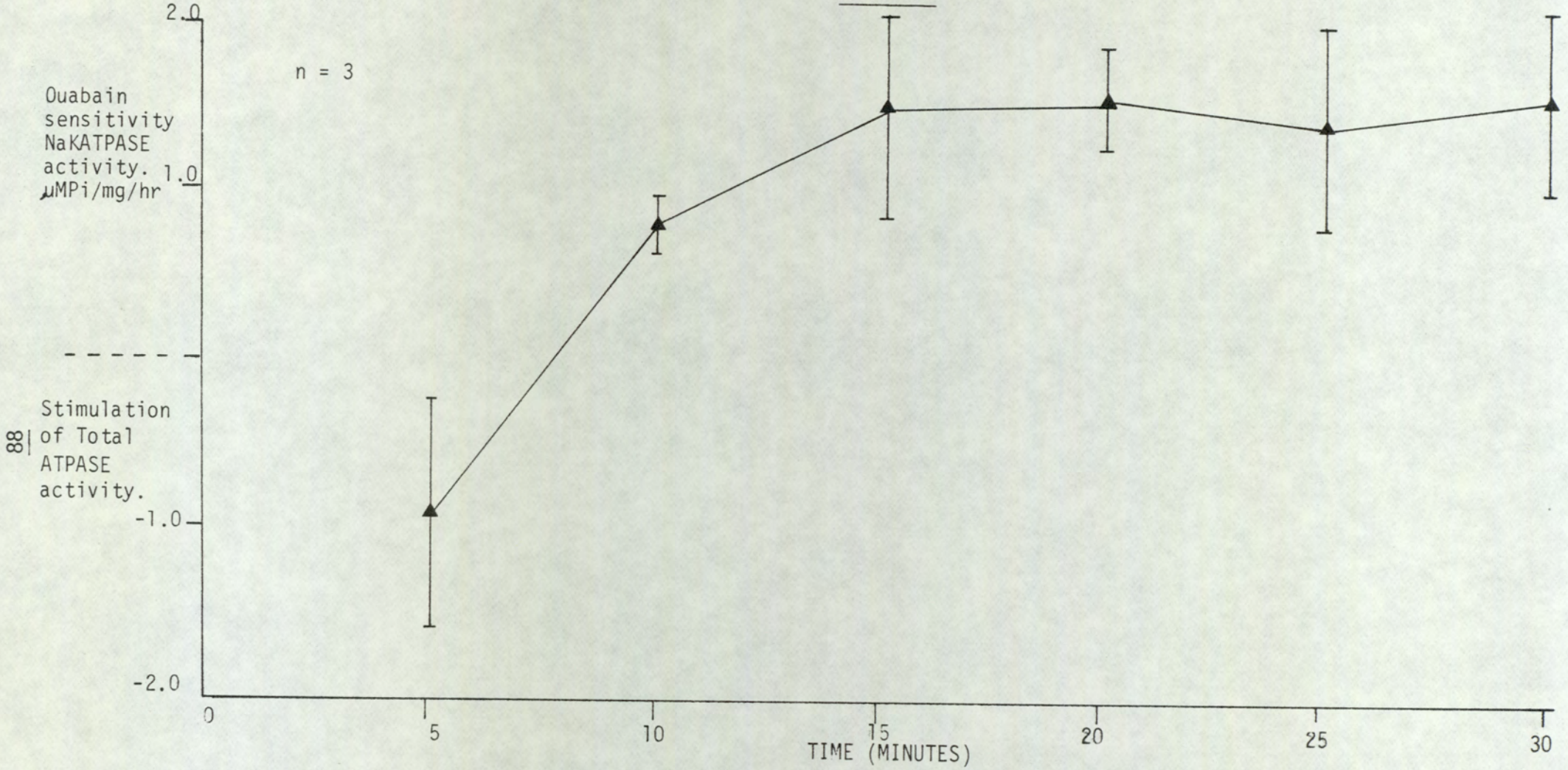


FIGURE 13.

EFFECT OF PREINCUBATION TIME WITH OUABAIN ON OUABAIN SENSITIVE NaKATPASE ACTIVITY



This result showed that a 15 minute preincubation with ouabain at 37°C prior to the addition of ATP was sufficient to allow the determination of ouabain sensitive NaKATPase activity.

7:14: THE INHIBITION OF OUABAIN SENSITIVE NaKATPase BY SULPHYDRYL REAGENTS (SEE METHODS 6:11:5:)

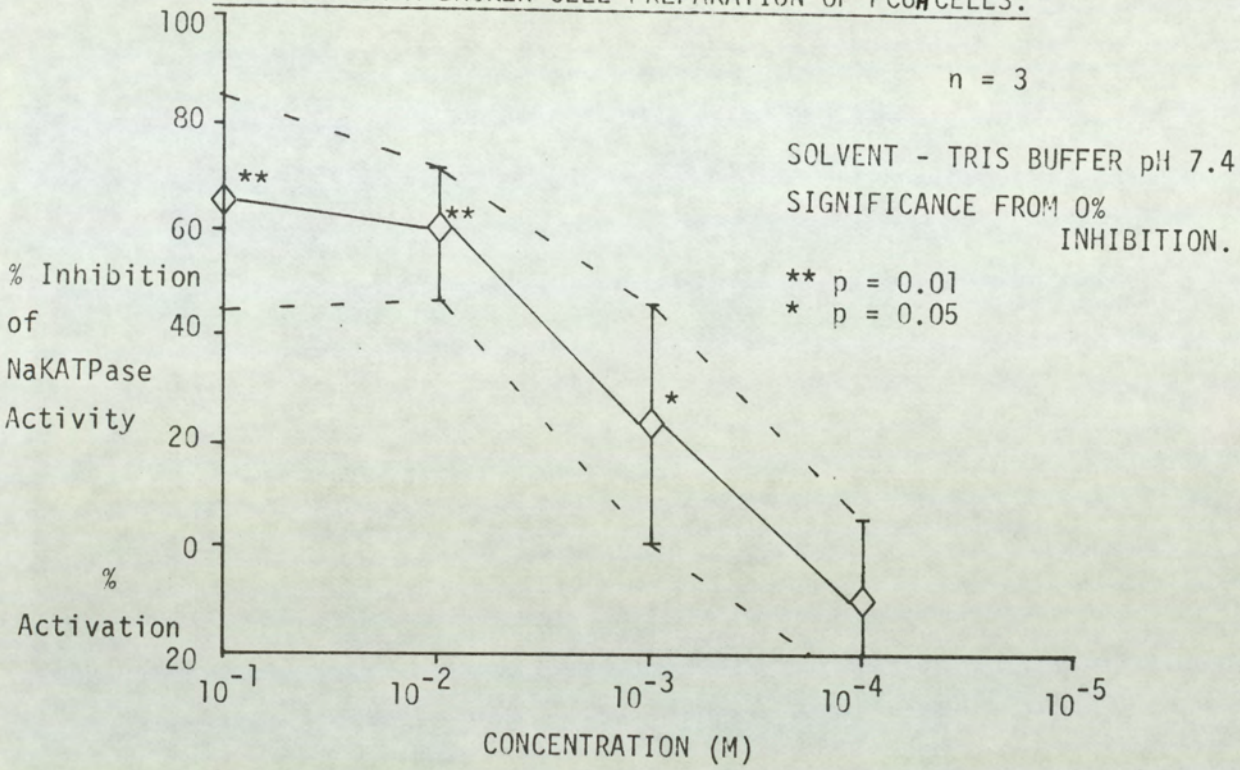
NaKATPase has at least two essential SH groups at the site of ATP hydrolysis (Schoot 1978). Figures14-16 show the effect of three classical sulphydryl reagents on the ouabain sensitive NaKATPase activity of a broken cell preparation.

Figure 14 shows that iodoacetic acid significantly inhibited NaKATPase at 10^{-3}M and above. Due to the variation in the assay the result at 10^{-1}M is not significantly different from 100% inhibition of ouabain sensitive NaKATPase ($p>0.3$). Figure 15 shows that N-ethylmaleimide significantly inhibited NaKATPase activity at 10^{-5}M and above, and again due to the variation in the assay the result at 10^{-5}M and above was not significantly different from 100% inhibition ($p>0.3$). Figure 16 shows that p-chloromercuribenzoic acid had no significant activity at 10^{-7}M or 10^{-8}M but at higher concentrations inhibition was significant:10^{-6}M and 10^{-4}M p-chloromercuribenzoic acid gave an inhibition that was not significantly different from 100% inhibition ($p>0.3$).

Figures 17-18 show the result of plotting the ratio of the percentage control activity on the addition of ouabain to the percentage control activity after a 30 minute preincubation with the sulphydryl reagents. A ratio greater than 1.0 indicates that in addition to 100% inhibition of ouabain sensitive NaKATPase there is a further inhibition which is possibly due to inhibition of the ouabain insensitive NaKATPase and MgATPase.

Figure 17 shows that iodoacetic acid had a ratio greater than 1.0 at a concentration of 10^{-2}M and above. On comparison with Figure 12, the inhibition of ouabain sensitive NaKATPase at 10^{-2}M was a mean value of 80%, which was significantly

FIGURE 14. THE EFFECT OF VARIOUS CONCENTRATIONS OF IODOACETIC ACID ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A BROKEN CELL PREPARATION OF PC6A CELLS.



THE EFFECT OF VARIOUS CONCENTRATIONS OF N-ETHYLMALEIMIDE ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A BROKEN CELL PREPARATION OF PC6A CELLS.

FIGURE 15

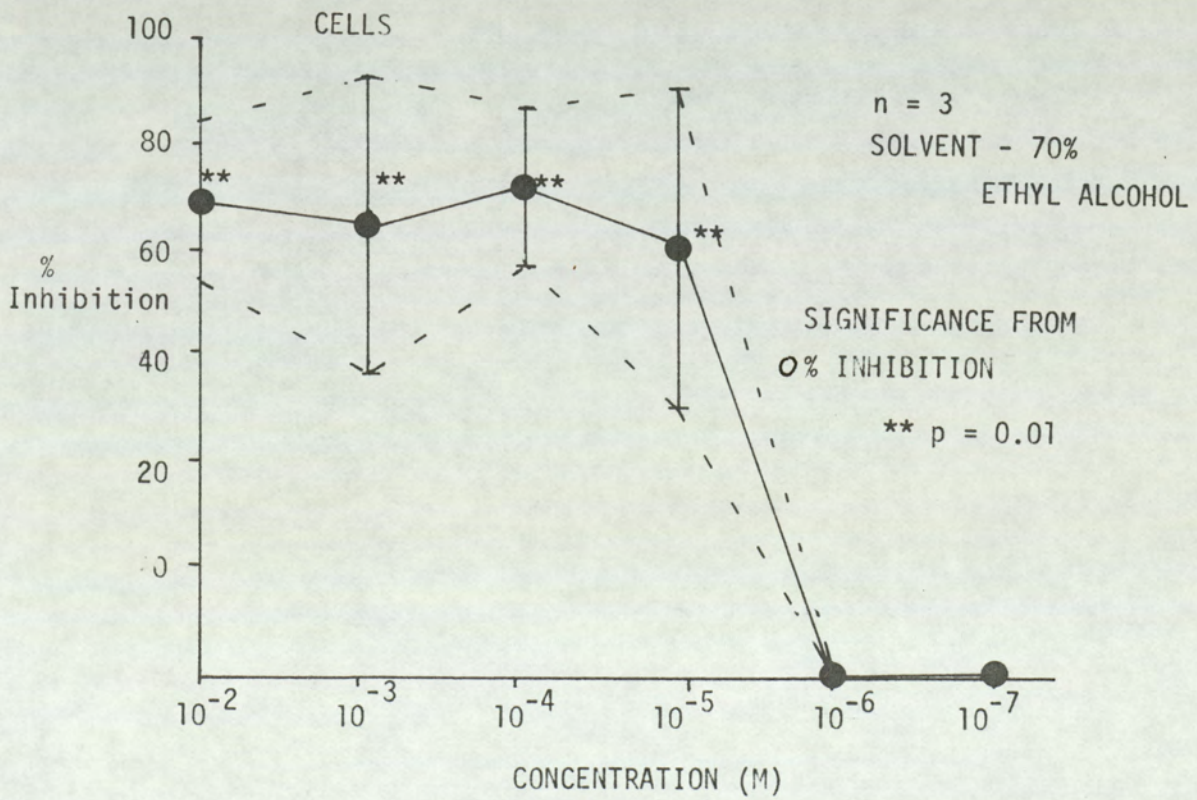


FIGURE 16

THE EFFECT OF VARIOUS CONCENTRATIONS OF p-CHLOROMERCURIBENZOIC ACID ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A BROKEN CELL PREPARATION FROM PC6A CELLS.

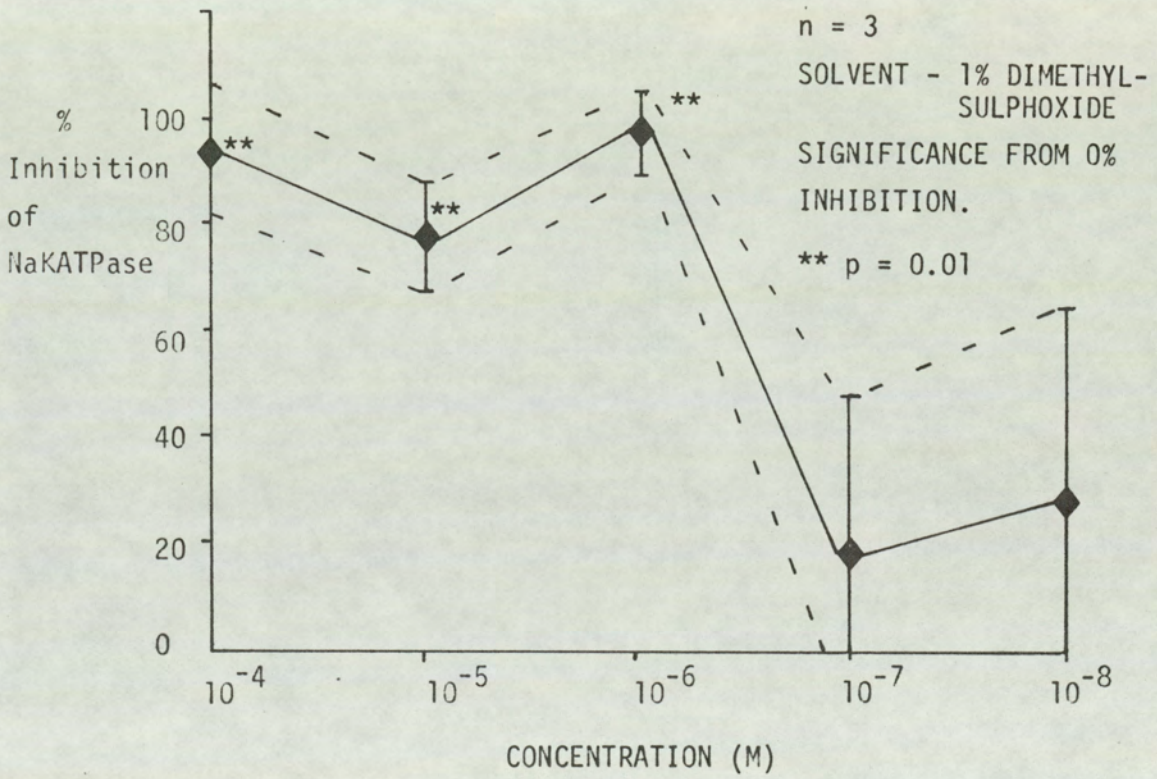


FIGURE 17

THE EFFECT OF INCUBATION FOR 30 MINUTES WITH VARIOUS CONCENTRATIONS OF IODOACETIC ACID ON THE RATIO OF PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH OUABAIN TO THE PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH IODOACETIC ACID.

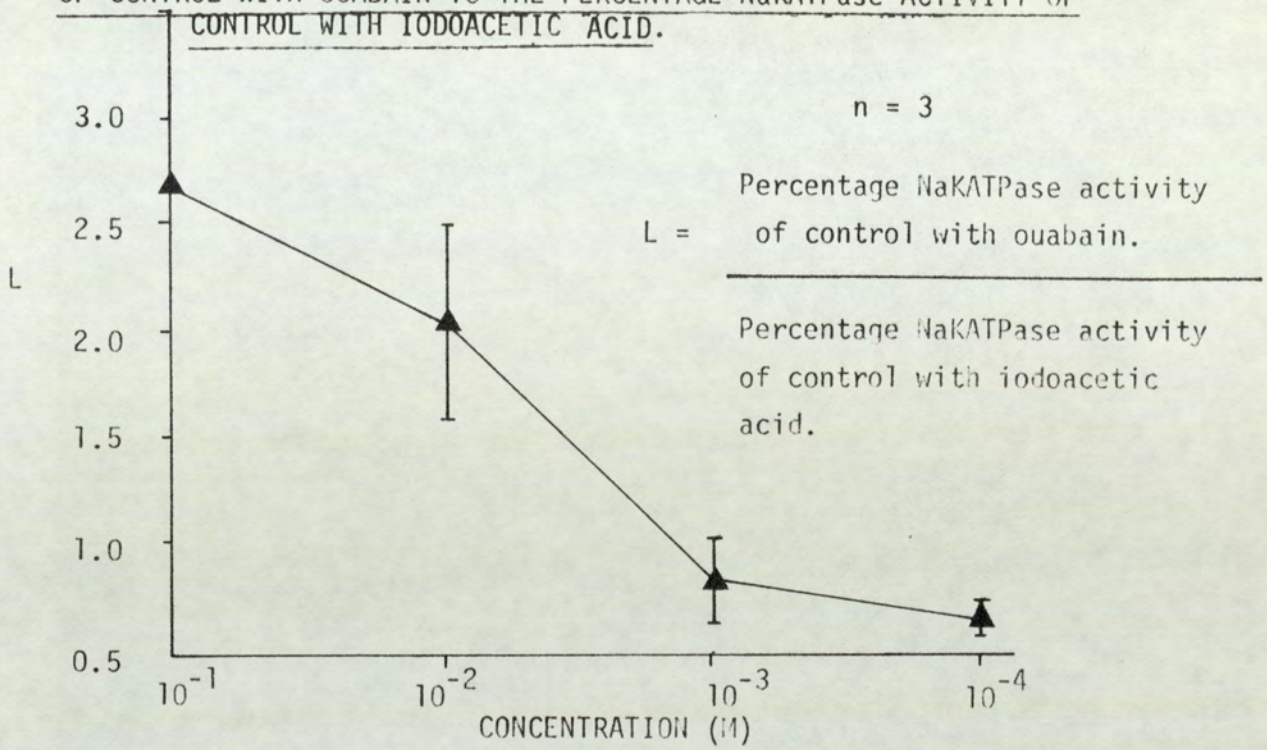
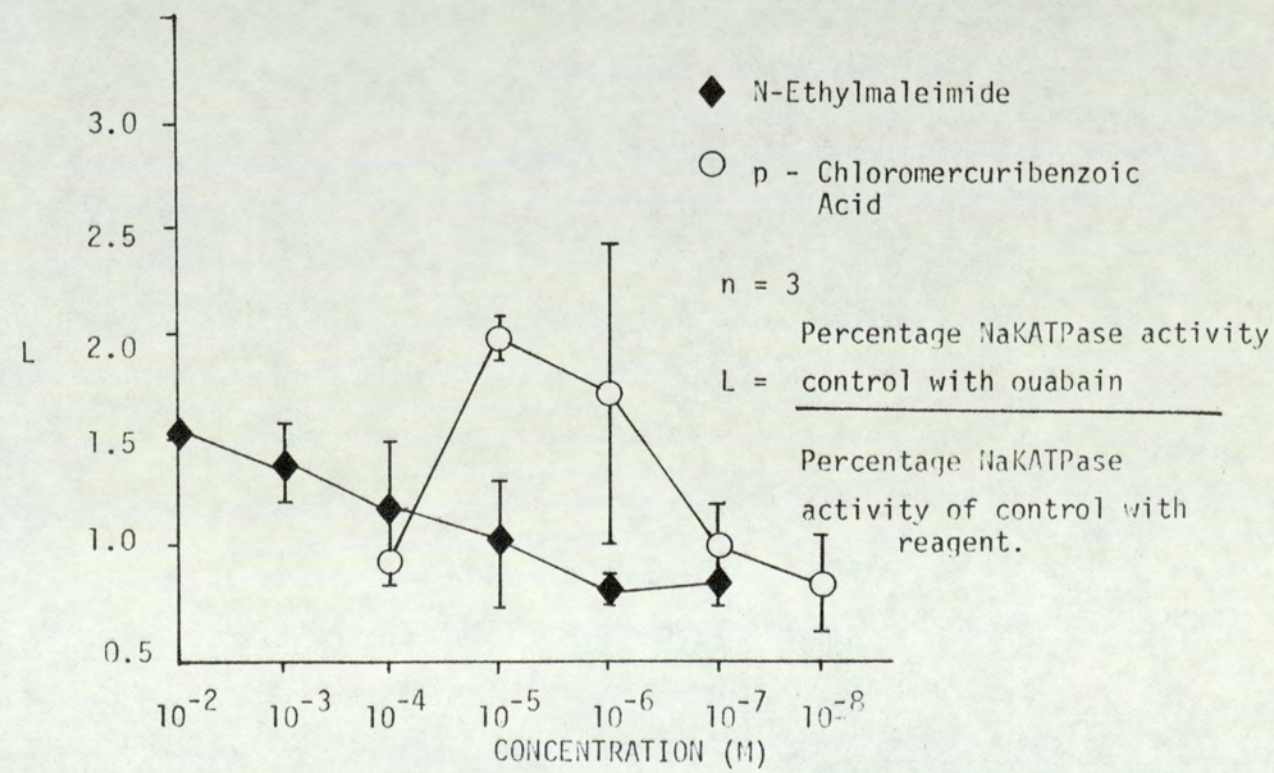


FIGURE 18

THE EFFECT OF INCUBATION FOR 30 MINUTES WITH VARIOUS CONCENTRATIONS OF N-ETHYLMALEIMIDE AND p-CHLOROMERCURIBENZOIC ACID ON THE RATIO OF PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH OUABAIN TO THE PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH REAGENT.



different from total inhibition ($p>0.05$). The discrepancy between the % inhibition of NaKATPase and the ratio of ouabain sensitive activity: reagent sensitive activity at 10^{-2}M was probably due to the significant inhibition of MgATPase at this concentration (Figure 28).

Figure 18 shows that at a conc. of 10^{-5}M N-ethylmaleimide and 10^{-7}M p-chloromercuribenzoic acid there was complete inhibition of ouabain sensitive NaKATPase, and at higher conc. than these there was inhibition of, for example, MgATPase and ouabain insensitive NaKATPase. The result with p-chloromercuribenzoate at 10^{-4}M was probably due to solubility problems with this reagent in 1% dimethylsulphoxide which reduced its availability to the enzyme systems.

The concentrations required to give 100% and 0% inhibition are summarised in Table 11 .

7:15:1: INHIBITION OF OUABAIN SENSITIVE NaKATPase BY ANTITUMOUR ALKYLATING AGENTS.

Table 10 shows the result of the determination of purity and half life in water of two samples of nitrogen mustard (see methods 6:13).

The purity of sample 1 at 0^{o} was 69.9% compared to 89.9% for sample 2. The half life determination showed that at 0^{o} it was 13.2 minutes compared to 87 seconds at 39^{o}C (Karnofsky et al 1947). Therefore all dilutions of nitrogen mustard were performed under ice cold conditions using sample 2, corrected for purity and calculated as the free base.

Figure 19 shows the effect of varying the preincubation time on the inhibition of Total ATPase by 10^{-12}M nitrogen mustard. The inhibition by the agent was time dependent and after 30 minutes the activity fell to 50% of that of the control incubated in the same way.

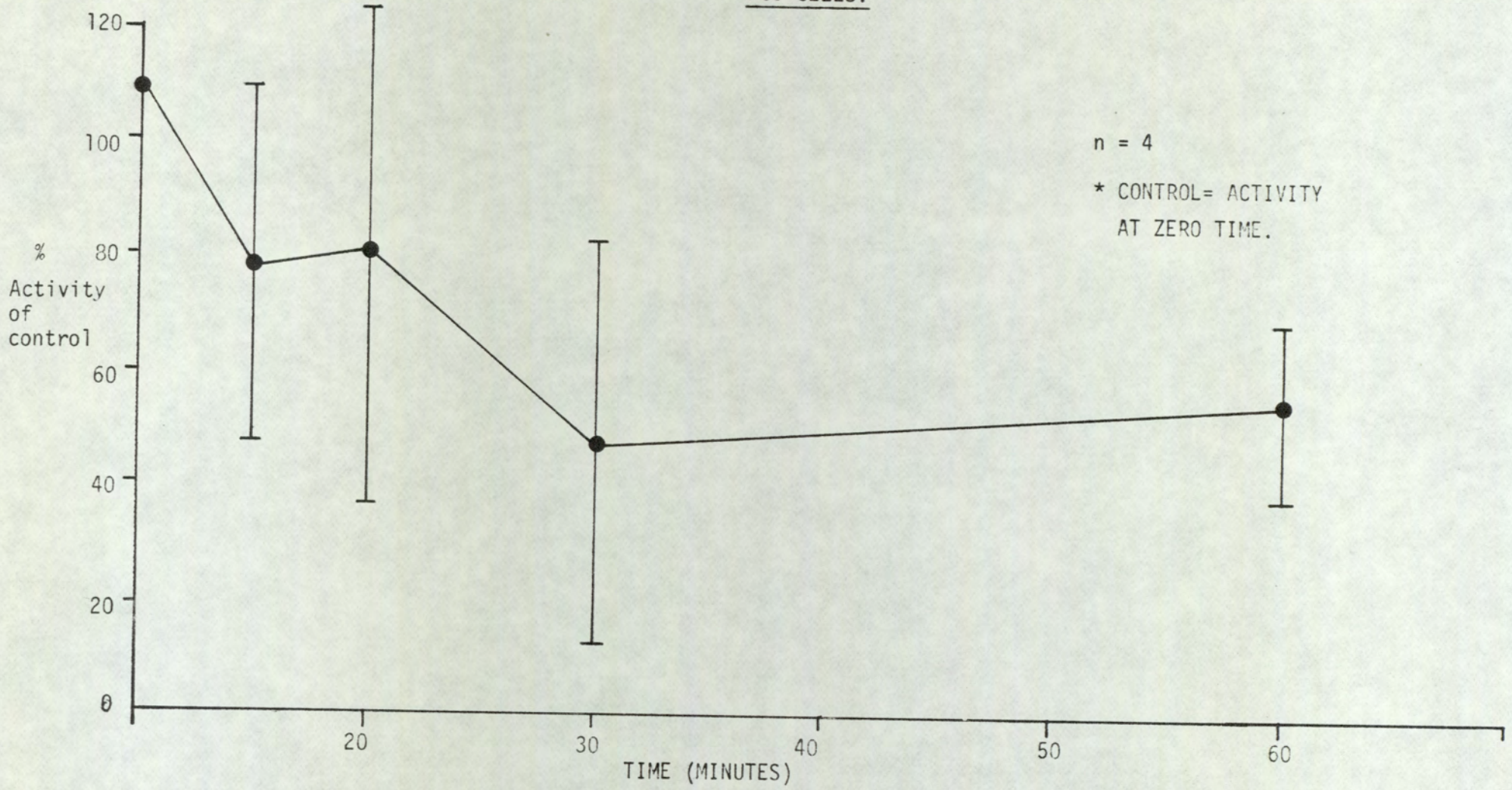
TABLE 10

PURITY OF NITROGEN MUSTARD SAMPLES AT 0^{o} AND HALF LIFE IN WATER (SEE METHODS 6:13:)

SAMPLE	% PURITY AT 0^{o}
1	69.91
2	89.87
TEMPERATURE	**HALF LIFE**
0^{o}	13.2 mins.
11^{o}	4.52 mins.
20^{o}	4.29 mins.
37^{o}	87 secs.

FIGURE 19

THE EFFECT OF INCUBATION WITH 10^{-12}M NITROGEN MUSTARD ON THE PERCENTAGE ACTIVITY IN COMPARISON TO A CONTROL* FROM A BROKEN CELL PREPARATION OF PC6 CELLS.



Comparison of Figure 19 with Figure 12 shows that to take into account any effect preincubation has on ATPase activity the control activity must be determined at the same time as the drug treated activity. Therefore all experiments were performed after a preincubation of 30 minutes at $37^{o}C$ prior to the addition of ATP (See methods 6:11:5:).

Figure 20 shows the concentration-inhibition curve for the effect of nitrogen mustard against an ouabain sensitive NaKATPase. The result at $10^{-10}M$ was not significantly different ($p>0.5$) from 100% inhibition of the enzyme, which corresponded to a ratio of ouabain sensitive activity : drug sensitive activity above 1.0 (Figure 33) which indicated that at this conc., in addition to inhibition of ouabain sensitive NaKATPase there was possible inhibition of ouabain insensitive NaKATPase. At this concentration there was negligible inhibition of the MgATPase (Figure 35).

Figure 21 shows the result for Chlorambucil. 100% inhibition is obtained at $10^{-11}M$ and above, a result which correlated with the result shown in Figure 23.

Figure 22 shows the effect of nitrogen mustard on a whole cell homogenate NaKATPase (see method 6:9:1). This was performed to see if the amount of cell protein normally removed by centrifugation had any effect on the potency of nitrogen mustard. In the experiment the cytosolic proteins were not removed by centrifugation and the results show that this had a significant effect on the potency of the agent. 100% inhibition was obtained at $10^{-8}M$, and at a concentration of $10^{-12}M$ the enzyme activity observed in the uncentrifuged cell homogenate was significantly different ($p=0.01$) from that of the broken whole cell preparation. The conc. required to give 100% inhibition were summarised in Table 11.

FIGURE 20

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARD ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A BROKEN CELL PREPARATION OF PC6A CELLS.

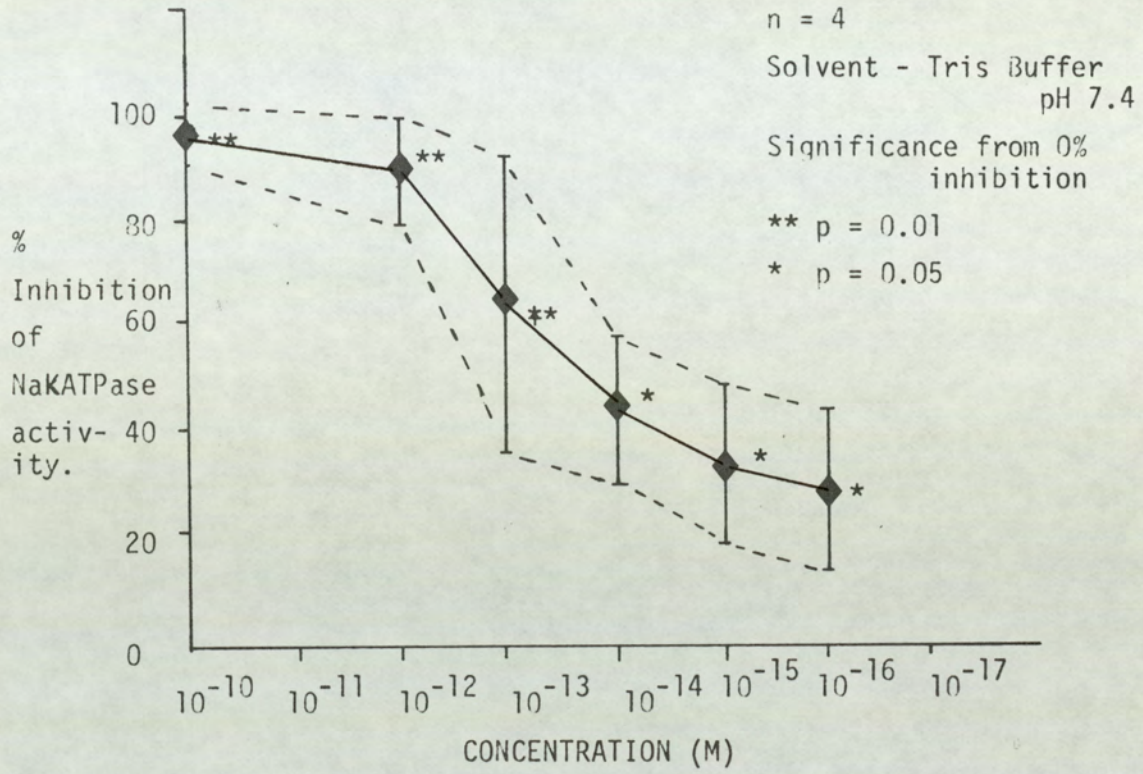


FIGURE 21

THE EFFECT OF VARIOUS CONCENTRATIONS OF CHLORAMBUCIL ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A BROKEN CELL PREPARATION OF PC6A CELLS.

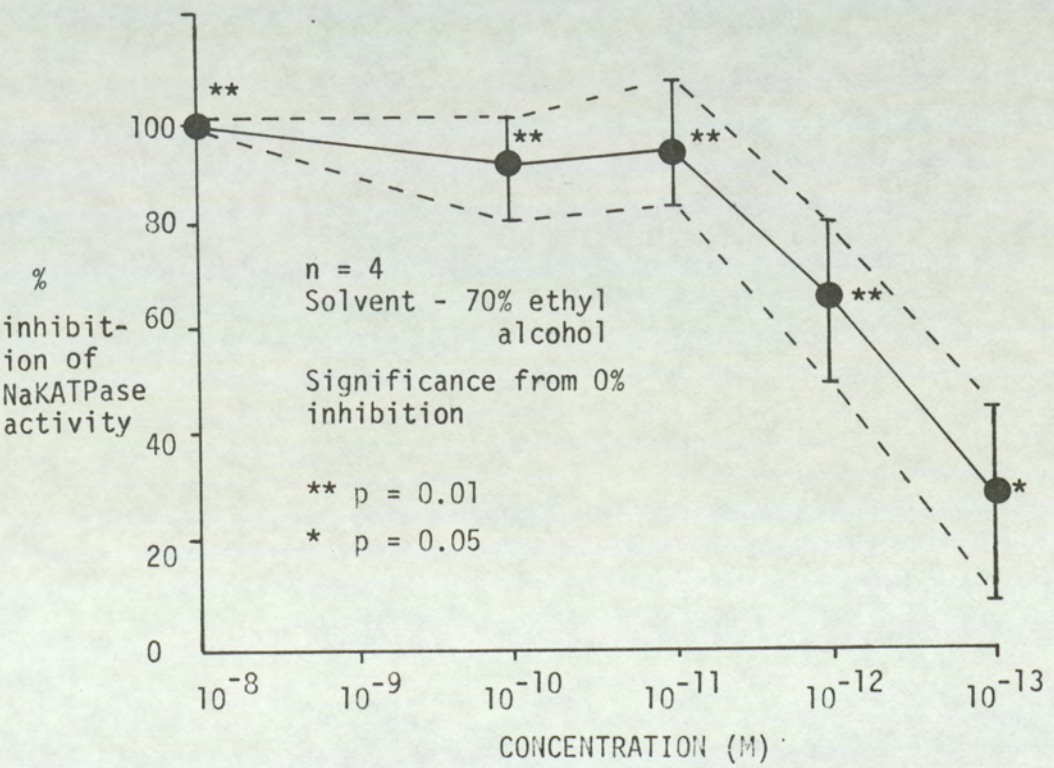


FIGURE 22

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARD ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A TOTAL CELL HOMOGENATE OF PC6A CELLS.

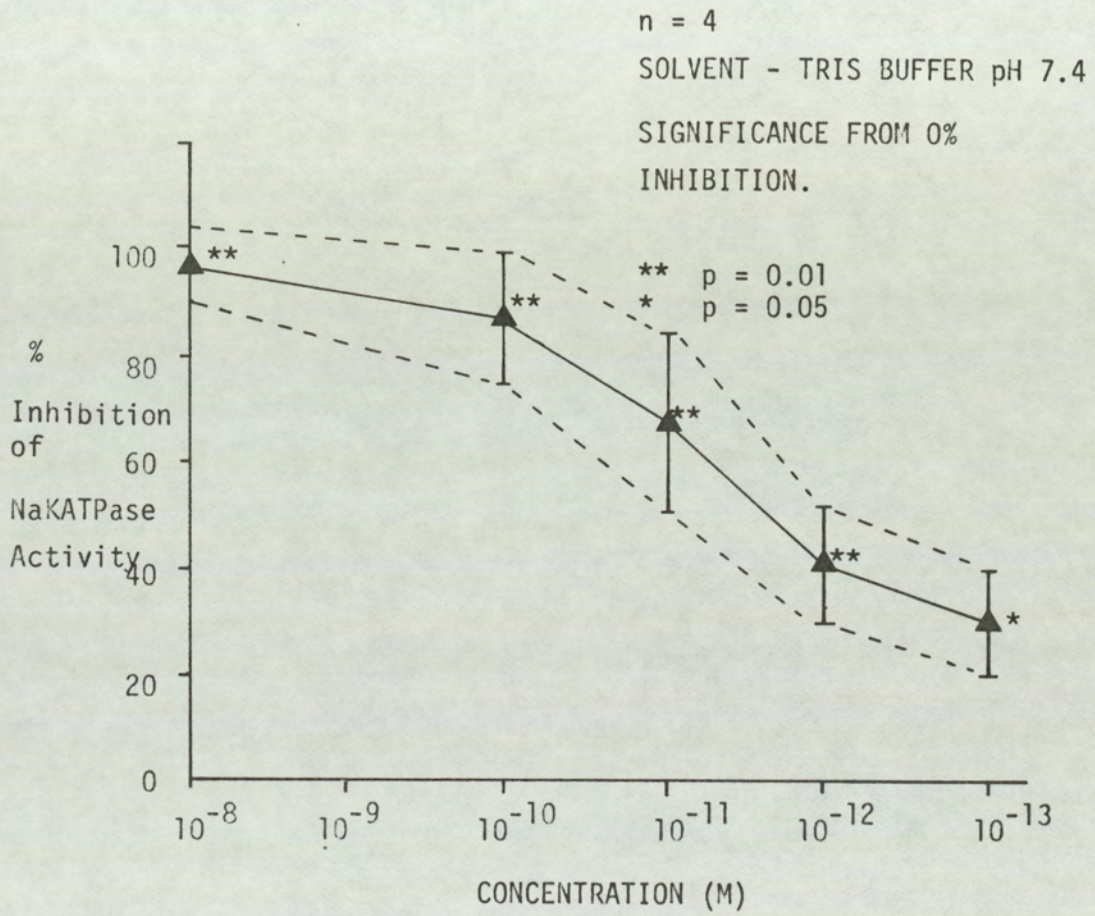
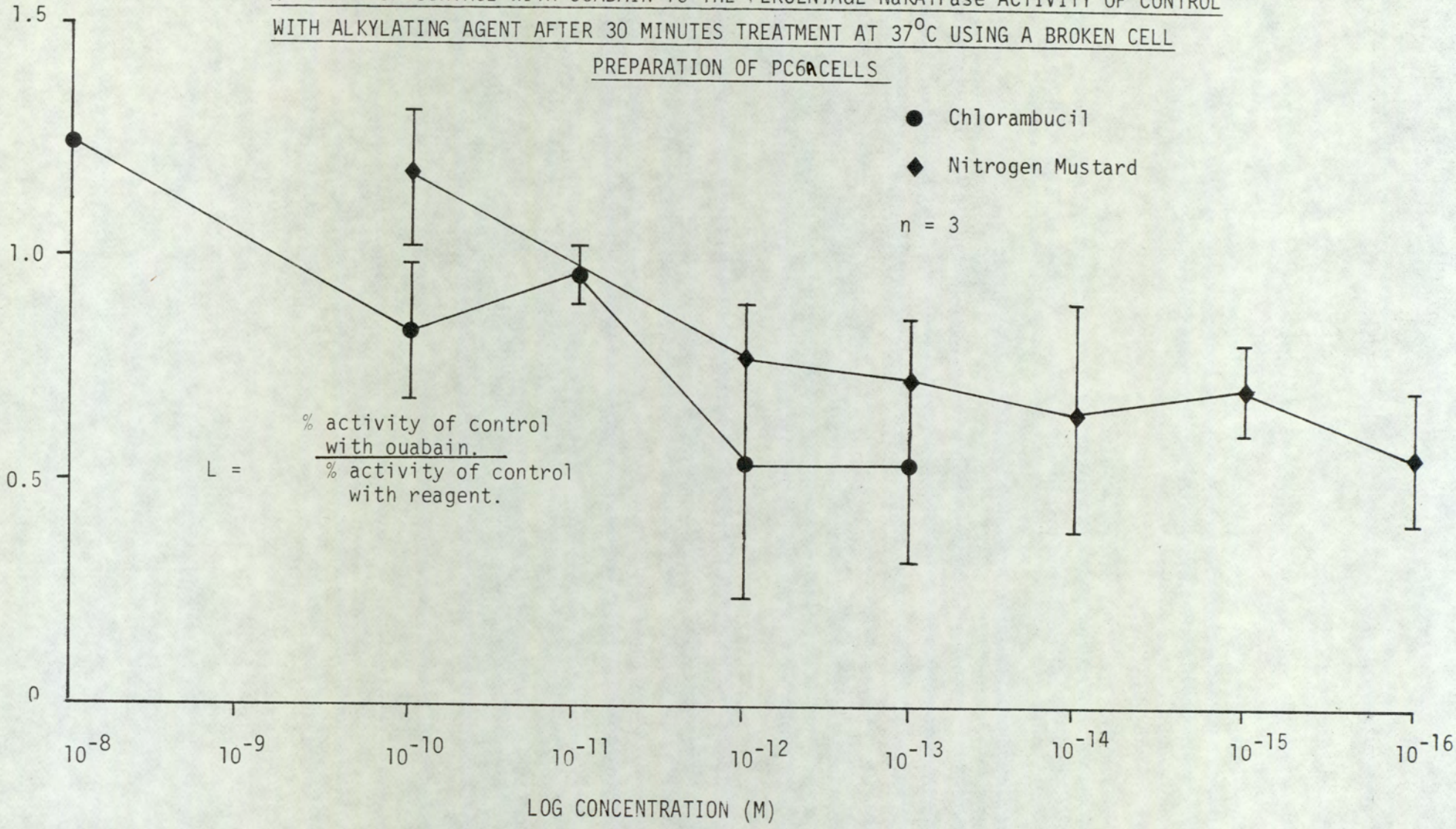


FIGURE 23

THE EFFECT OF ALKYLATING AGENT CONCENTRATION ON THE RATIO OF PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH OUABAIN TO THE PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH ALKYLATING AGENT AFTER 30 MINUTES TREATMENT AT 37°C USING A BROKEN CELL PREPARATION OF PC6A CELLS



7:15:2: EFFECT OF ANALOGUES OF ANTITUMOUR ALKYLATING AGENTS ON OUABAIN SENSITIVE NaKATPase

Figure 24 shows the result of the incubation of N,N dimethylchloroethylamine on ouabain sensitive NaKATPase. At concentration of 10^{-5}M and above, the inhibition obtained is not significantly different ($p>0.3$) from 100% of this enzyme system. This correlates with the ratio of ouabain sensitive : drug treated activity (Figure 26) which indicates that at 10^{-4}M in addition to inhibition of ouabain sensitive NaKATPase, there was also an induction of inhibition of ouabain insensitive NaKATPase and/or MgATPase.

Monofunctional chlorambucil remained a highly potent inhibitor (Figure 26). 100% inhibition was obtained at 10^{-7}M, which correlated with the result shown in Figure 26, but it was less potent than the difunctional compound ($p>0.05$).

To check that it was not the hydrolysed product that was giving the delayed toxicity, nitrogen mustard hydrolysed for 24 hours at pH 8.6 at 37^{o} showed that 100% inhibition of ouabain sensitive NaKATPase activity after a 30 minute preincubation required conc. higher than 10^{-4}M (Table 11).

Table 11 summarises the conc. required to obtain 100% inhibition of the ouabain sensitive NaKATPase.

7:15:3: INHIBITION OF MgATPase BY SULPHYDRYL REAGENTS

Figures 28-30 show the effect of sulphydryl reagents on MgATPase activity. Only in the case of iodoacetic acid (Figure 30) was inhibition obtained which was not significant from 100% ($p>0.3$). N-ethylmaleimide,p-chloromercuribenzoic acid and nitrogen mustard failed to give 100% inhibition values at the concentrations used, which in the case of p-chloromercuribenzoic acid was the limit of its solubility in 1% dimethylsulphoxide.

TABLE 11

THE CONCENTRATION RANGE REQUIRED TO GIVE 0% AND 100% INHIBITION OF THE OUABAIN SENSITIVE NaKATPase ($p>0.3$)

AGENT	100% INHIBITION	0% INHIBITION
Iodoacetic Acid	10^{-1}M	10^{-4}M
p-Chloromercuribenzoic Acid	10^{-6}M	10^{-7}M
N-Ethylmaleimide	10^{-5}M	10^{-6}M
Nitrogen Mustard	10^{-10}M	n.d.
Chlorambucil	10^{-11}M	n.d.
Nitrogen Mustard (1)	10^{-8}M	n.d.
N,N Dimethylchloroethylamine	10^{-5}M	10^{-7}M
Monofunctional Chlorambucil	10^{-7}M	10^{-9}M
Hydrolysed Nitrogen Mustard	$>10^{-4}$M	n.d.

1. Cell homogenate NaKATPase used (See methods 6:9:1:)

2. n.d. - not determined.

FIGURE 24

THE EFFECT OF VARIOUS CONCENTRATIONS OF N,N DIMETHYLCHLORO-ETHYLAMINE ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY FROM A BROKEN CELL PREPARATION OF PC6A CELLS

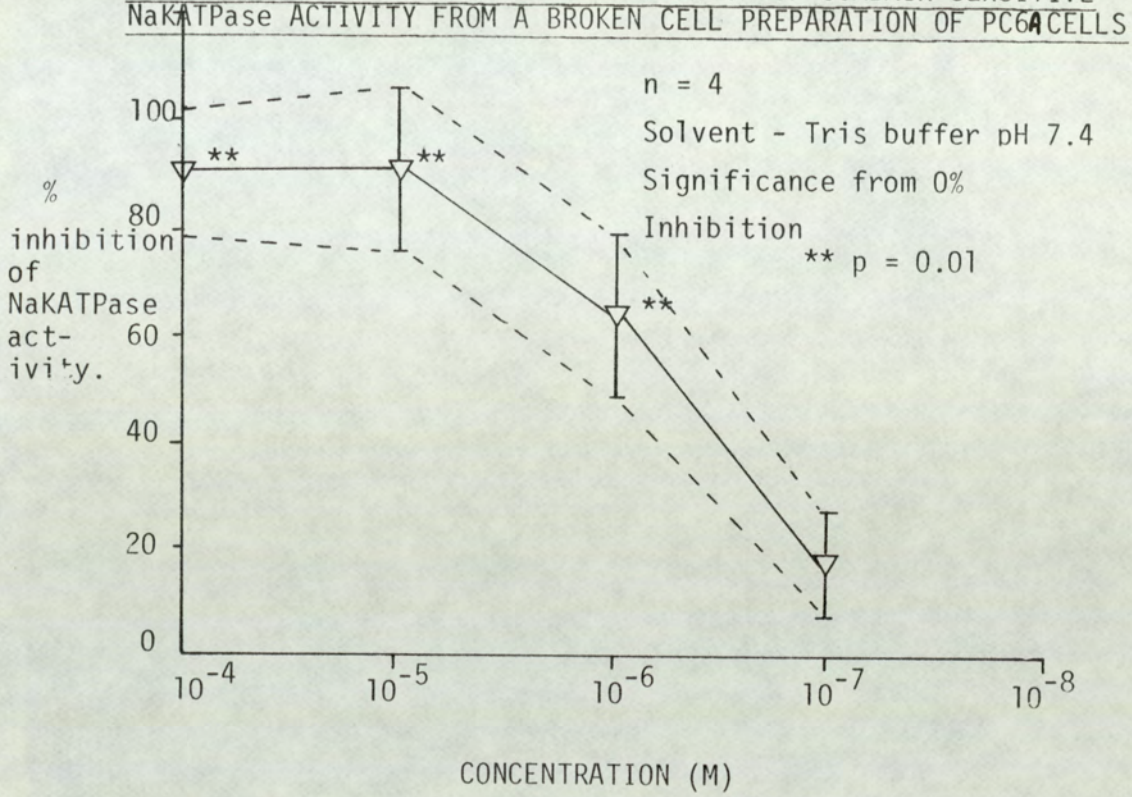


FIGURE 25

THE EFFECT OF VARIOUS CONCENTRATIONS OF MONOFUNCTIONAL CHLORAMBUCIL ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY FROM A BROKEN CELL PREPARATION OF PC6A CELLS.

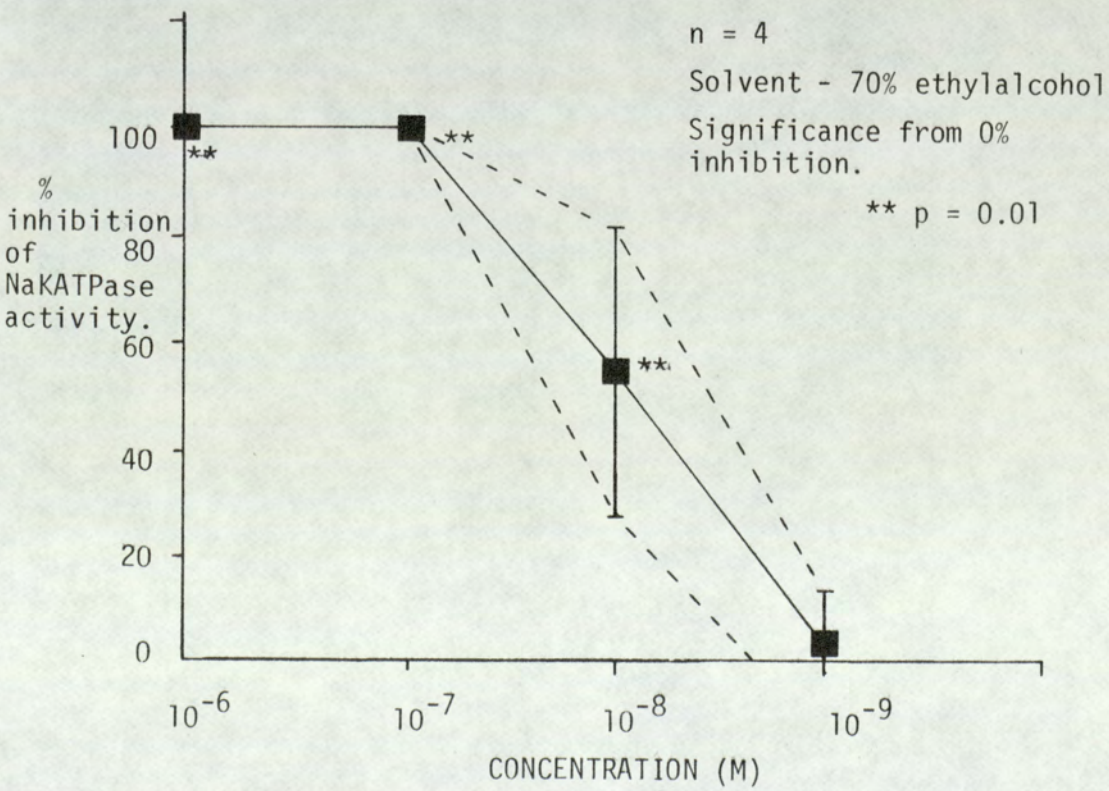


FIGURE 26

THE EFFECT OF THE INCUBATION FOR 30 MINUTES WITH N, N DIMETHYL-CHLOROETHYLAMINE ON THE RATIO OF PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH OUABAIN TO THE PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH N,N DIMETHYLCHLOROETHYLAMINE.

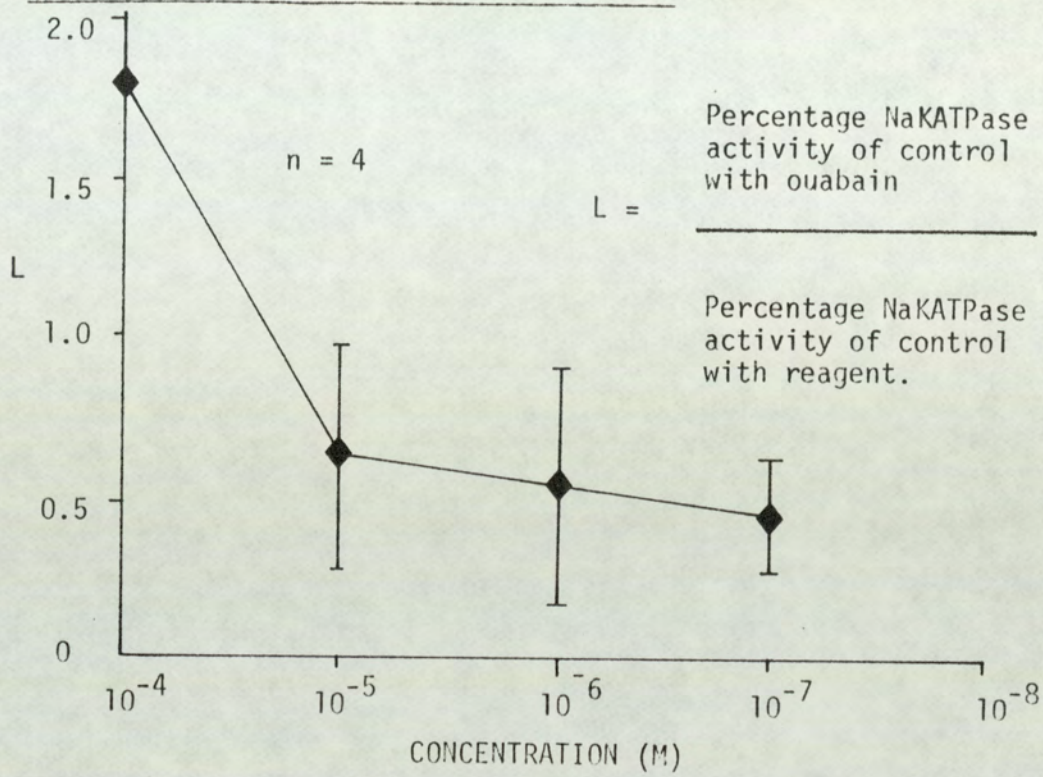


FIGURE 27

THE EFFECT OF THE INCUBATION FOR 30 MINUTES WITH VARIOUS CONCENTRATIONS OF MONOFUNCTIONAL CHLORAMBUCIL ON THE RATIO OF PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH OUABAIN TO THE PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH MONOFUNCTIONAL CHLORAMBUCIL.

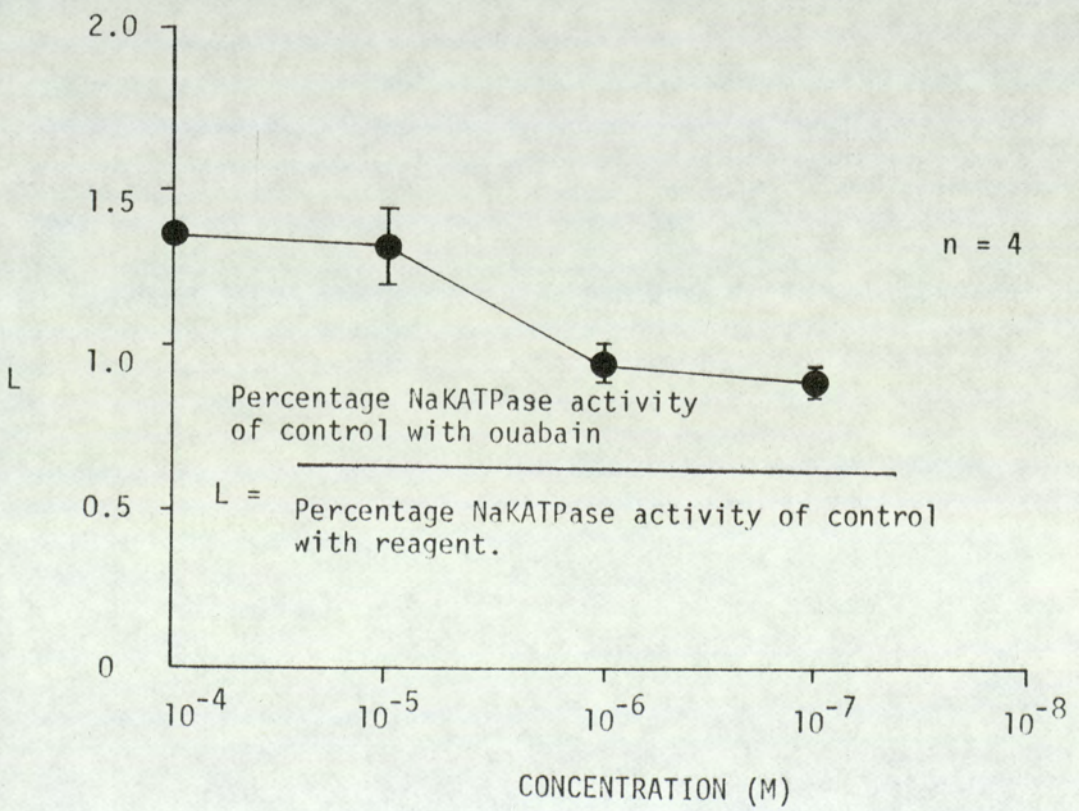


FIGURE 28

THE EFFECT OF VARIOUS CONCENTRATIONS OF IODOACETIC ACID ON THE PERCENTAGE INHIBITION OF MgATPase ACTIVITY OF A BROKEN CELL PREPARATION OF PC6A CELLS.

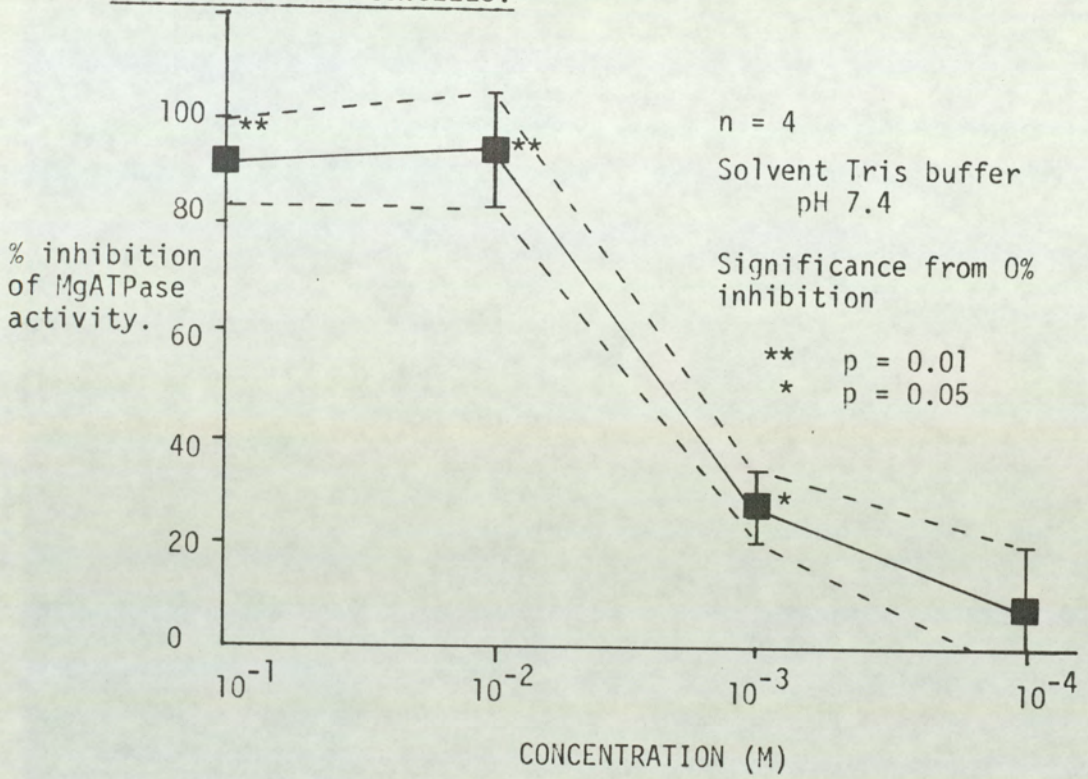


FIGURE 29

THE EFFECT OF VARIOUS CONCENTRATIONS OF p-CHLOROMERCURIBENZOIC ACID ON THE PERCENTAGE INHIBITION OF MgATPase ACTIVITY OF A BROKEN CELL PREPARATION OF PC6A CELLS.

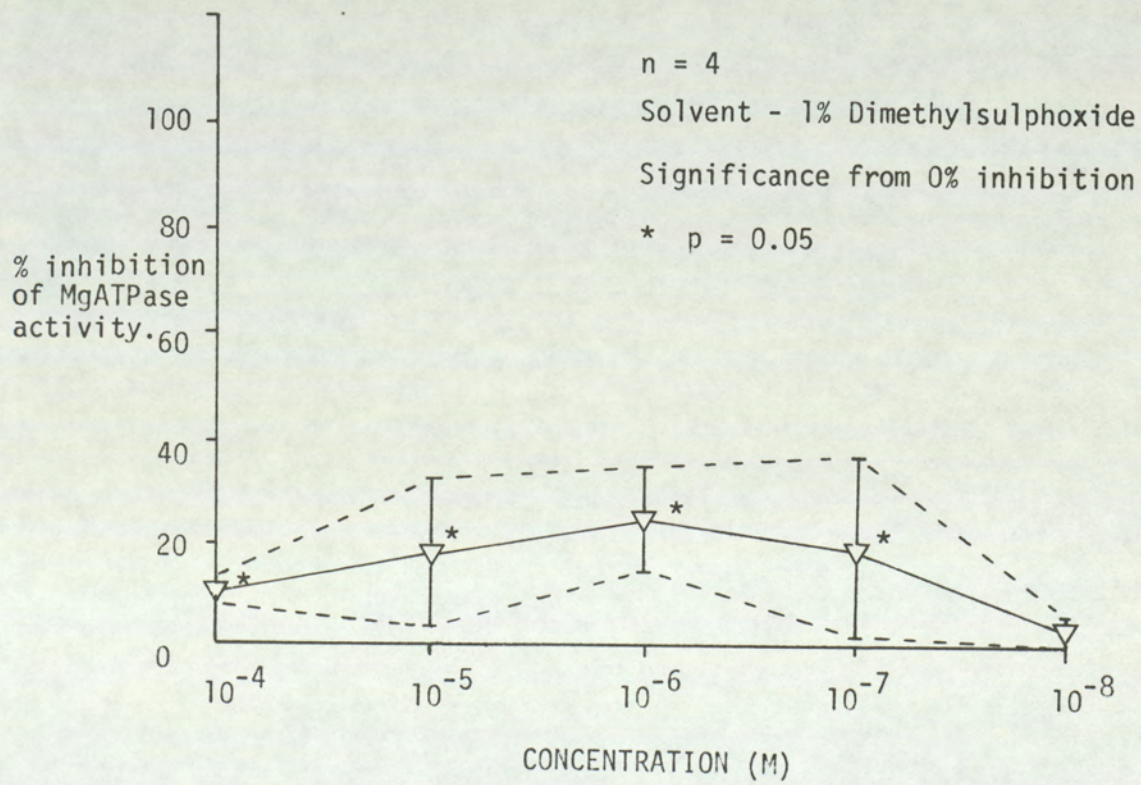


FIGURE 30

THE EFFECT OF VARIOUS CONCENTRATIONS OF N-ETHYLMALEIMIDE ON THE PERCENTAGE INHIBITION OF MgATPase ACTIVITY OF A BROKEN CELL PREPARATION OF PC6A CELLS.

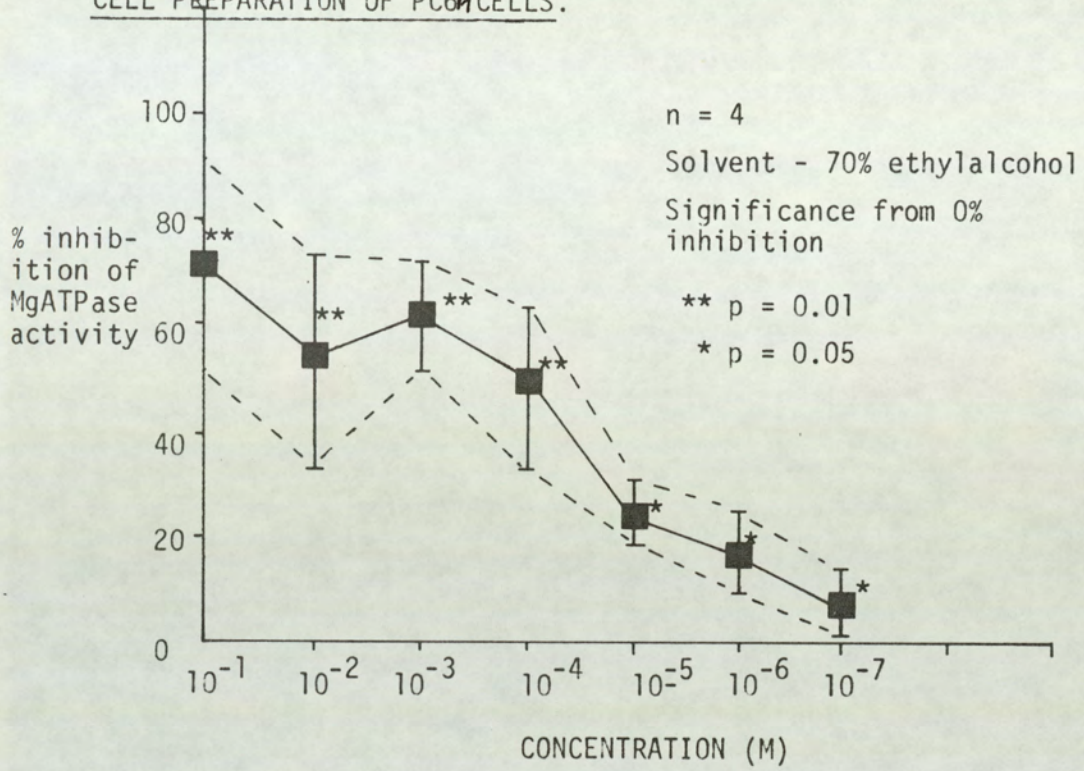


FIGURE 31

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARD ON THE PERCENTAGE INHIBITION OF MgATPase ACTIVITY OF A BROKEN CELL PREPARATION OF PC6A CELLS.

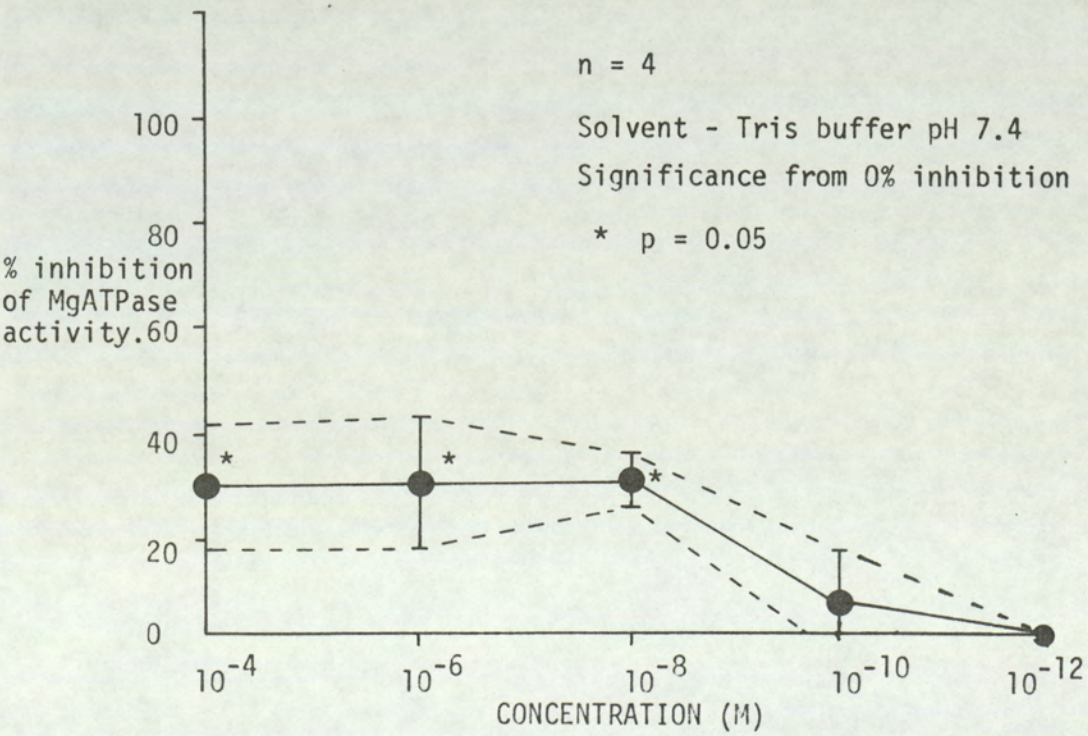


Figure 31 suggested that the ratio of ouabain sensitive activity : nitrogen mustard sensitive activity which was greater than 1.0 in Figure 23 at 10^{-10}M was possibly due to the inhibition of ouabain insensitive NaKATPase and not to MgATPase which was not significantly affected.

7:16 THE EFFECT OF PLATINUM COMPOUNDS ON NaKATPase

The platinum agents used were cis diammine dichloro platinum and its trans isomer. The method used was as for the sulphydryl reagents (6:11:5:)

Figure 32 shows the effect of cis diammine dichloro platinum on ouabain sensitive NaKATPase inhibition which was not apparently significant from 100% was obtained at 10^{-8}M. In comparison the trans isomer gave 100% inhibition at 10^{-4}M (Figure 33). Figure 34 shows the plot of the ratio of ouabain inhibition : inhibition with reagent. Cis diammine dichloro platinum gave a ratio significantly above 1.0 at 10^{-8}M whilst the trans isomer gave a ratio significantly above 1.0 at 10^{-4}M.

Full statistics on the data were not performed since the work was only repeated once.

7:17:1: EFFECT OF IONIC CONTENT ON p-NITROPHENOLPHOSPHATASE ACTIVITY (See methods 6:11;7)

Studies were performed on the activity of p-nitrophenol-phosphatase of the broken cell preparation at pH 9.5. Figure 35 shows the effect of ionic content on this activity. 1 molar tris gave a basal activity which was not significantly altered (p 0.5) by the addition of 2omM K^+ and 16mM Mg^{2+} Reductions of the Mg^{2+} content from 16mM to 8mM caused a significant (p 0.01) increase in activity and all experiments were then performed at this concentration of Mg^{2+}.

FIGURE 32

THE EFFECT OF VARIOUS CONCENTRATIONS OF CISPLATINUM ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A BROKEN CELL PREPARATION OF PC6A CELLS.

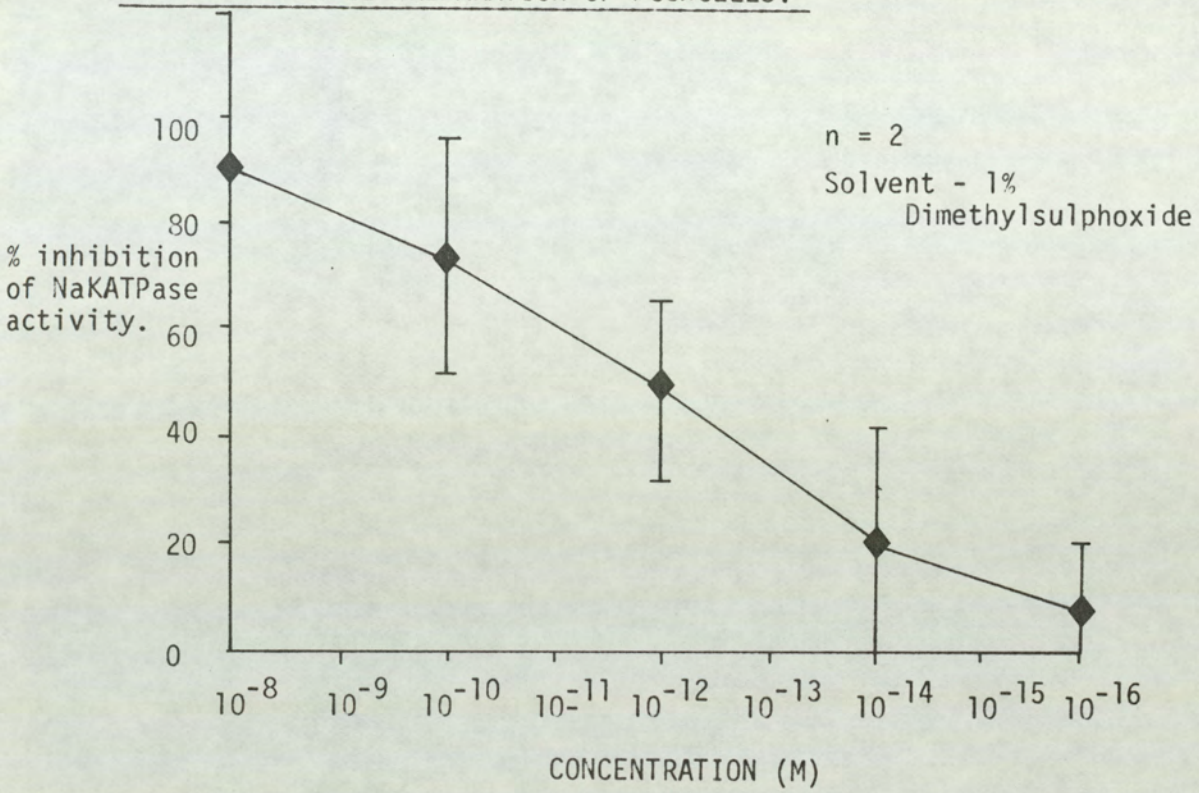


FIGURE 33

THE EFFECT OF VARIOUS CONCENTRATIONS OF TRANSPLATINUM ON THE PERCENTAGE INHIBITION OF OUABAIN SENSITIVE NaKATPase ACTIVITY IN A BROKEN CELL PREPARATION OF PC6A CELLS.

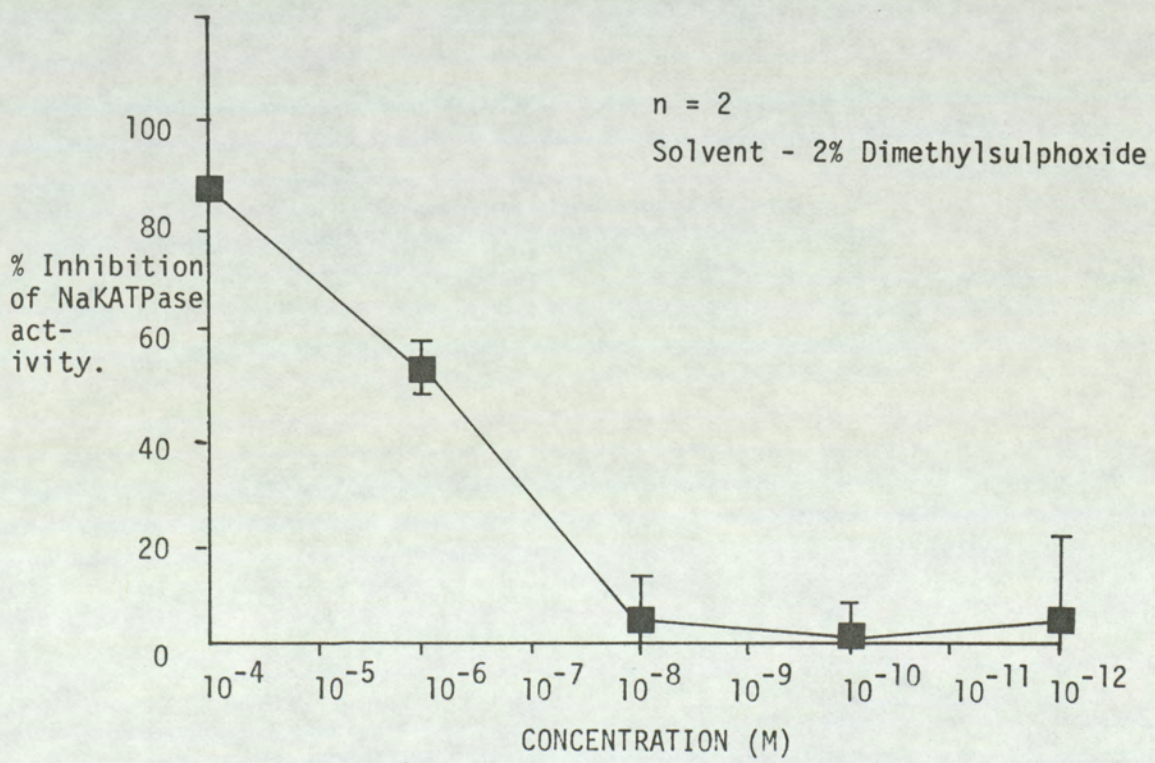


FIGURE 34

THE EFFECT OF PLATINUM COMPOUNDS ON THE RATIO OF PERCENTAGE NaKATPase ACTIVITY OF CONTROL WITH OUABAIN TO THE PERCENTAGE NaKATPase ACTIVITY ACTIVITY OF CONTROL WITH PLATINUM COMPOUNDS AFTER 30 MINUTES TREATMENT AT 37°C USING A BROKEN CELL PREPARATION OF PC6A CELLS.

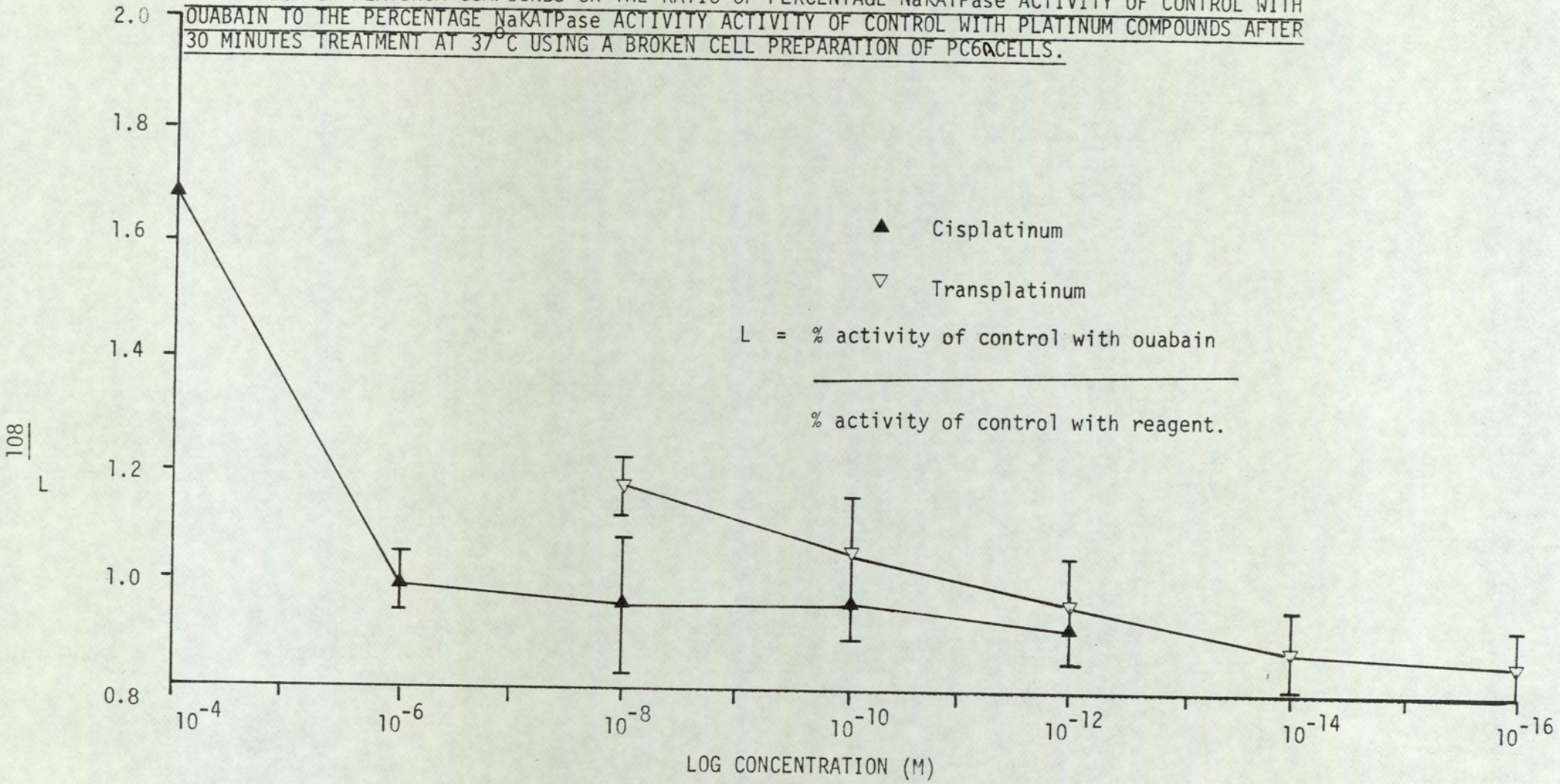
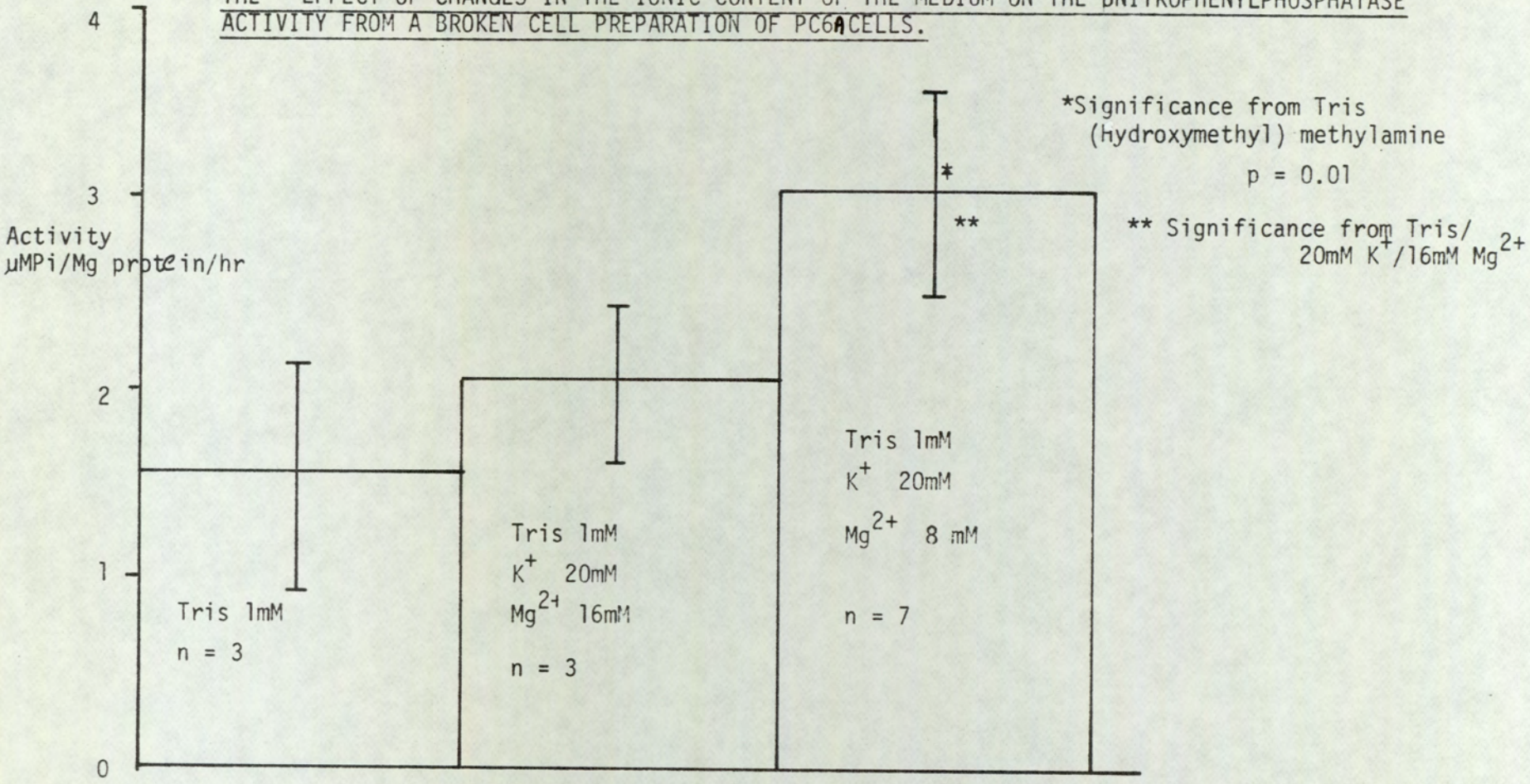


FIGURE 35

THE EFFECT OF CHANGES IN THE IONIC CONTENT OF THE MEDIUM ON THE pNITROPHENYLPHOSPHATASE ACTIVITY FROM A BROKEN CELL PREPARATION OF PC6A CELLS.



7:17:2: EFFECT OF SULPHYDRYL REAGENTS

Figures 36-38 show the effect of three classical sulphydryl reagents on p-nitrophenolphosphatase activity. p-Chloromercuribenzoate showed no effect over the range tested and neither did N-ethylmaleimide. Iodoacetic acid inhibited activity at 10^{-1}M by about 40% which was significant at P=0.2.

Therefore all three agents tested failed to show 100% inhibition of the activity under the conditions of the assay Figure 39 shows the effect of 10^{-4}M nitrogen mustard on p-nitrophenolphosphatase activity. This agent had no effect at high concentrations even with a preincubation time of up to 90 minutes. This result is not to be unexpected due to the very short half life of the agent under alkaline conditions.

7:18: EXTERNAL ATPASE (RONQUIST AND AGREN 1975)

7:18:1 EFFECT OF IONIC CONTENT (See methods 6:11:8)

This enzyme resides on the external surface of the plasma membrane.Assays are therefore performed on whole cells and the activity is shown as μMP./10^6 cells/hour. Figure 40 shows the effect of ionic content on this activity . 5mM Mg^{2+} shows a basal activity, which is not increased significantly by the addition of 0.2mM Ca^{2+}. Addition of 100mM Na^+ and 20mM K^+ does increase activity (p=0.05) and by the further addition of 0.2mM Ca^{2+} the activity is also increased so that it is significantly different from the basal activity (p=0.05).

This activity is not due to NaKATPase since the addition of $2x10^{-4}$M ouabain has no significant effect on the activity obtained with 100mM Na^+, 20mM K^+ and 5mM Mg^{2+}.

7:18:2: EFFECT OF NITROGEN MUSTARD

Figure 41 shows the effect of 10^{-4}M nitrogen mustard on this external ATPase. It shows that over a 60 minute preincubation period this agent has no significant effect

FIGURE 36

THE EFFECT OF VARIOUS CONCENTRATIONS OF p-CHLOROMERCURIBENZOIC ACID ON THE p-NITROPHENYLPHOSPHATASE ACTIVITY OF A BROKEN CELL PREPARATION OF PC6A CELLS.

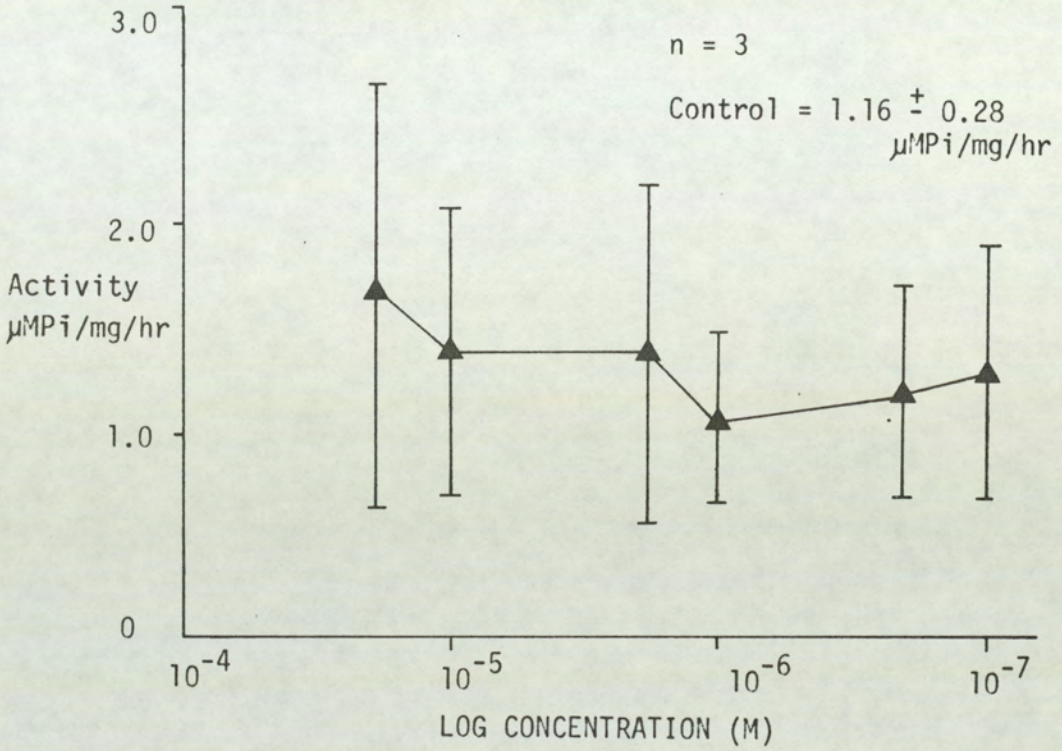


FIGURE 37

THE EFFECT OF VARIOUS CONCENTRATIONS OF N-ETHYLMALEIMIDE ON THE p-NITROPHENYLPHOSPHATASE ACTIVITY OF A BROKEN CELL PREPARATION OF PC6A CELLS.

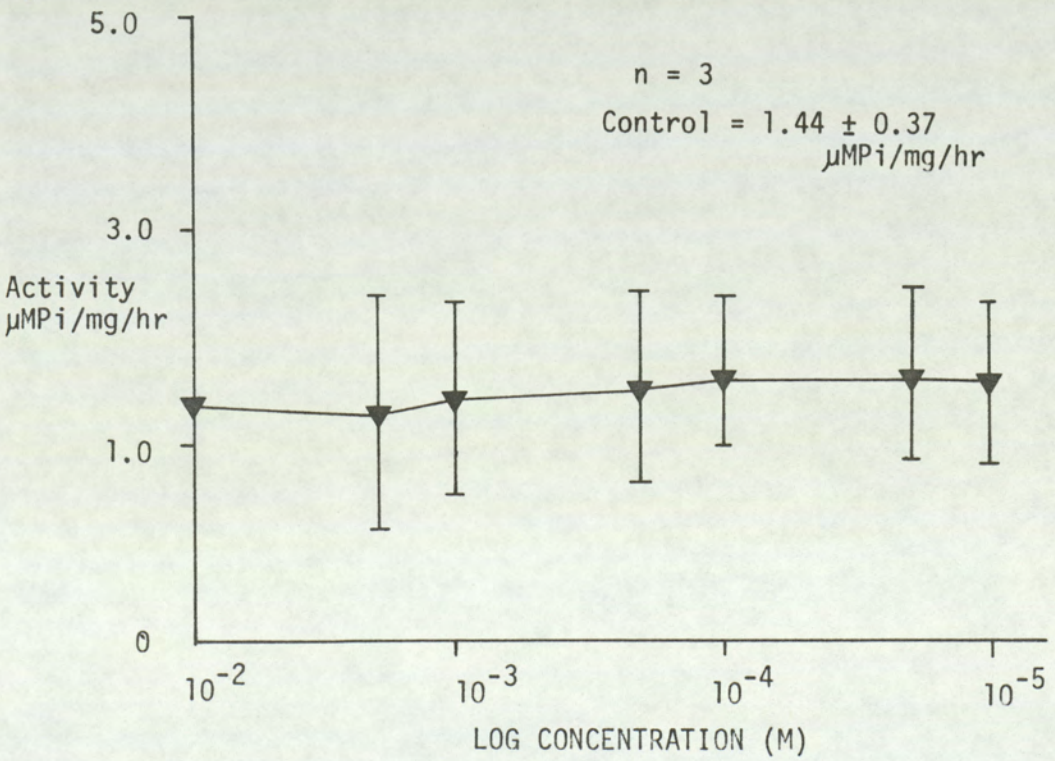


FIGURE 38

THE EFFECT OF VARIOUS CONCENTRATIONS OF IODOACETIC ACID ON THE ACTIVITY AND PERCENTAGE ACTIVITY OF CONTROL OF THE p-NITROPHENYL-PHOSPHATASE ACTIVITY OF BROKEN CELL PREPARATION OF PC6A CELLS.

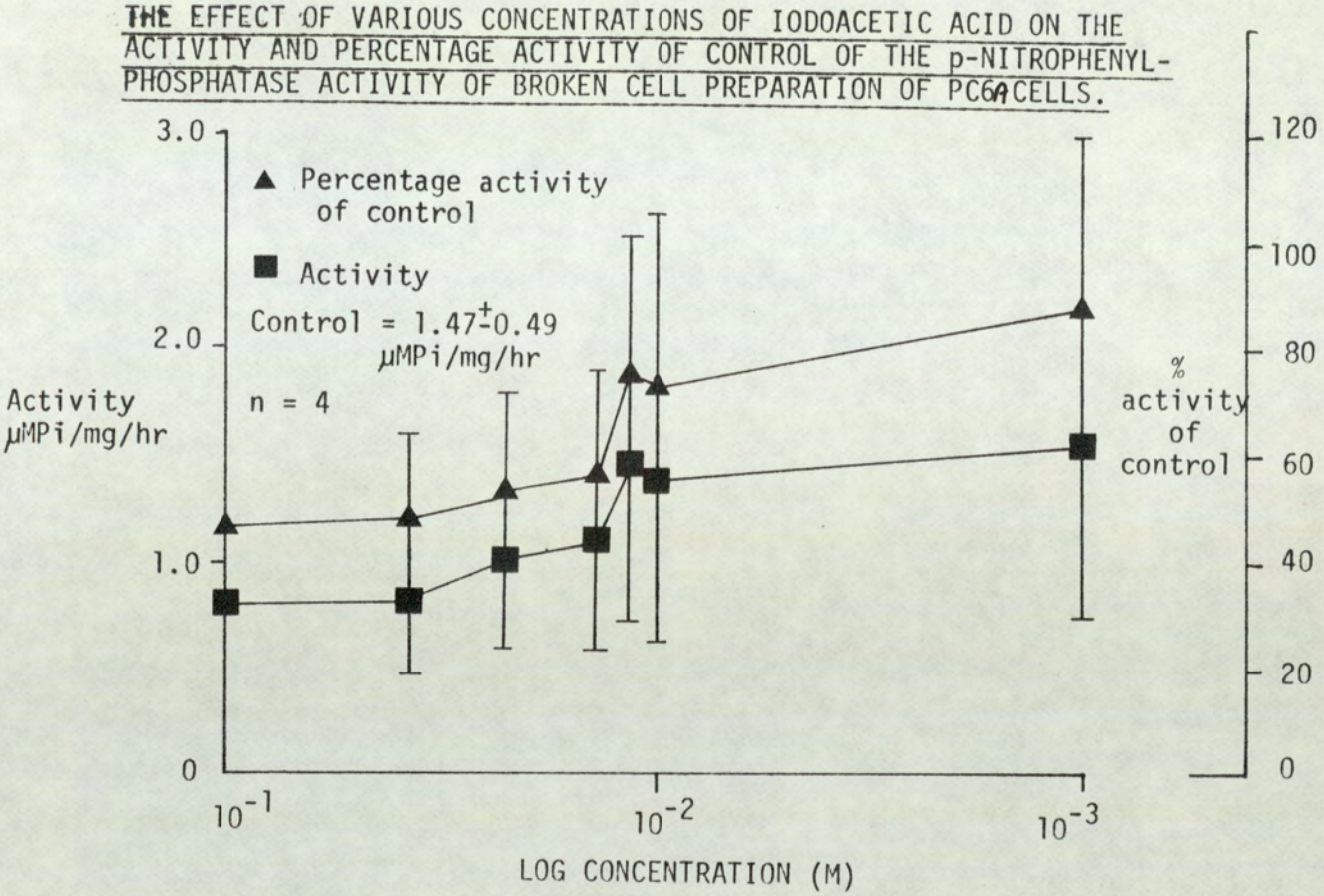


FIGURE 39

THE EFFECT OF INCUBATION TIME WITH 10^{-4}M NITROGEN MUSTARD ON THE p-NITROPHENYLPHOSPHATASE ACTIVITY OF A BROKEN CELL PREPARATION OF PC6A CELLS.

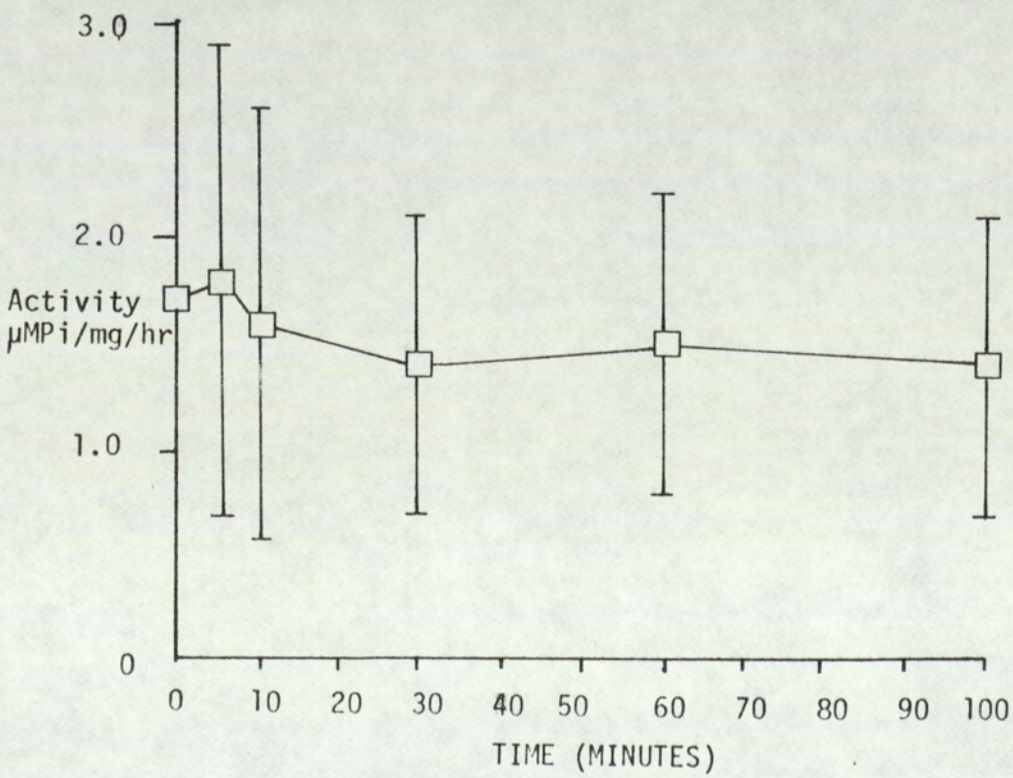


FIGURE 40

THE EFFECT OF CHANGES IN IONIC CONTENT OF THE MEDIUM ON THE ACTIVITY OF AN EXTERNAL CaMgATPase FROM PC6A CELLS.

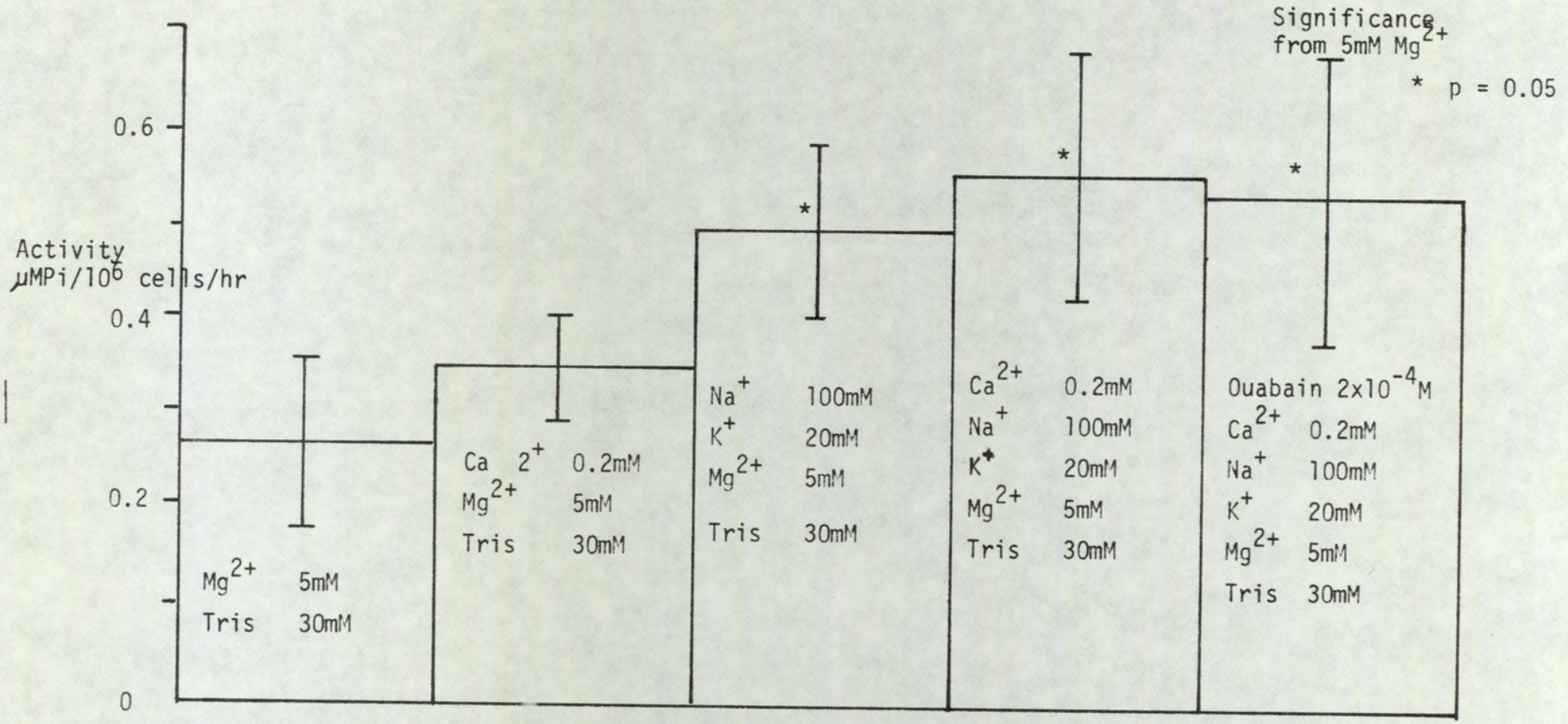
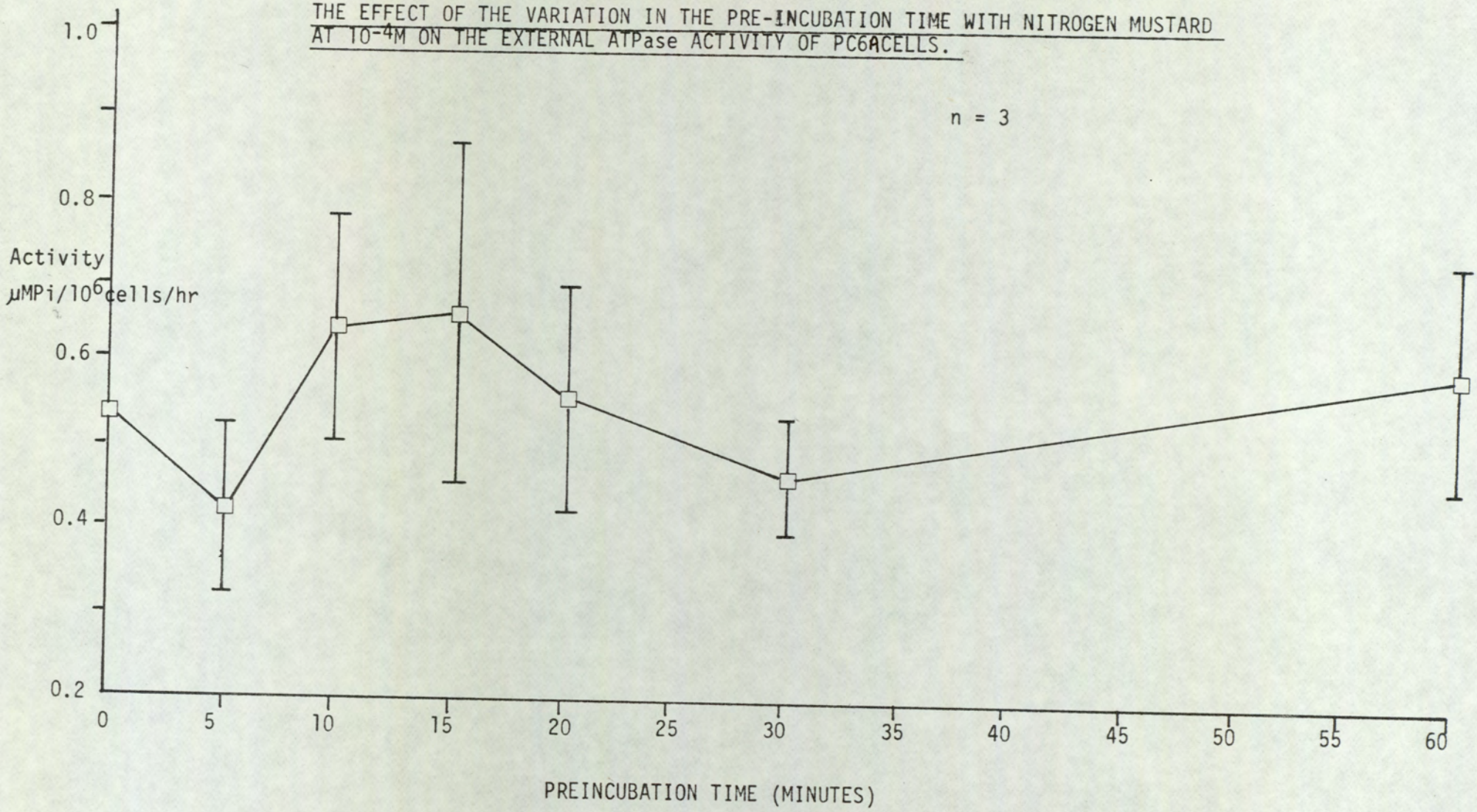


FIGURE 41

THE EFFECT OF THE VARIATION IN THE PRE-INCUBATION TIME WITH NITROGEN MUSTARD AT 10^{-4}M ON THE EXTERNAL ATPase ACTIVITY OF PC6A CELLS.



on the ATPase activity under the conditions of the assay.

7:19: STUDIES ON INTACT PC6A CELLS

7:19:1: THE STUDY OF THE GROWTH OF WASHED INTACT PC6A CELLS WITH AN IN VITRO - IN VIVO TECHNIQUE USING AN ASCITIC PC6A TUMOUR (SEE METHODS 6:8:)

Figure 42 shows the relationship between the number of cells injected into an animal on day 0 and the number harvested on the seventh day from the animal (methods 6:8:) The increase in cell number corresponded approximately to an *in vivo* doubling time of between 19 and 21 hours when between 10^3 and 10^6 cells were injected, but this increased when 10^7 cells were injected to a time of approximately 28 hours.

Table 12 shows the effect of the variation of the preincubation time in the RPMI 1640 media prior to injection (see methods 6:8:). It can be seen that after 6 hours preincubation there was a 40% fall in the number of cells harvested. Preincubation of longer time periods had no apparent effect above this 40% fall, except for the result at 10 hours.

7:19:2: THE ESTIMATION OF THE CYTOTOXICITY OF NITROGEN MUSTARD BY THE USE OF AN IN VITRO-IN VIVO TECHNIQUE USING AN ASCITIC PC6A TUMOUR

Figures 43 and 44 show that the concentration of nitrogen mustard required to inhibit by 90% the total number of cells harvested after 7 days compared to the control was 0.238 μg/ml By reference to the standard curve (Figure 42) this approximated to an apparent 3 log cell kill.

7:19:3: THE EFFECT OF N,N DIMETHYLCHLOROETHYLAMINE AND OUABAIN AS MEASURED BY THE PC6A ASCITIC IN VITRO-IN VIVO GROWTH TECHNIQUE.

Table 13 shows the results for the cytotoxicity in a bioassay of the monofunctional analogue of nitrogen mustard and for ouabain. In both cases there was no significant

TABLE 12

THE EFFECT OF THE VARIATION IN PREINCUBATION TIME ON THE TOTAL NUMBER OF CELLS HARVESTED AS MEASURED BY THE IN VITRO-IN VIVO ASSAY WITH AN ASCITIC PC6A TUMOUR

PREINCUBATION TIME (HOURS)	NUMBER OF CELLS	% OF CONTROL AT 0 TIME
0	6.02×10^8	100
2	5.38×10^8	89.37
4	6.11×10^8	101.50
6	3.59×10^8	59.63
8	3.79×10^8	62.96
10	9.88×10^7	16.41
12	3.38×10^8	56.15

FIGURE 42

THE EFFECT OF THE VARIATION IN THE NUMBER OF PC6A CELLS INJECTED ON THE NUMBER OF CELLS HARVESTED 7 DAYS LATER USING THE IN VITRO-IN VIVO ASSAY WITH AN ASCITIC PC6A TUMOUR

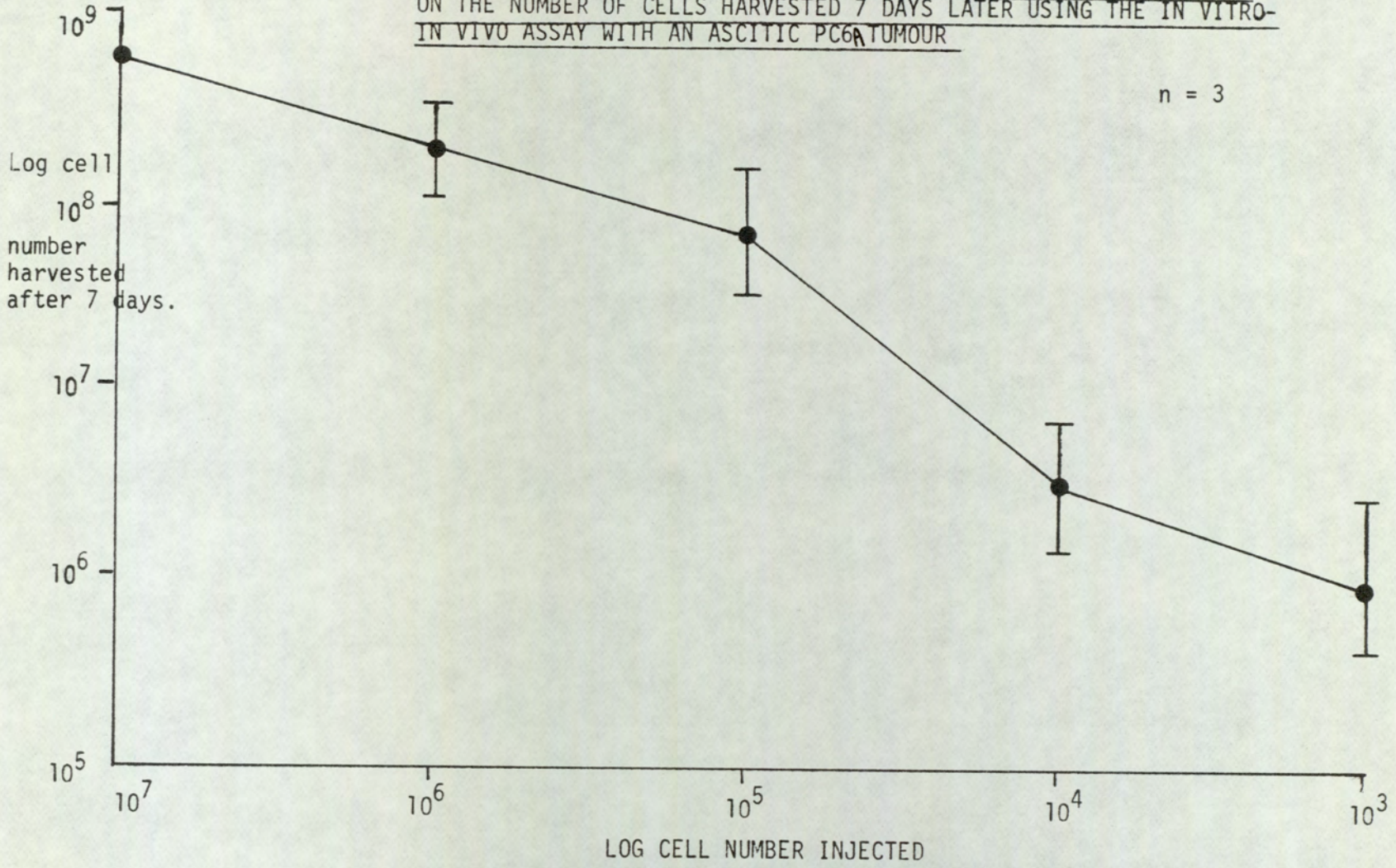


FIGURE 43

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARD ON THE PERCENTAGE INHIBITION OF CELL NUMBERS COMPARED TO A CONTROL AS DETERMINED BY AN IN VITRO-IN VIVO ASSAY WITH AN ASCITIC PC6A TUMOUR

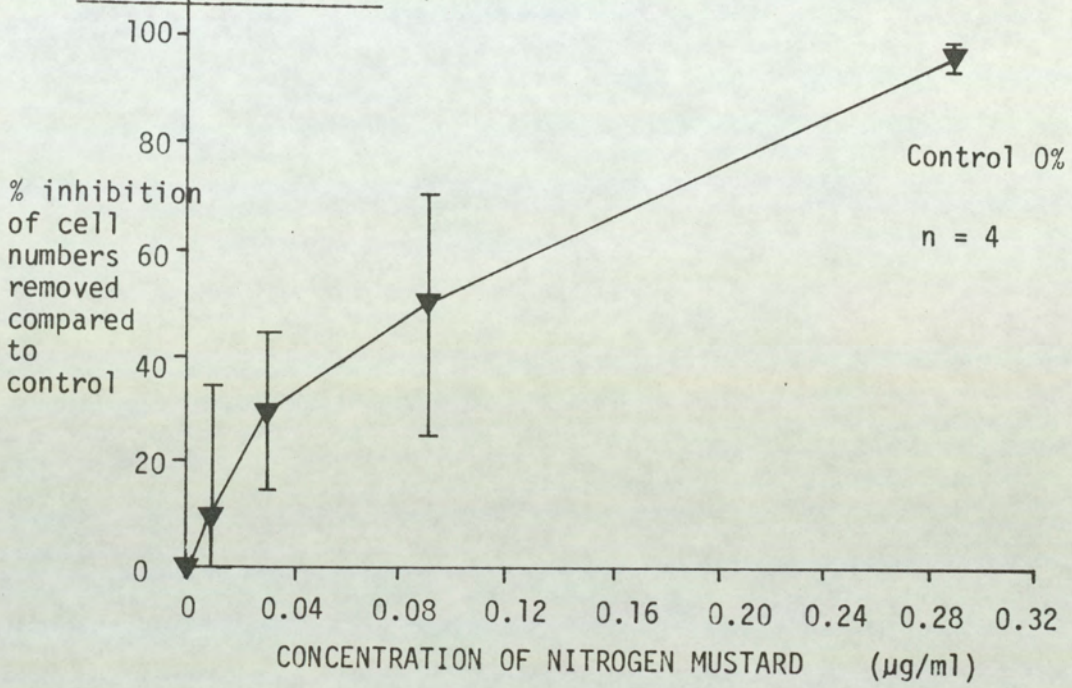


FIGURE 44

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARD ON THE NUMBER OF CELLS REMOVED AS DETERMINED BY AN IN VITRO IN VIVO ASSAY WITH AN ASCITIC PC6A TUMOUR

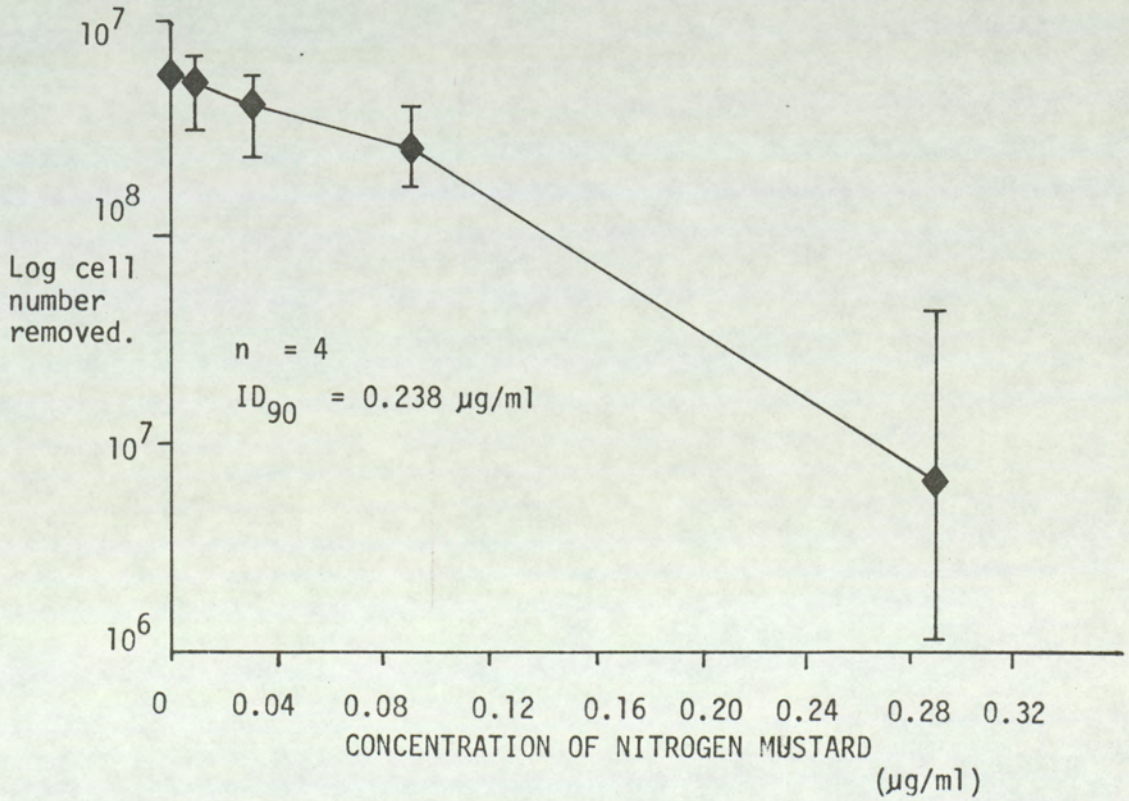


TABLE 13

EFFECT OF N, N DIMETHYLCHLOROETHYLAMINE AND OUABAIN(1) ON THE INCREASE IN CELL NUMBERS AS MEASURED BY THE IN VITRO-IN VIVO ASSAY WITH AN ASCITIC PC6A TUMOUR

AGENT	DOSE	CELL NUMBER $\times 10^8$	MEAN n=3
N,N Dimethyl-chloroethylamine	Control	5.63 4.96 6.11	5.57 ± 0.58
	0.09 µg/ml	4.24 3.47 5.16	4.29 ± 0.85
	0.27 µg/ml	5.83 5.16 3.42	4.80 ± 1.24
	0.81 µg/ml	7.47 4.24 3.66	5.12 ± 2.45
Ouabain (1)	Control	5.34 9.17 6.02	7.05 ± 2.38
	10^{-3}M(2)	7.56 2.59 3.79	4.65 ± 2.59
	10^{-4}M	6.26 6.05 3.38	5.23 ± 1.61

1. Preincubation time increased to 4 hours. Cells washed twice before injection to remove the ouabain, and so reduce toxicity to the animal.

2. 10^{-2}M ouabain was toxic to Balb/C mice. Injection of cells treated with this dose, without washing, led to death within 5 minutes.

difference in the control and treated cells.

Ouabain although it is cytotoxic in vitro over a longer period of exposure (Shank and Smith 1976) appeared to be a reversible inhibitor under these conditions. It is possible therefore that the washing process removed the drug and hence allowed regrowth.

7:19:4: THE ATTEMPTED FORMATION OF A RESISTANT LINE OF PC6 CELLS TO NITROGEN MUSTARD

An attempt was made to make a line of PC6A cells resistant to nitrogen mustard. Cells were treated with an ID_{90} concentration (Figure 43) for two hours and then injected and left as before (See methods 6:8:). On Day 7 the cells were harvested and 10^6 cells were injected into each of three female Balb/ C mice. On Day 7 the cells were again harvested, treated with nitrogen mustard, as above, and injected into three mice. This cycle was repeated four times but no resistance to nitrogen mustard was detected (Table 14).

7:19:5: THE STUDY OF WASHED INTACT PC6A CELLS BY THE IN VITRO - IN VIVO ASSAY WITH A SOLID PC6A TUMOUR (SEE METHODS 6:5:)

Due to problems in the interpretation of the results using this technique with an ascitic PC6A tumour (see discussion), a study was performed using an in vitro - in vivo technique with a solid PC6A tumour.

Table 15 shows the effect of a 2 hour preincubation of cells prior to injection on the resultant solid tumour. It can be seen that there was no significant effect on the size of tumour.

7:19:6: THE EFFECT OF NITROGEN MUSTARD ON THE RESULTANT TUMOUR MASS AS MEASURED BY AN IN VITRO - IN VIVO TECHNIQUE USING A SOLID PC6A TUMOUR

Figures 45 and 46 show the effect of the changes in concentration of nitrogen mustard on the weight of the tumour

FIGURE 45

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARD ON TUMOUR WEIGHT AS DETERMINED BY AN IN VITRO IN VIVO TECHNIQUE WITH A SOLID PC6A TUMOUR

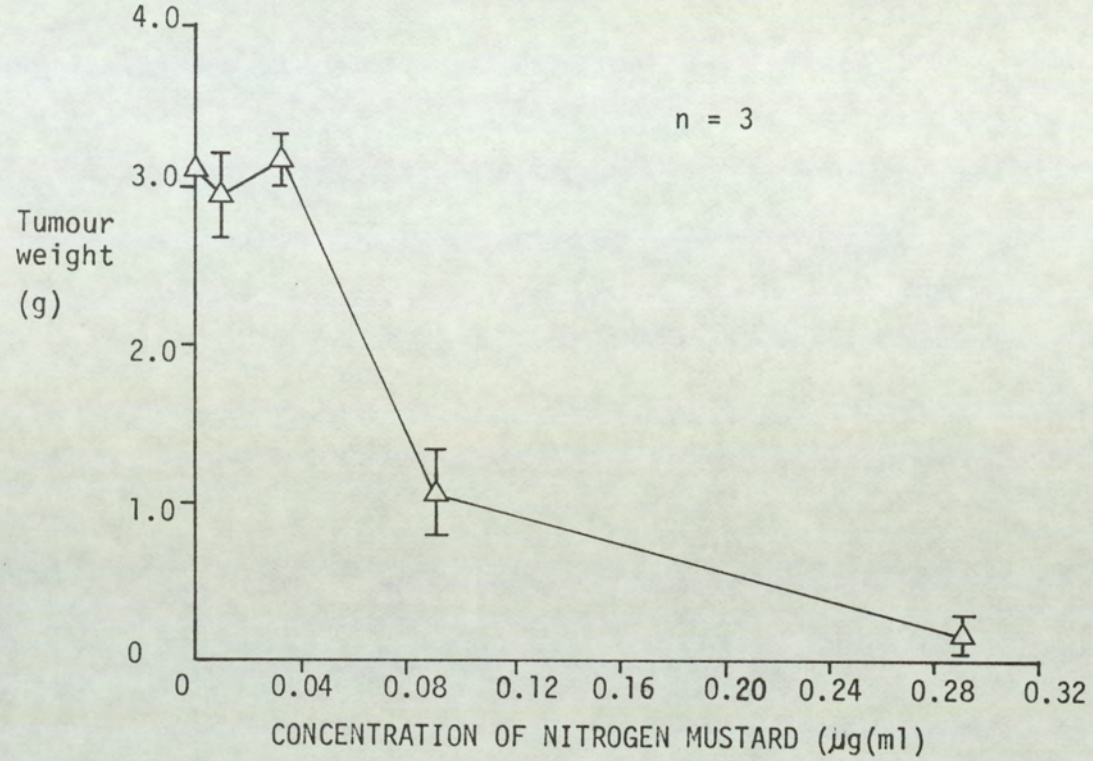


FIGURE 46

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARD ON THE PERCENTAGE INHIBITION OF TUMOUR WEIGHT COMPARED TO A CONTROL AS DETERMINED BY AN IN VITRO IN VIVO TECHNIQUE WITH A SOLID PC6A TUMOUR

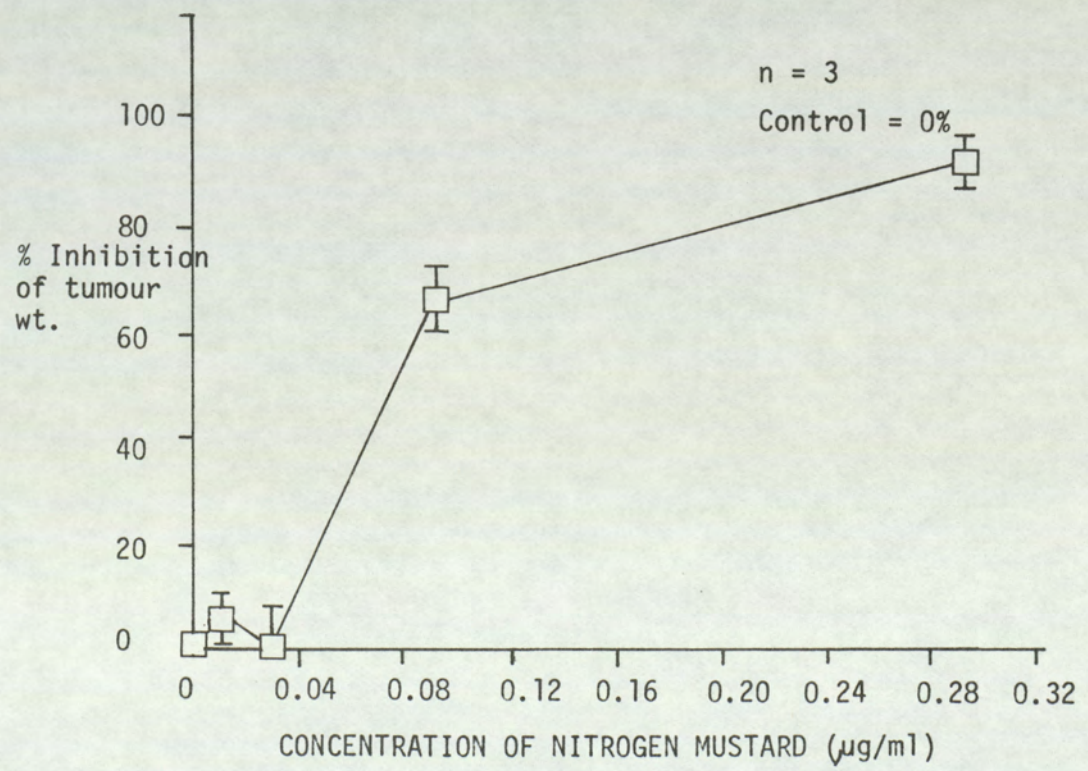


TABLE 14

THE EFFECT OF REPEATED TREATMENT WITH NITROGEN MUSTARD ON THE CELL NUMBERS AS MEASURED BY THE PC6A IN VITRO-IN VIVO ASSAY WITH AN ASCITIC PC6A TUMOUR

TREATMENT NUMBER(1)	CELL NUMBER SEVEN DAYS AFTER A 2 HOUR INCUBATION AT 0.238 µg/ml
1	1.04×10^7
2	1.92×10^2
3	9.82×10^6
4	2.96×10^7

1. Relates to the number of treatments with nitrogen mustard (see 7:19:4:)

Table 15

EFFECT OF PREINCUBATION TIME ON THE RESULTANT TUMOUR AFTER 28 DAYS GROWTH MEASURED BY THE PC6A IN VITRO - IN VIVO ASSAY WITH A SOLID PC6 TUMOUR

PREINCUBATION TIME (HOURS)	AVERAGE WEIGHT OF TUMOUR
0	2.625g n=3
2	2.476g n=2(1)

(1) Only two out of the three test animals survived the 28 days.

after growth in a mouse for 28 days. The ID_{90}, that is, the dose required to reduce by 90% the weight of the control tumour was 0.248 µg/ml.

7:20: STUDIES ON INTACT PC6A CELLS

7:20:1: STUDIES ON THE REGULATION OF CELL VOLUME (SEE METHODS 6:11:9:)

Table 16 shows the results obtained with ouabain using the hypotonic shock assay described by Shank and Smith (1976).

An R value was calculated which was the ratio of the number of cells immediately after the hypotoxic swelling divided by the number of cells counted 10 minutes after the hypotoxic shock. A value greater than 1.0 indicated that the cell volume had reduced over 10 minutes, and hence by definition (Shank and Smith 1976) the sodium pump was operable. A value of 1.0 indicated that there was complete inhibition of the sodium pump.

The results show a time dependent inhibition of the sodium pump up to 30 minutes with 10^{-3}M ouabain (Figure 47). A levelling off and a decrease in inhibition was seen with 10^{-4}M and 10^{-5}M ouabain (Figures 48-49).

The 10^{-3}M ouabain result (Figure 47) showed that after 30 minutes there was only a 50% inhibition of the pump. This suggests that at all concentrations of ouabain the PC6A cells are not especially sensitive to the effects of ouabain. This low affinity for rodent NaKATPase has been reported previously (Detweilker 1967) and was considered to be due to the low strength of glycoside binding to the receptor.

In Table 17 and Figure 50 the effect of 10^{-4}M nitrogen mustard on the regulation of cell volume is shown. The results show a time dependent inhibition of cell volume regulation, up to an approximate 45% inhibition.

TABLE 16

CELL VOLUME REGULATION ASSAY - EFFECT OF OUABAIN

n=3

CONCENTRATION (M)	TIME[1]	R VALUE[2]	MEAN
10^{-3}M	0	1.14 1.16 1.14	1.15 ± 0.01
	5	1.08 1.15 1.10	1.11 ± 0.04
	10	1.09 1.12 1.09	1.10 ± 0.02
	15	1.10 1.09 1.08	1.09 ± 0.01
	30	1.07 1.06 1.09	1.07 ± 0.02
10^{-4}M	0	1.52 1.35 1.30	1.39 ± 0.12
	5	1.55 1.24 1.25	1.35 ± 0.18
	10	1.46 1.18 1.19	1.27 ± 0.17
	15	1.42 1.15 1.16	1.24 ± 0.15
	30	1.34 1.14 1.19	1.22 ± 0.10
10^{-5}M	0	1.73 1.26 1.63	1.54 ± 0.25
	5	1.57 1.23 1.51	1.44 ± 0.18
	10	1.44 1.21 1.43	1.36 ± 0.13
	15	1.39 1.18 1.42	1.33 ± 0.13
	30	1.45 1.19 1.50	1.38 ± 0.17

1. Preincubation time prior to assay.
2. $R = \frac{\text{Mean cell number at start of assay}}{\text{Mean cell number 10 minutes after assay.}}$

TABLE 17

CELL VOLUME REGULATION ASSAY - EFFECT OF 10^{-4}M HN2

n = 2

TIME (MINS.)	R VALUE	MEAN[1]
0	1.25 1.29	1.27
10	1.22 1.32	1.27
25	1.22 1.21	1.22
30	1.19 1.22	1.21
60	1.17 1.10	1.14
90	1.15 1.14	1.15
120	1.16 1.15	1.16
180	1.16 1.15	1.16

1. Standard deviation is not shown since n = 2

FIGURE 47

THE EFFECT OF INCUBATION WITH 10^{-3}M OUABAIN ON THE PERCENTAGE INHIBITION OF CELL VOLUME REGULATION AS DETERMINED BY THE METHOD OF SHANK AND SMITH (1976).

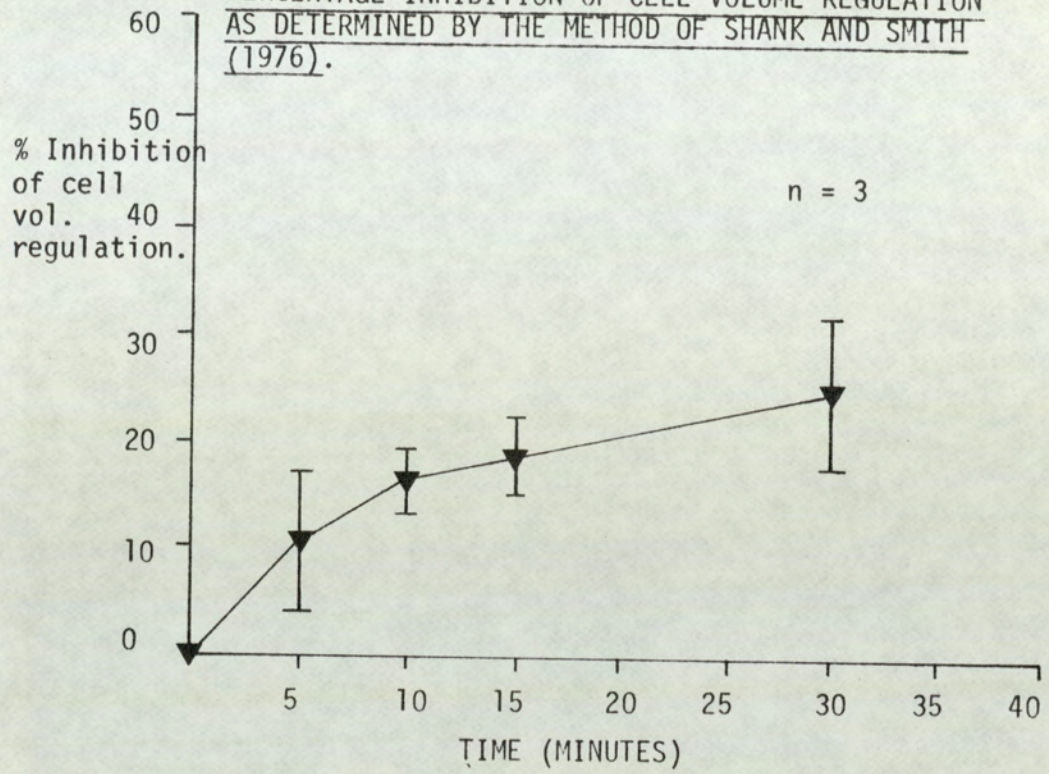


FIGURE 48

THE EFFECT OF INCUBATION WITH 10^{-4}M OUABAIN ON THE PERCENTAGE INHIBITION OF CELL VOLUME REGULATION AS DETERMINED BY THE METHOD OF SHANK AND SMITH (1976)

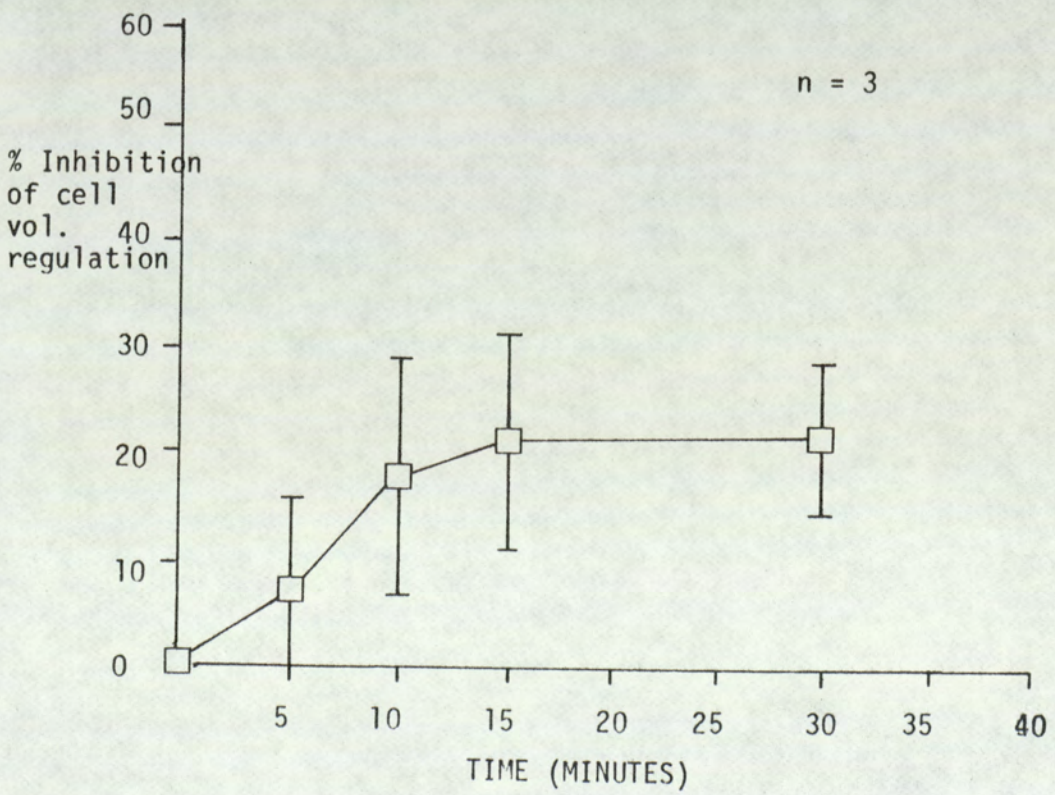


FIGURE 49

THE EFFECT OF INCUBATION WITH 10^{-5}M OUABAIN ON THE PERCENTAGE INHIBITION OF CELL VOLUME REGULATION AS DETERMINED BY THE METHOD OF SHANK AND SMITH (1976)

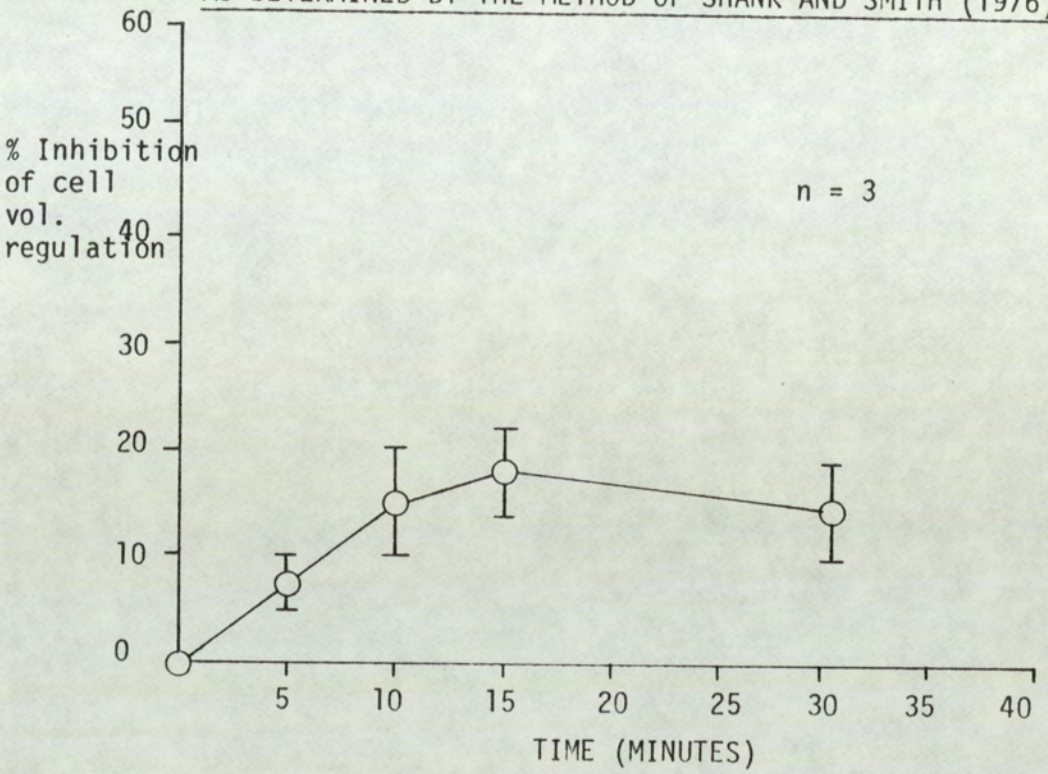
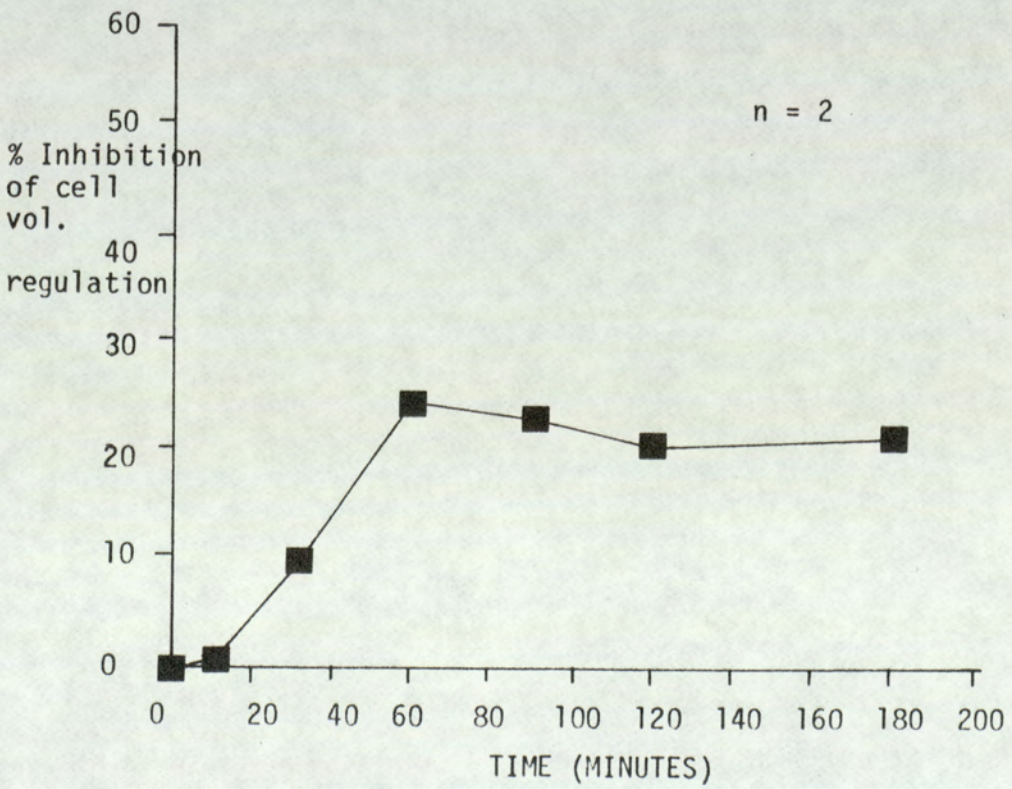


FIGURE 50

THE EFFECT OF INCUBATION WITH 10^{-4}M NITROGEN MUSTARD ON THE PERCENTAGE INHIBITION OF CELL VOLUME REGULATION AS DETERMINED BY THE METHOD OF SHANK AND SMITH (1976)



7:21: STUDIES ON THE UPTAKE OF RADIOLABELLED RUBIDIUM INTO INTACT PC6 CELLS

7:21:1: PREPARATORY WORK

Oil Density: In Plate 1 the result is shown of the effect of the alteration of the oil density on the passage of PC6 cells through the layer (see methods 6:12:2:). The oil densities used were:-

Tube Number	(parts) Silicon Oil	+	Corn Oil
1	5		0
2	5		0.5
3	5		1
4	5		1.5
5	5		2

Plate 1 illustrates that a high proportion of silicon oil i.e. tubes 1 and 2, gave a layer too dense to allow passage of cells through the oil. A ratio of 5 parts silicon oil plus 1.5 parts corn oil was the maximum density to allow the complete passage of the cells through the oil to the oil/formic acid interface. Although not clear from the photograph, there was some suggestion of the oil and supernatent inverting when the corn oil was increased to 2 parts. Therefore, the ratio of 5 parts silicon oil plus 1.5 parts corn oil was used throughout the subsequent experiments.

7:21:2: ^{3}H INULIN CARRYOVER (SEE METHODS 6:12:4:)

Table 18 gives the result of the experiments to determine the percentage carryover of ^{3}H inulin from the supernatant into the formic acid layer by the PC6 cells after rapid centrifugation. It was found by trial and error that a cell number of 10^{7}/ml corrected for coicidence gave a carryover of 0.24% and this was corrected for in all subsequent experiments.

PLATE 1

EFFECT OF OIL DENSITY ON PASSAGE OF PC6A CELLS THROUGH THE OIL LAYER

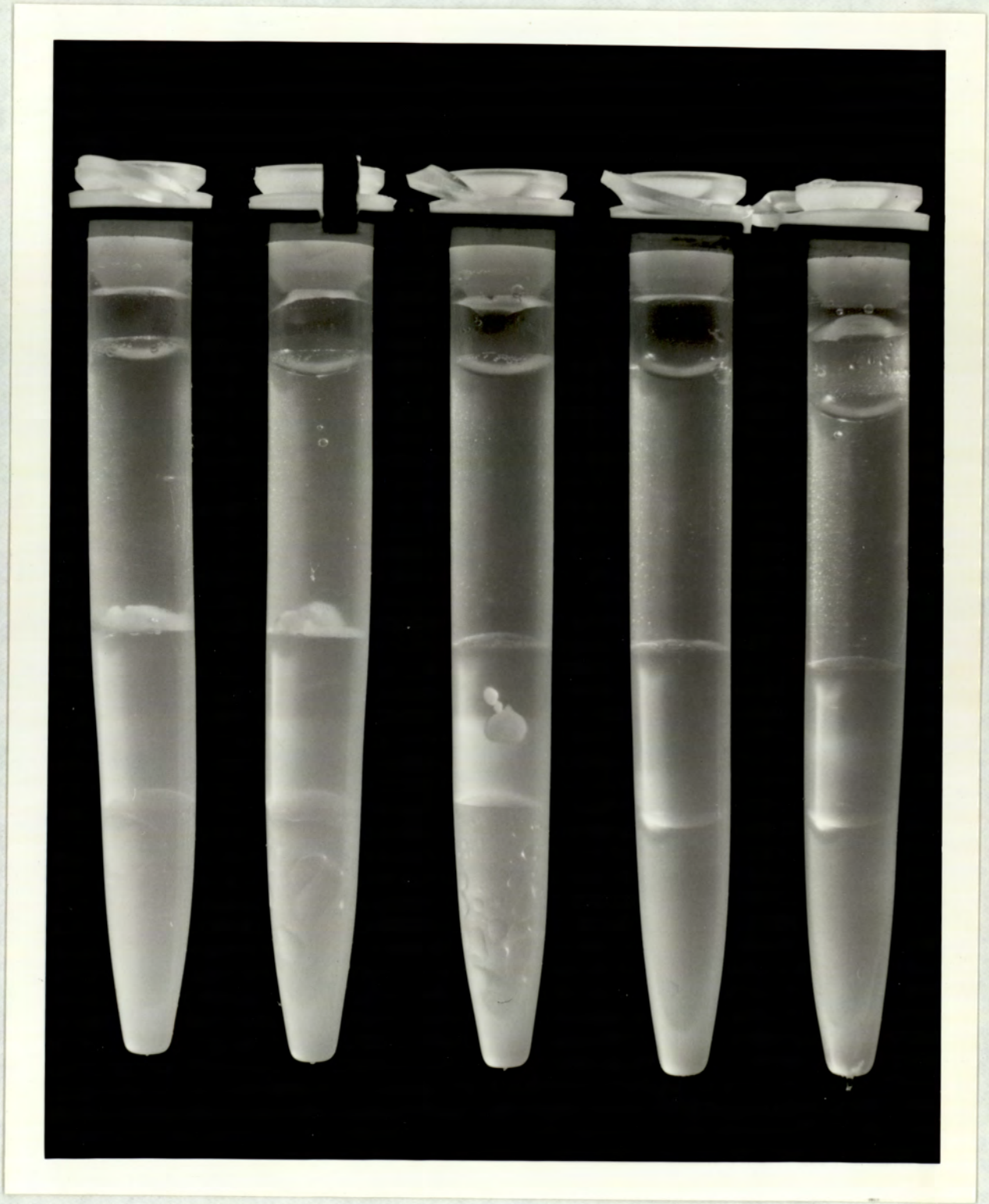


TABLE 18

^{3}H INULIN CARRY OVER STUDIES

DISINTEGRATIONS PER MINUTE		% CARRY OVER	n = 3 MEAN
892	Pellet	0.23	
390673	Control		
949	Pellet	0.24	0.24
390673	Control		±0.015
1051	Pellet	0.26	
399767	Control		

TABLE 19

^{86}RUBIDIUM CARRYOVER FROM SUPERNATANT

COUNTS PER 10 MINUTES		% CARRY OVER	n = 3 MEAN
107	Formic Acid	0.45	
23924	Supernatant		
8	Formic Acid	0.03	0.18
24661	Supernatant		± 0.23
15	Formic Acid	0.06	
24038	Supernatant		

1. Corrected for background count.

7:21:3: DETERMINATION OF THE PERCENTAGE OF CELLS PASSING THROUGH THE OIL LAYER ON CENTRIFUGATION

Table 20 shows that on each centrifugation 92.4% of the ^{51}Cr labelled cells passed through the oil layer. This was repeated with cells immediately after treatment with 10^{-4}M HN2 and the result in Table 20 shows that there was no significant alteration.

7:21:4: DETERMINATION OF THE PERCENTAGE OF $^{86}Rb^{+}$ LABELLED MEDIA PASSING THROUGH THE OIL LAYER ON CENTRIFUGATION

To make sure that no supernatant passed through the oil layer during centrifugation $^{86}Rb^{+}$ labelled media was used in a control experiment with no cells present. The result obtained in Table 19 indicated that there was negligible carryover of label from the layer of medium across the oil layer to the formic acid.

7:21:5: THE EFFECT OF AGENTS ON $^{86}Rb^{+}$ INFLUX

In Figure 51 the percentage of $^{86}Rb^{+}$ contained in the pellet formed by centrifugation divided by the percentage $^{86}Rb^{+}$ contained in the supernatant is plotted against the incubation time of the cell preparation with radiolabelled $^{86}Rb^{+}$. This shows a linearity in the rate of uptake of label up to 30 minutes, then a plateau. This reduction in rate of influx has been reported before (Brown and Lamb 1978) and was considered to be due to significant back efflux of $^{86}Rb^{+}$. The maximum time that $^{86}Rb^{+}$ was in contact with the cells was thus limited to 60 minutes.

7:21:6: EFFECT OF OUABAIN ON THE INFLUX OF ^{86}Rb

On the addition of 10^{-4}M ouabain after 10 minutes of labelling with $^{86}Rb^{+}$ there was an immediate inhibition of influx (Figure 51). The decrease in rate of $^{86}Rb^{+}$ influx suggests that a

TABLE 20

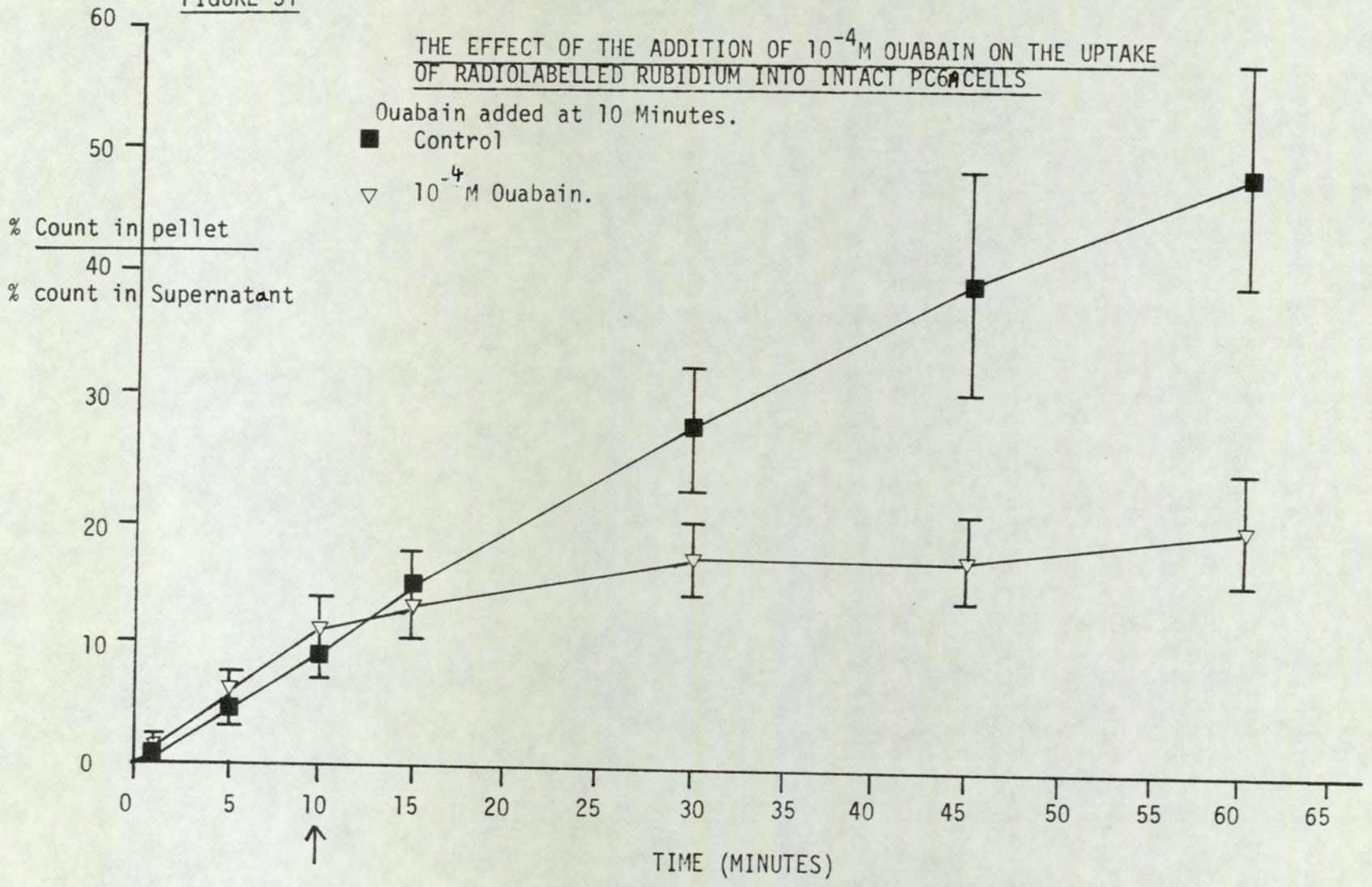
THE PERCENTAGE CARRYOVER OF ^{51}Cr LABELLED PC6 CELLS

COUNTS/10 MINUTES		% CARRYOVER	n = 4 MEAN
853	Pellet	93.84	
56	Supernatant		
846	Pellet	94.74	
47	Supernatant		92.4 ± 2.74
820	Pellet	92.56	
66	Supernatant		
850	Pellet	88.54	
110	Supernatant		
HN2 10^{-4}M			n = 3
816	Pellet	91.48	
109	Supernatant		
833	Pellet	92.49	91.4
109	Supernatant		± 1.14
855	Pellet	90.22	
110	Supernatant		

1. Corrected for background count

FIGURE 51

THE EFFECT OF THE ADDITION OF 10^{-4}M OUABAIN ON THE UPTAKE OF RADIOLABELLED RUBIDIUM INTO INTACT PC6A CELLS



dynamic equilibrium is set up and that the remaining active pump sites pump in $^{86}Rb^{+}$ at the same rate as the ion leaves by passive diffusion. The inhibition of the uptake of $^{86}Rb^{+}$ by ouabain suggests that $^{86}Rb^{+}$ influx is thus via the sodium pump and this technique may therefore show whether an agent effects NaKATPase in intact cells.

7:21:7: EFFECT OF N-ETHYLMALEIMIDE ON THE INFLUX OF $^{86}Rb^{+}$

Figure 52 shows the effect on the influx of $^{86}Rb^{+}$ of the addition after 10 minutes preincubation with N-ethylmaleimide. 10^{-2} M N-ethylmaleimide immediately inhibited $^{86}Rb^{+}$ influx. 10^{-3}M N-ethylmaleimide inhibited the rate of $^{86}Rb^{+}$ influx more slowly, but after 30 minutes there was a similar fall in $^{86}Rb^{+}$ influx. 10^{-4}M N-ethylmaleimide had no significant effect on $^{86}Rb^{+}$ influx over the time period of the assay.

7:21:8: EFFECT OF NITROGEN MUSTARD ON THE INFLUX OF $^{86}Rb^{+}$

The effects of nitrogen mustard on the uptake of $^{86}Rb^{+}$ are shown in Figures 53-59. The time dependence of nitrogen mustard action on enzyme activity in a broken cell preparation (7:15:1:) suggested that to show inhibition of $^{86}Rb^{+}$ influx the washed PC6A cells should be preincubated with nitrogen mustard prior to the addition of $^{86}Rb^{+}$. This preincubation ranges from zero to 6 hours and in all cases the control activity is shown for comparison.

On the immediate addition of 10^{-2}M nitrogen mustard (Figure 53) the rate of $^{86}Rb^{+}$ influx was inhibited within 15 minutes. The same effect was observed with 10^{-3}M nitrogen mustard, but the time of onset of inhibition was delayed to 30 minutes. 10^{-4}M - 10^{-6}M nitrogen mustard had no significant effect without a preincubation period.

The dose response relationship between the concentration of nitrogen mustard and the inhibition of the rate of $^{86}Rb^{+}$ influx was determined after preincubation of nitrogen mustard with intact cells for 1, 2, 4 and 6 hours. The longer the preincubation time, the lower was the concentration of

FIGURE 52

THE EFFECT OF THE ADDITION OF N-ETHYLMALEIMIDE ON THE RUBIDIUM INFLUX INTO PC6A CELLS

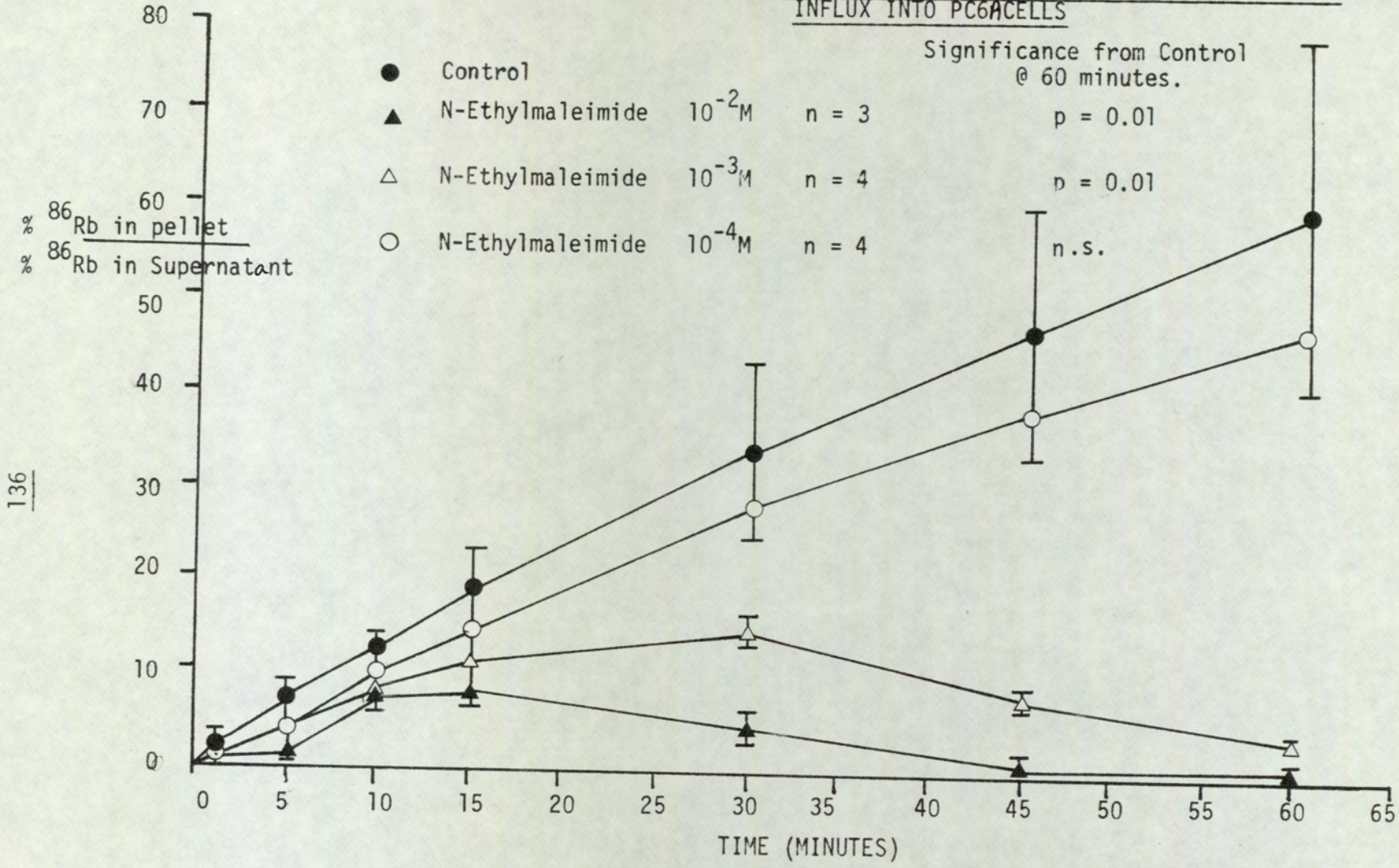
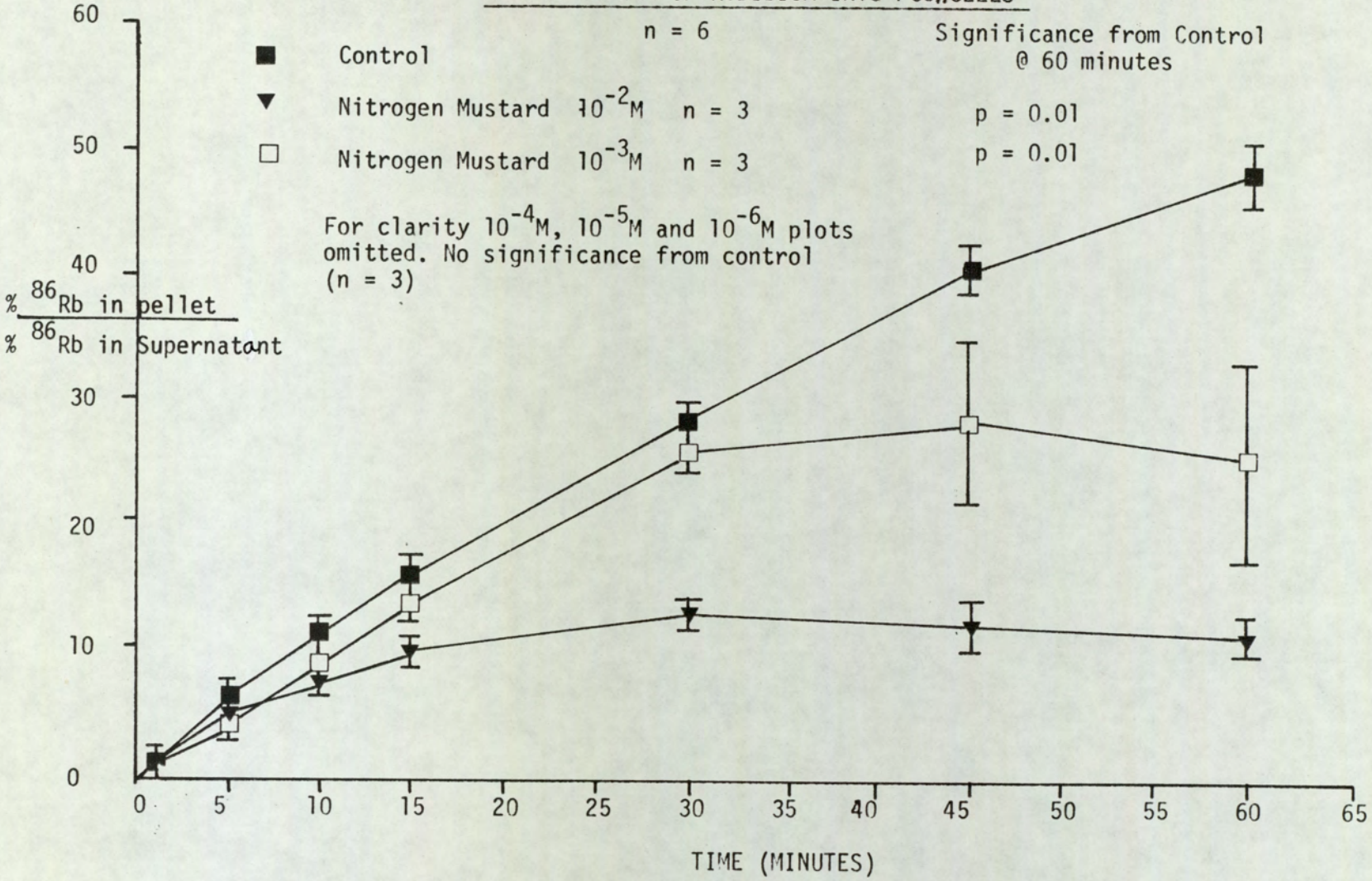


FIGURE 53

THE EFFECT OF THE ADDITION OF NITROGEN MUSTARD AT TIME ZERO ON THE INFLUX OF RUBIDIUM INTO PC6A CELLS



nitrogen mustard required for inhibition to be significantly different from control ($p>0.05$) Figures 54-59.

The control activity after 6 hours pretreatment fell by approximately 20%, which corresponded to an approximate 40% decrease in cell viability as determined by the *in vitro in vivo* ascitic cell suspension bioassay (Table 12). Therefore the preincubation was not increased over this time due to the problems of maintenance of cells in a viable state in the media used (See Table 12).

Figures 58-59 show the results as a percentage activity $^{86}Rb^{+}$ influx compared to the control. Nitrogen mustard at various concentrations inhibited $^{86}Rb^{+}$ influx, and by inference, the sodium pump, in a time dependent manner, 10^{-2}M nitrogen mustard drastically inhibited $^{86}Rb^{+}$ influx to reach a maximum effect within 1 hour. 10^{-3}M nitrogen mustard also had a rapid effect, but up to 4 hours preincubation did not increase the effect above an 85% inhibition. The same effects were observed at lower doses, until at 10^{-6}M nitrogen mustard there was no effect over the time period measured.

1.24×10^{-6}M, a concentration which gave a 90% inhibition of cell growth as measured by the *in vitro in vivo* assay with an ascitic tumour equivalent to an apparent three log kill (Figure 43) had no effect on the influx of $^{86}Rb^{+}$ even after 6 hours incubation.

7:21:9: EFFECT OF N,N DIMETHYLCHLOROETHYLAMINE ON $^{86}Rb^{+}$ INFLUX INTO PC6A CELLS

The monofunctional analogue of nitrogen mustard showed a similar pattern of inhibition but was less potent (Figures 60-65). At a preincubation of zero hours, up to 10^{-3}M of this agent had no effect. As the preincubation time was increased there was a concentration dependent inhibition of $^{86}Rb^{+}$ influx, (Figure 65).

FIGURE 54 THE EFFECT OF THE ADDITION OF NITROGEN MUSTARD ONE HOUR PRIOR TO THE ADDITION OF THE RUBIDIUM, ON THE RUBIDIUM INFLUX INTO PC6A CELLS.

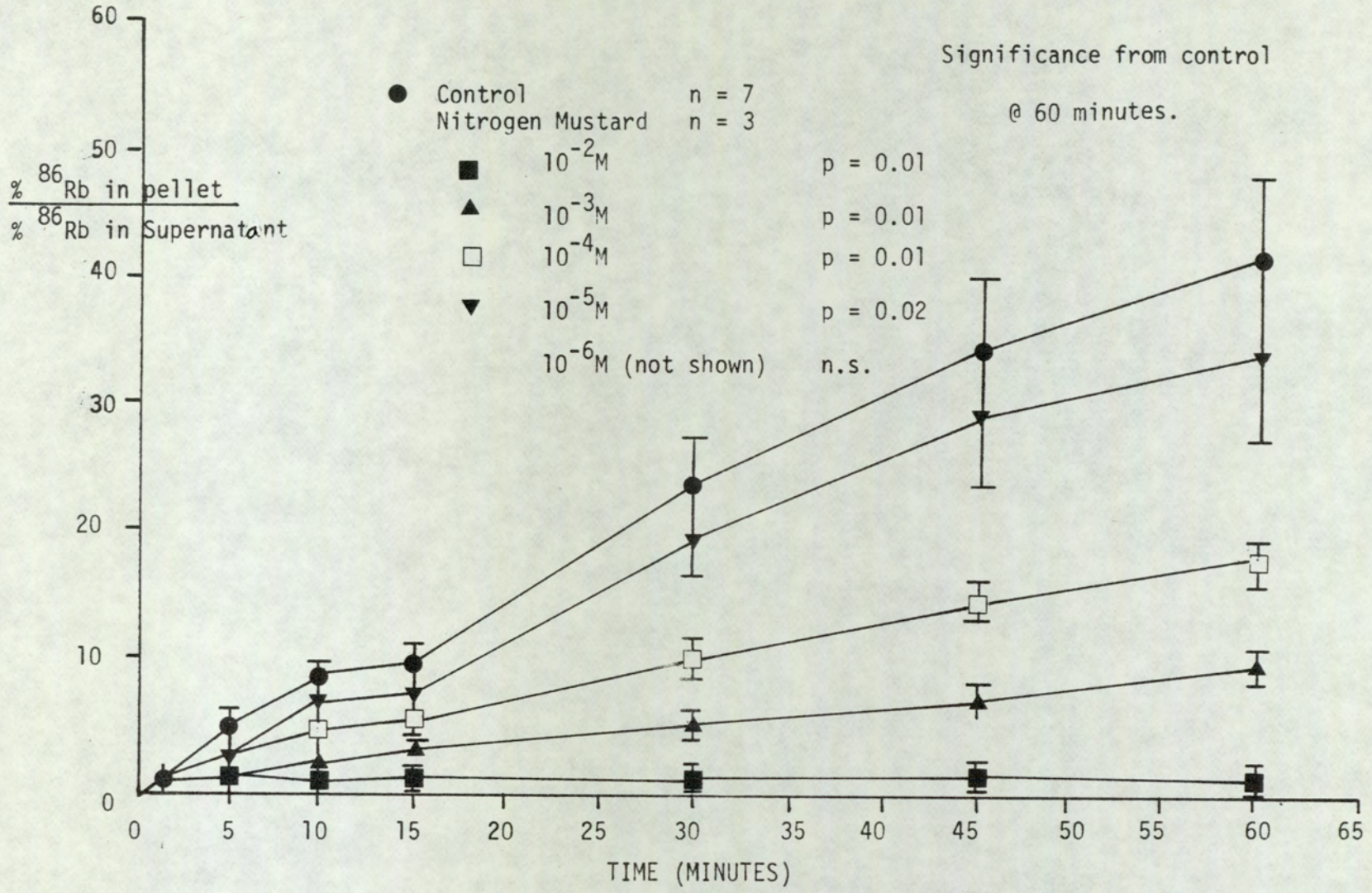


FIGURE 55 THE EFFECT OF ADDITION OF NITROGEN MUSTARD TWO HOURS PRIOR TO THE ADDITION OF RUBIDIUM ON THE UPTAKE OF RUBIDIUM INTO PC6A CELLS

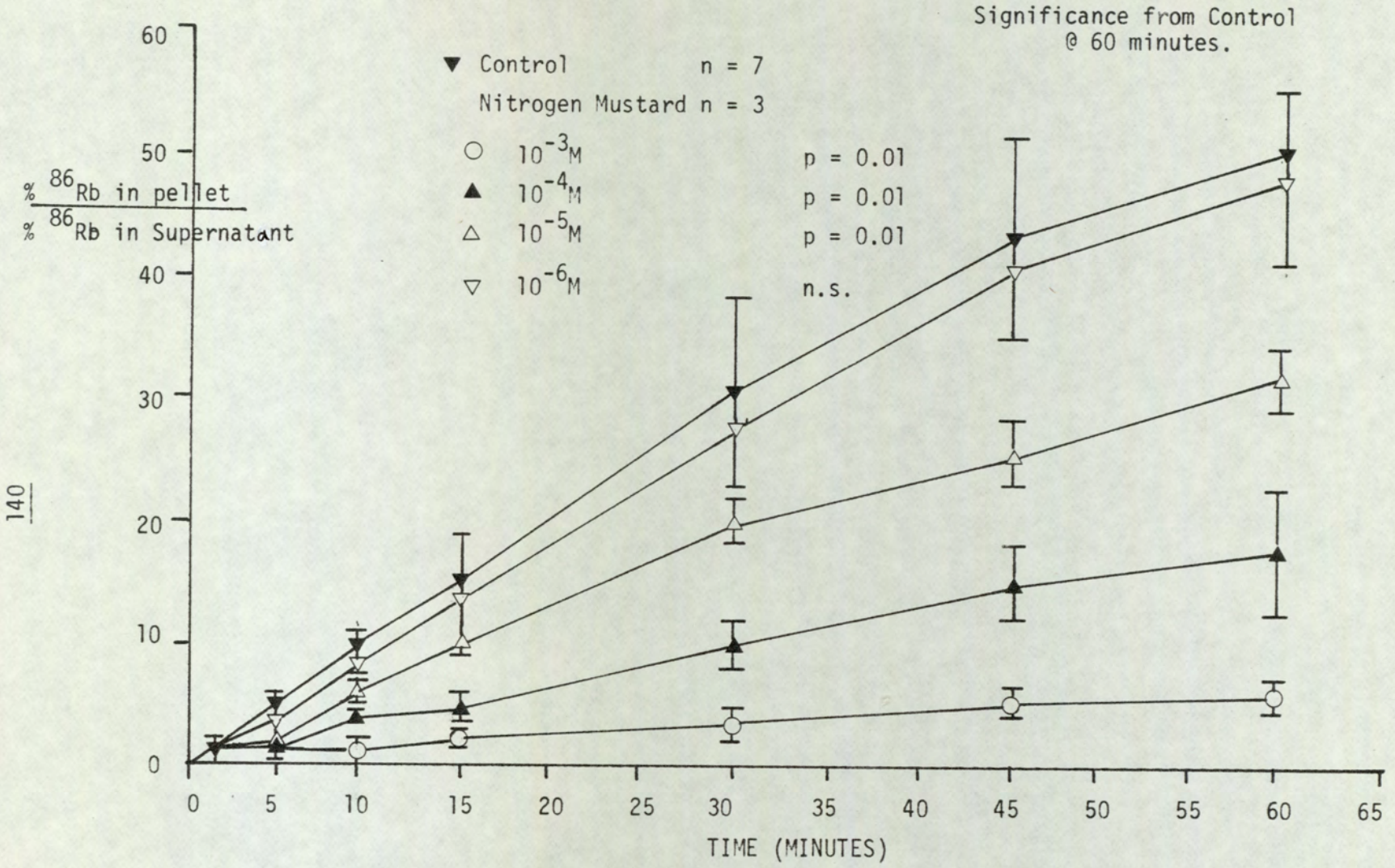


FIGURE 56 THE EFFECT OF THE ADDITION OF NITROGEN MUSTARD FOUR HOURS PRIOR TO THE ADDITION OF RUBIDIUM, ON THE UPTAKE OF RUBIDIUM BY PC6ACELLS.

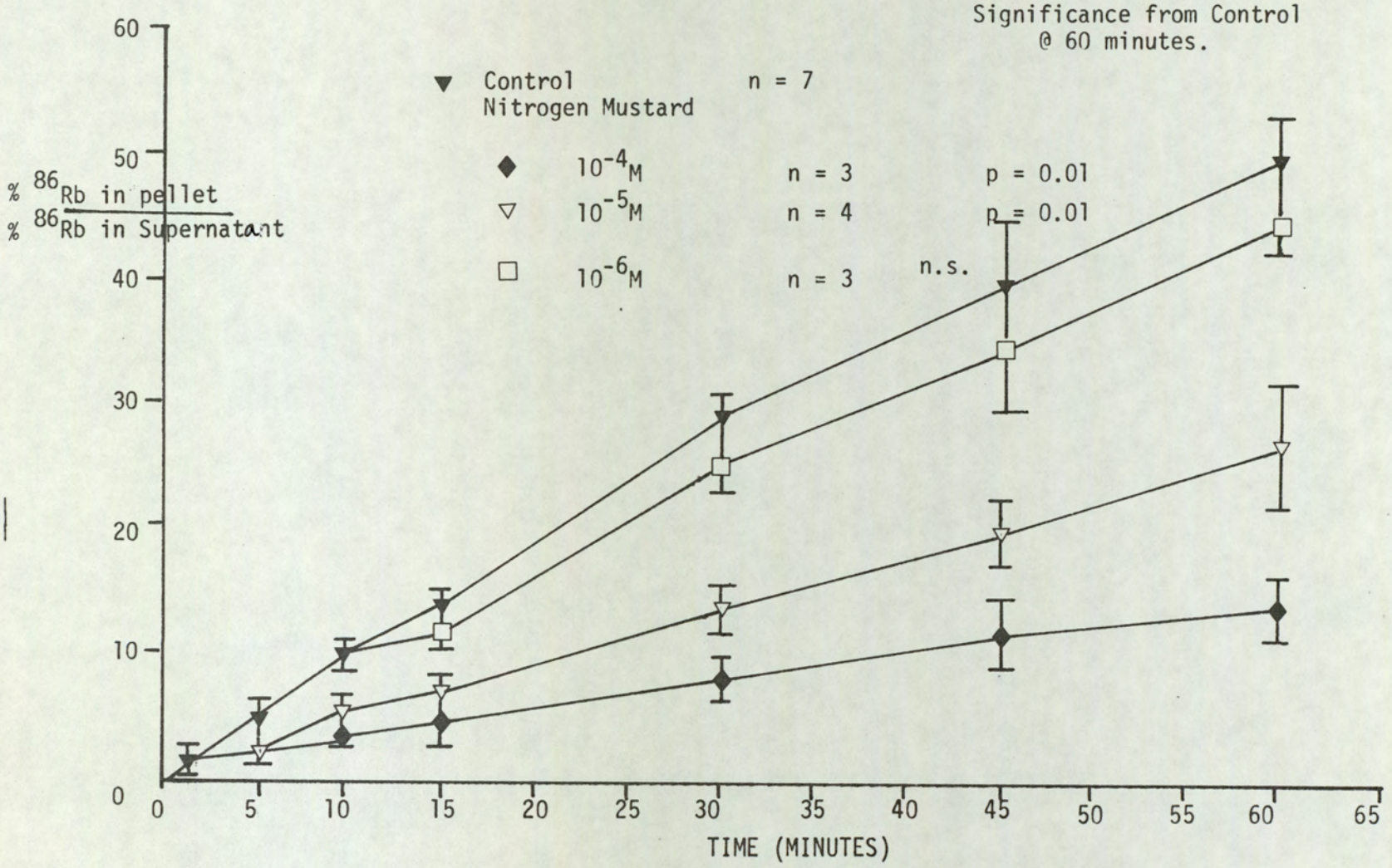


FIGURE 57

THE EFFECT OF THE ADDITION OF NITROGEN MUSTARD 6 HOURS PRIOR TO THE ADDITION OF RUBIDIUM ON THE INFLUX OF RUBIDIUM INTO PC6ACELLS

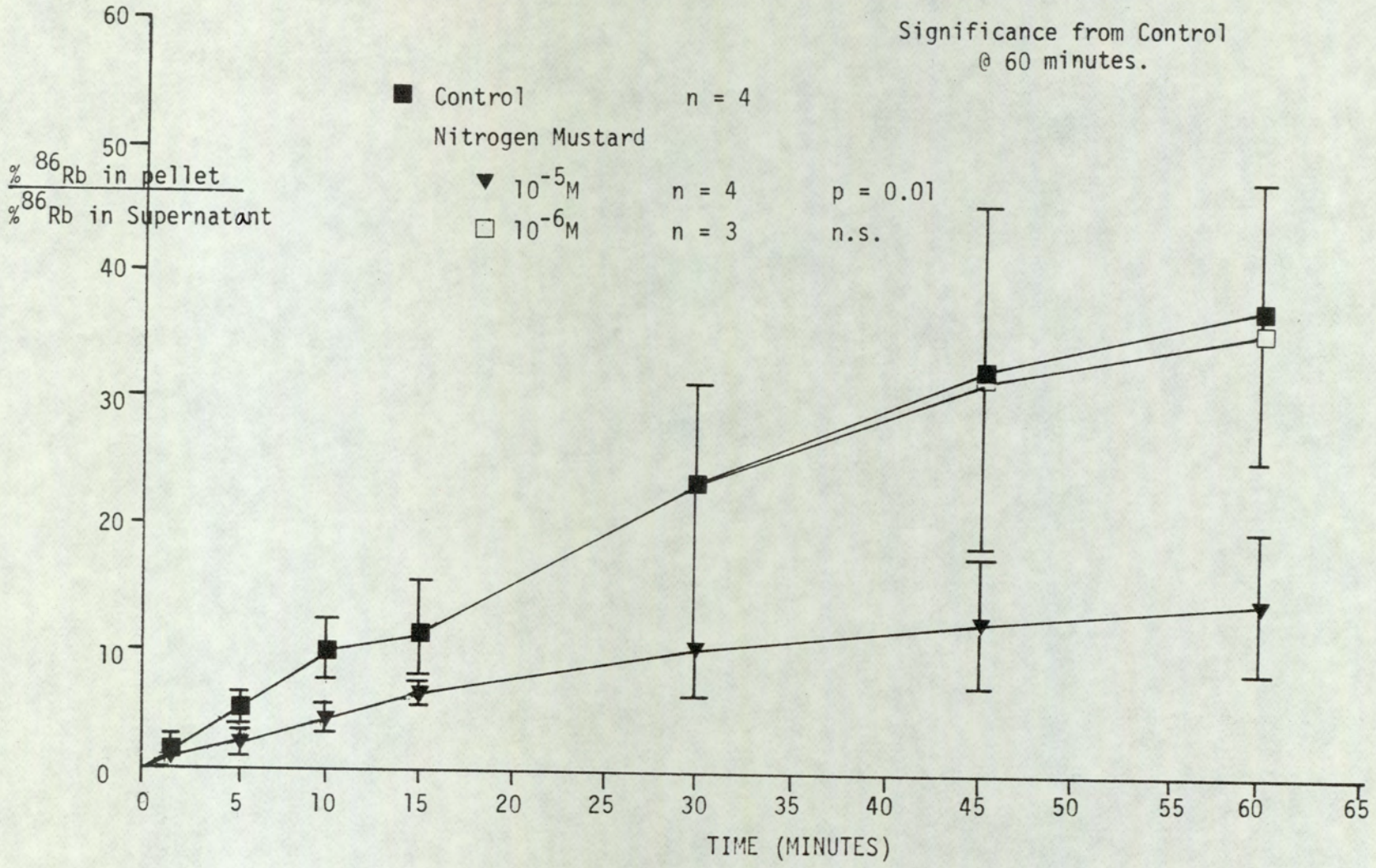


FIGURE 58

THE EFFECT OF INCUBATION TIME WITH NITROGEN MUSTARD ON THE PERCENTAGE ACTIVITY AS MEASURED BY RUBIDIUM INFLUX IN COMPARISON TO THE CONTROL.

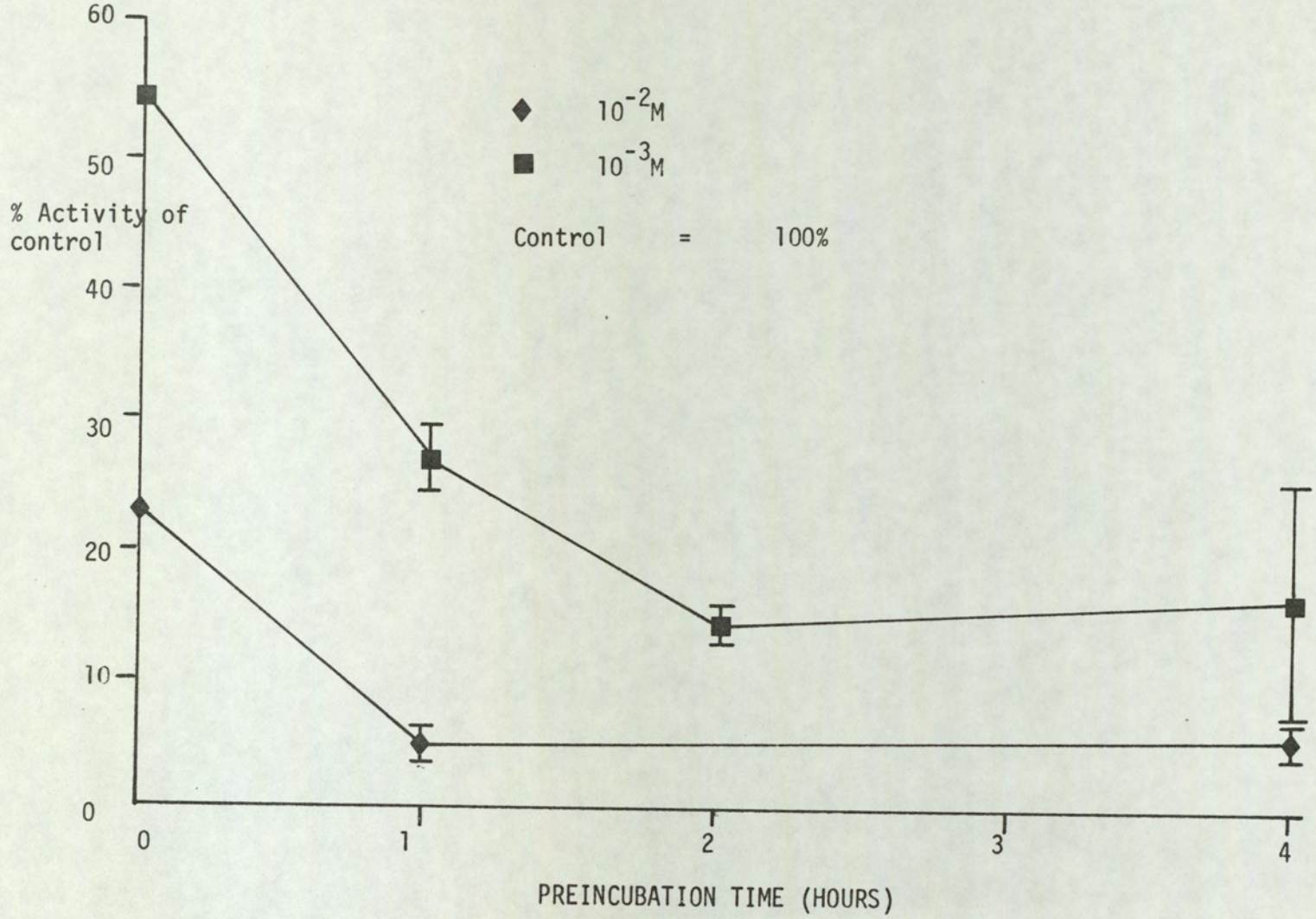


FIGURE 59

THE EFFECT OF INCUBATION TIME WITH NITROGEN MUSTARD ON THE PERCENTAGE ACTIVITY AS MEASURED BY RUBIDIUM INFLUX IN COMPARISON TO THE CONTROL

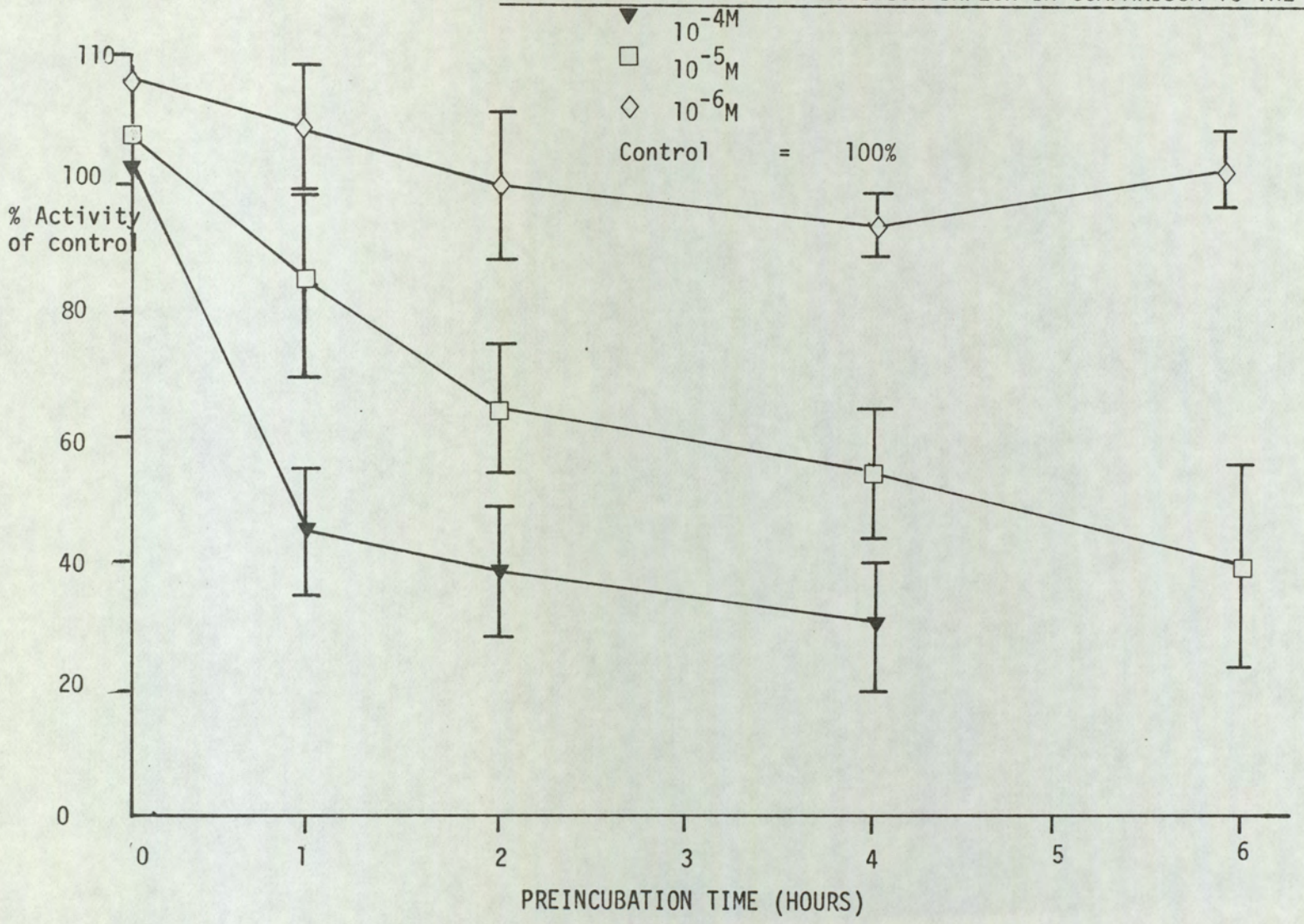


FIGURE 60

THE EFFECT OF THE ADDITION OF N,N DIMETHYLCHLORO ETHYLAMINE AT TIME ZERO PRIOR TO THE ADDITION OF RUBIDIUM ON RUBIDIUM INFLUX INTO PC6A CELLS.

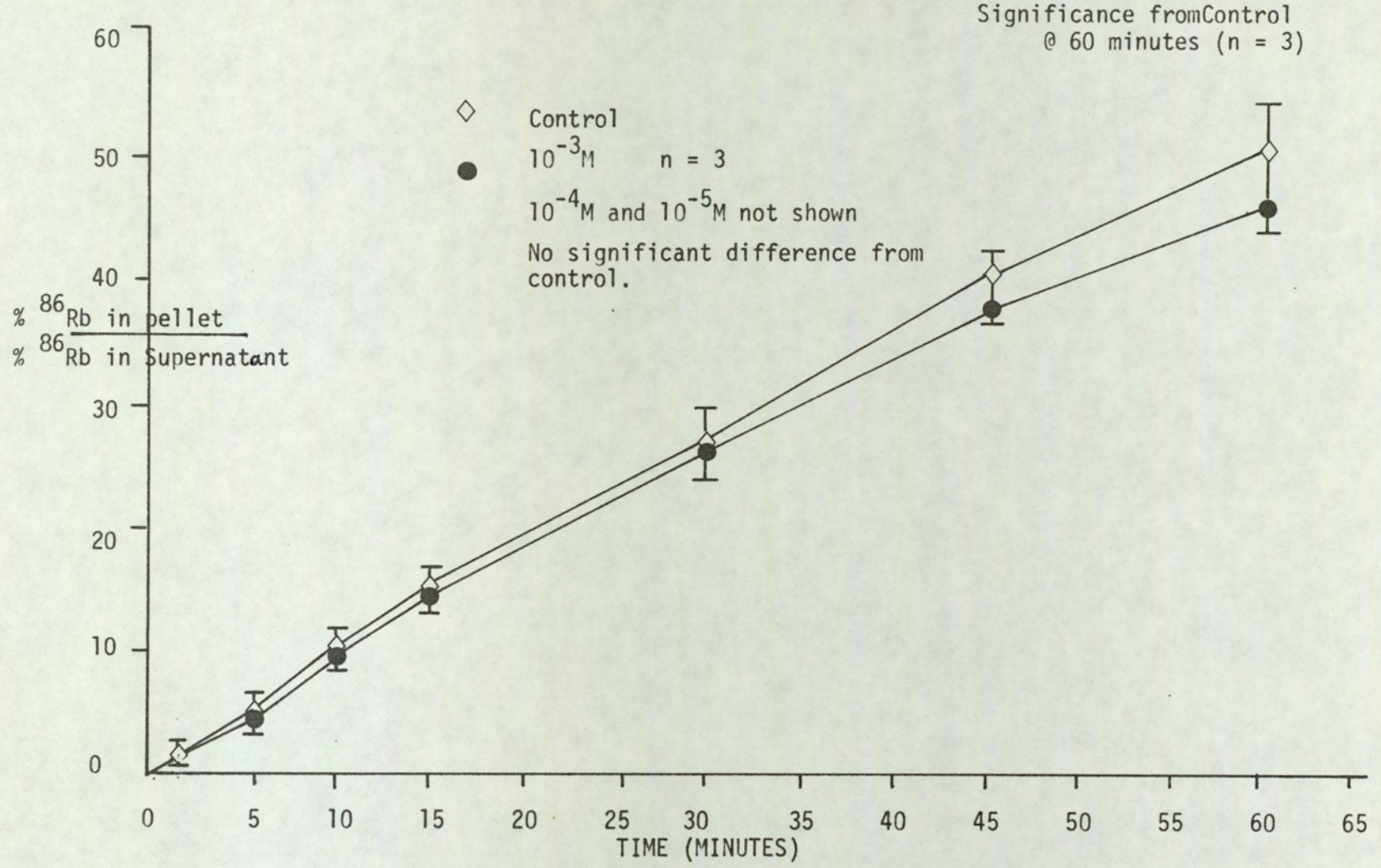


FIGURE 61

THE EFFECT OF THE ADDITION OF N, N DIMETHYLCHLOROETHYLAMINE ONE HOUR PRIOR TO THE ADDITION OF RUBIDIUM, ON THE RUBIDIUM INFLUX INTO PC6A CELLS

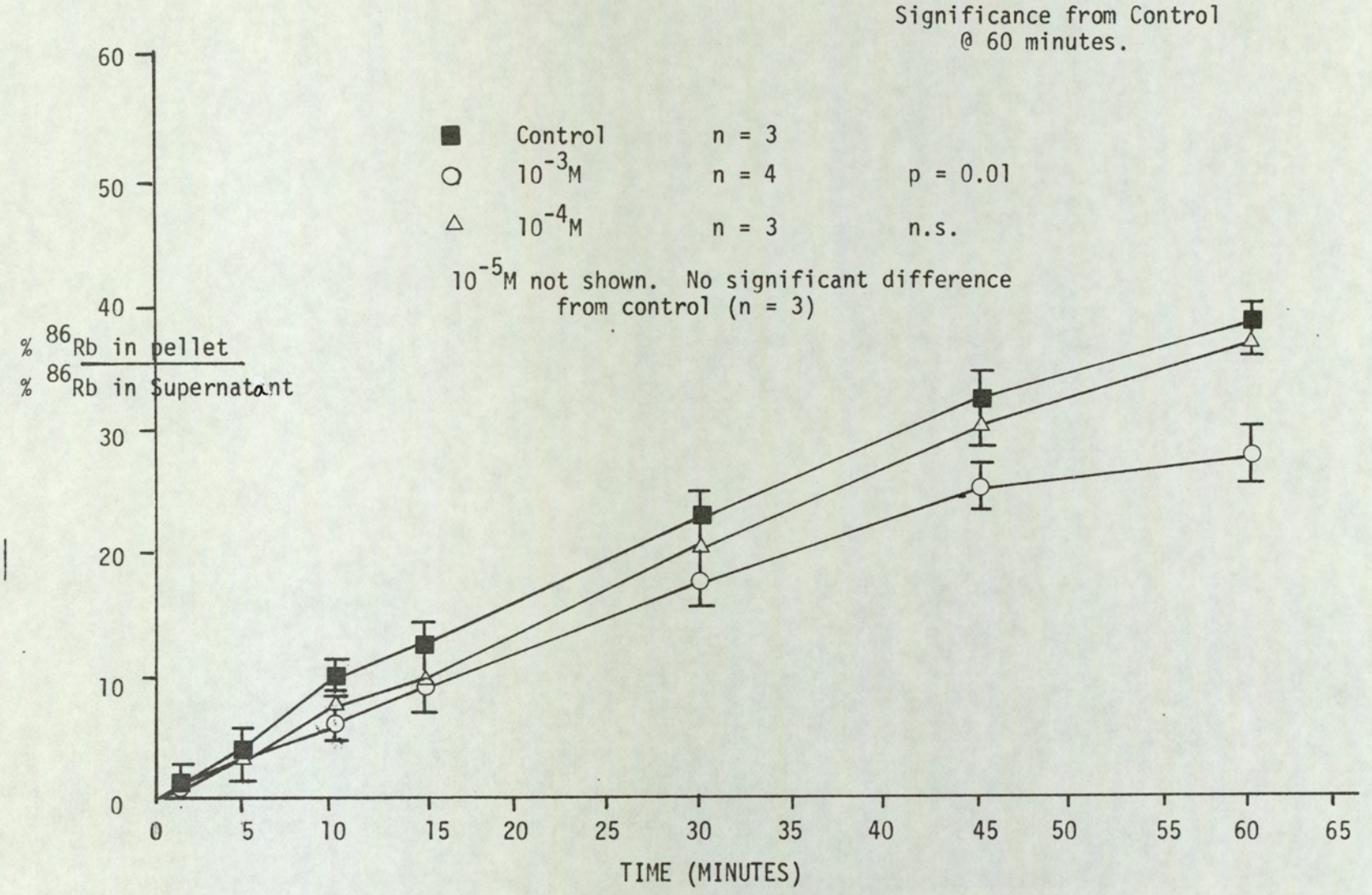


FIGURE 62

THE EFFECT OF THE ADDITION OF N, N-DIMETHYLCHLOROETHYLAMINE TWO HOURS PRIOR TO THE ADDITION OF RUBIDIUM, ON RUBIDIUM INFLUX INTO PC6A CELLS

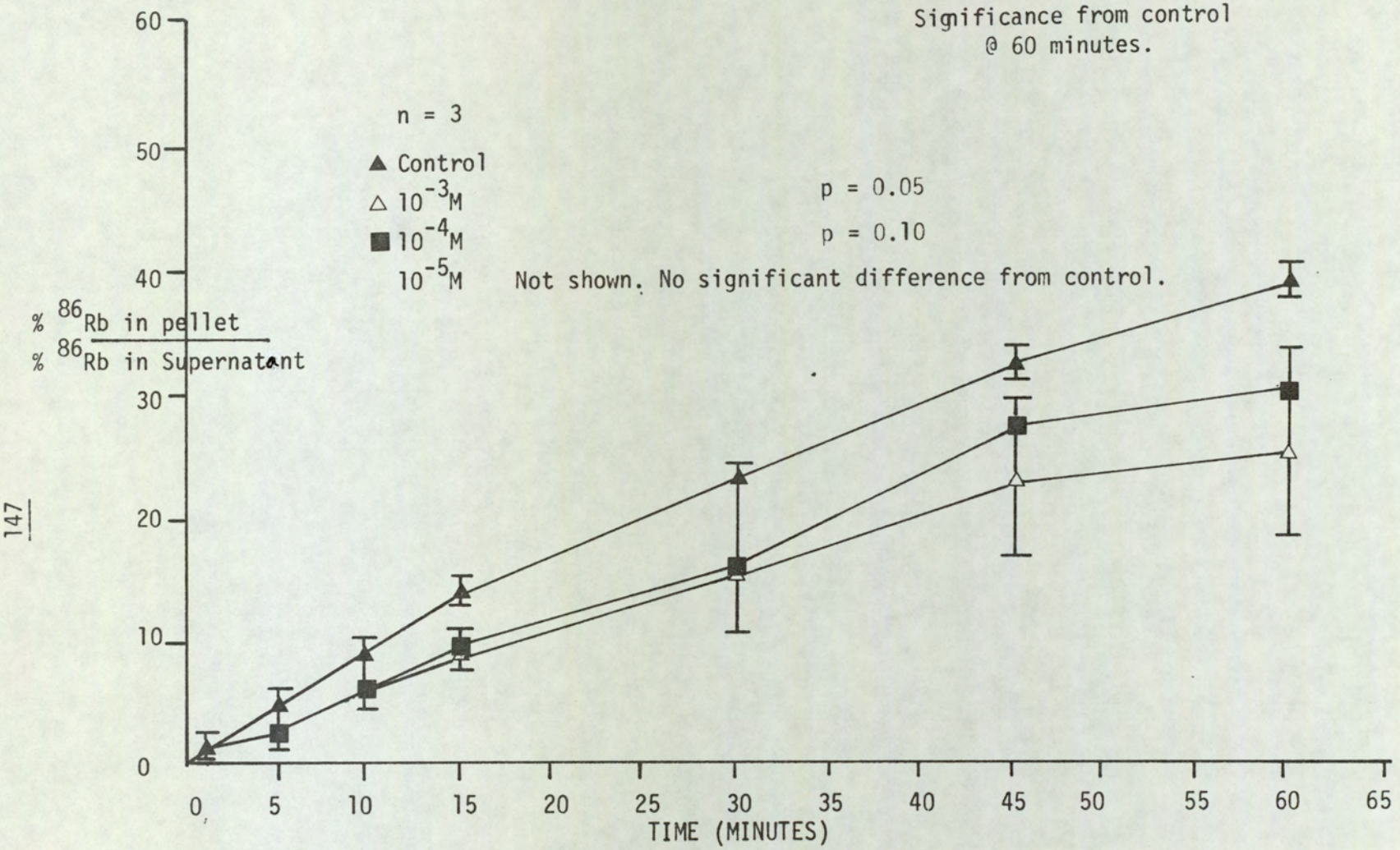


FIGURE 63

THE EFFECT OF THE ADDITION OF N, N DIMETHYLCHLOROETHYLAMINE FOUR HOURS PRIOR TO THE ADDITION OF RUBIDIUM, ON THE INFLUX OF RUBIDIUM INTO PC6A CELLS

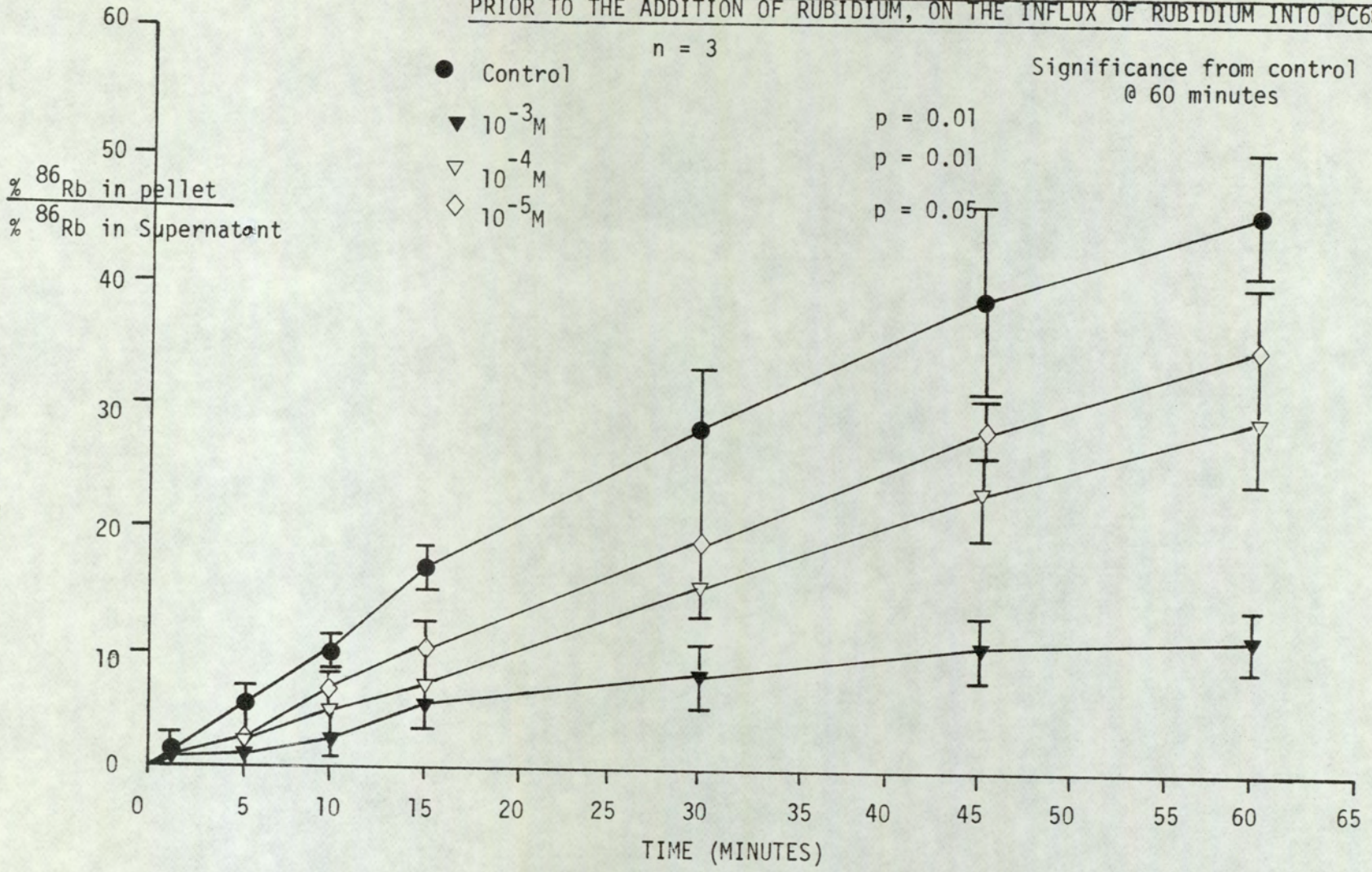


FIGURE 64

THE EFFECT OF THE ADDITION OF N, N DIMETHYLCHLOROETHYLAMINE SIX HOURS PRIOR TO THE ADDITION OF RUBIDIUM, ON THE INFLUX OF RUBIDIUM INTO PC6A CELLS

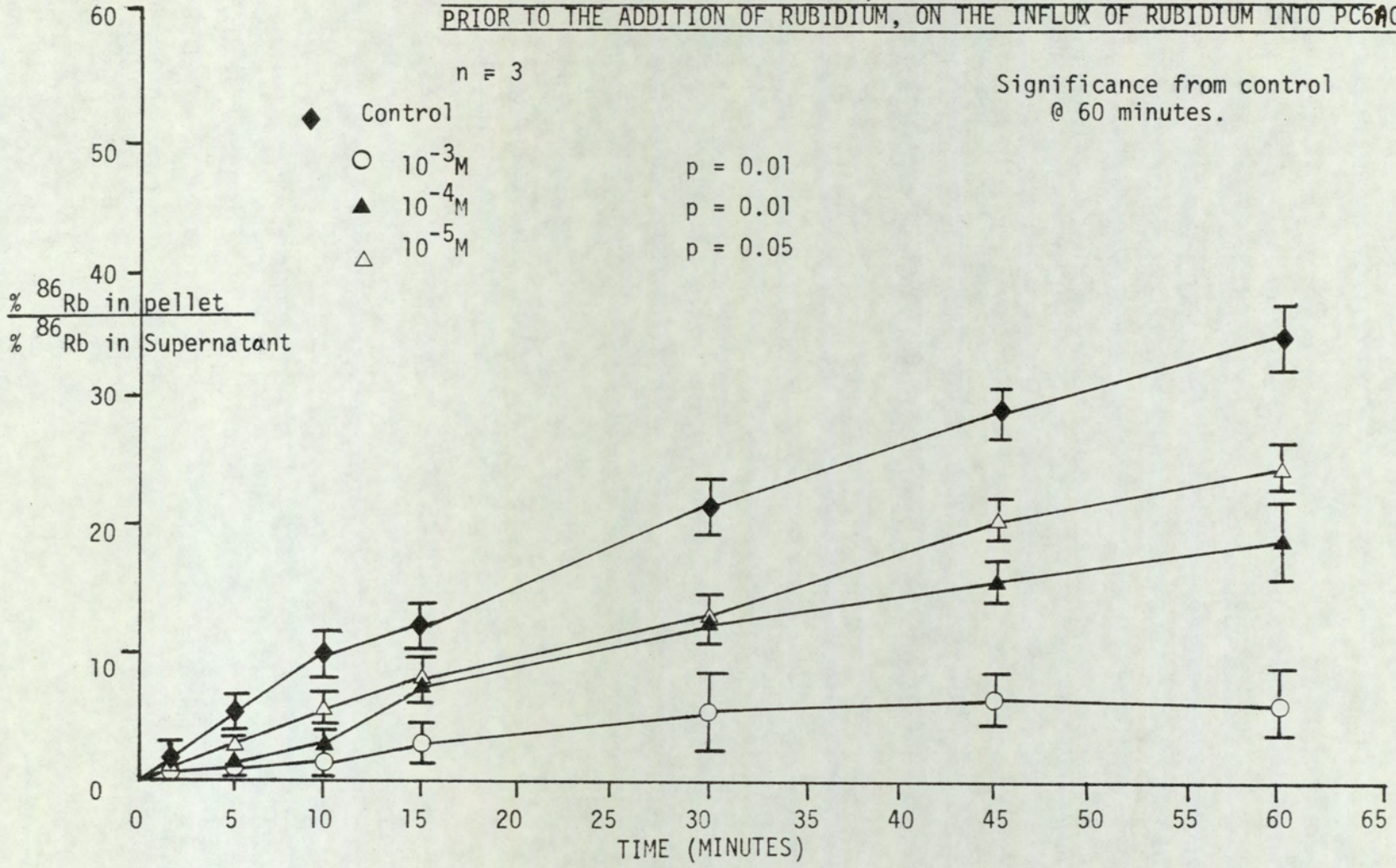
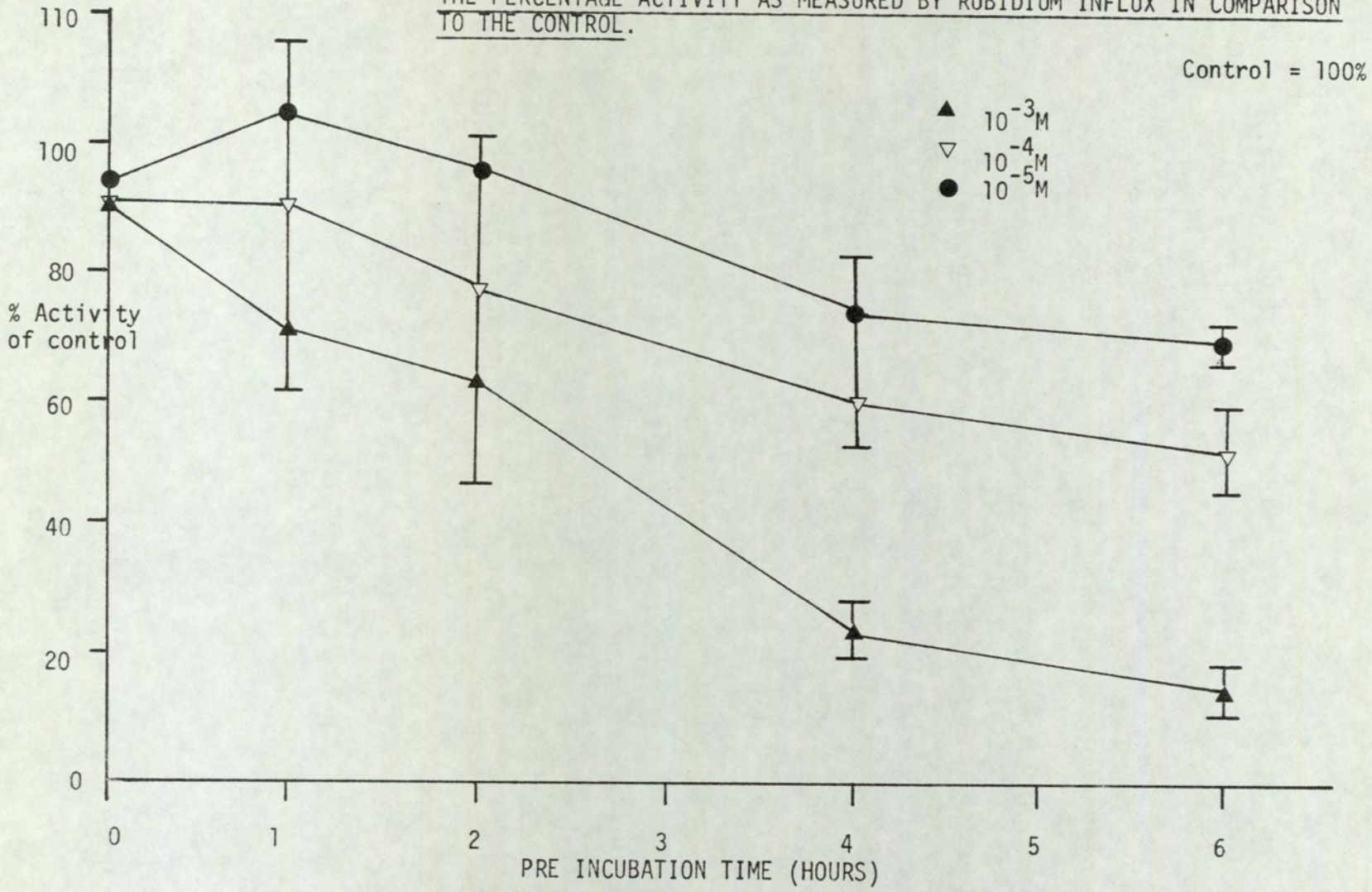


FIGURE 65

THE EFFECT OF INCUBATION TIME WITH N, N-DIMETHYLCHLOROETHYLAMINE ON THE PERCENTAGE ACTIVITY AS MEASURED BY RUBIDIUM INFLUX IN COMPARISON TO THE CONTROL.



A time dependent inhibition was apparent at 10^{-3}, 10^{-4} and 10^{-5}M N,N-dimethylchloroethylamine, as for nitrogen mustard, but the monofunctional analogue was approximately 10 times less potent (Figure 72).

7:21:10: EFFECT OF HYDROLYSED NITROGEN MUSTARD ON THE INFLUX OF $^{86}Rb^{+}$

Figures 66-71 show the effect of hydrolysed nitrogen mustard on the influx of $^{86}Rb^{+}$. Figure 72 compares the percentage activity after 4 hours preincubation with nitrogen mustard, its monofunctional analogue and hydrolysed nitrogen mustard. It is shown that the hydrolysed analogue had little toxicity in comparison to the other agents, and also that the monofunctional analogue was less toxic than the difunctional nitrogen mustard. At a concentration of 10^{-4}M of both the hydrolysed and monofunctional analogues of nitrogen mustard, there was a significant difference from the inhibition caused by nitrogen mustard ($p=0.01$).

7:21:11: EFFECT OF CYCLOPHOSPHAMIDE ON THE INFLUX OF $^{86}Rb^{+}$

Figure 73 shows the effect of cyclophosphamide on the influx of $^{86}Rb^{+}$ after a 2 hour preincubation. 10^{-3}M cyclophosphamide had no significant effect on $^{86}Rb^{+}$ influx. This compared with an 85% inhibition of $^{86}Rb^{+}$ influx with 10^{-3}M nitrogen mustard after 2 hour preincubation (Figure 55).

7:21:12: THE EFFECT OF DIBUTRYL CYCLIC AMP ON THE INFLUX OF $^{86}Rb^{+}$

Figure 74 shows the effect of 10^{-6}M dibutryl cAMP on $^{86}Rb^{+}$ influx. These results show that this concentration had no significant effect on $^{86}Rb^{+}$ influx after two hours preincubation.

FIGURE 66

THE EFFECT OF THE ADDITION OF HYDROLYSED NITROGEN MUSTARD AT ZERO TIME ON THE INFLUX OF RUBIDIUM INTO PC6A CELLS.

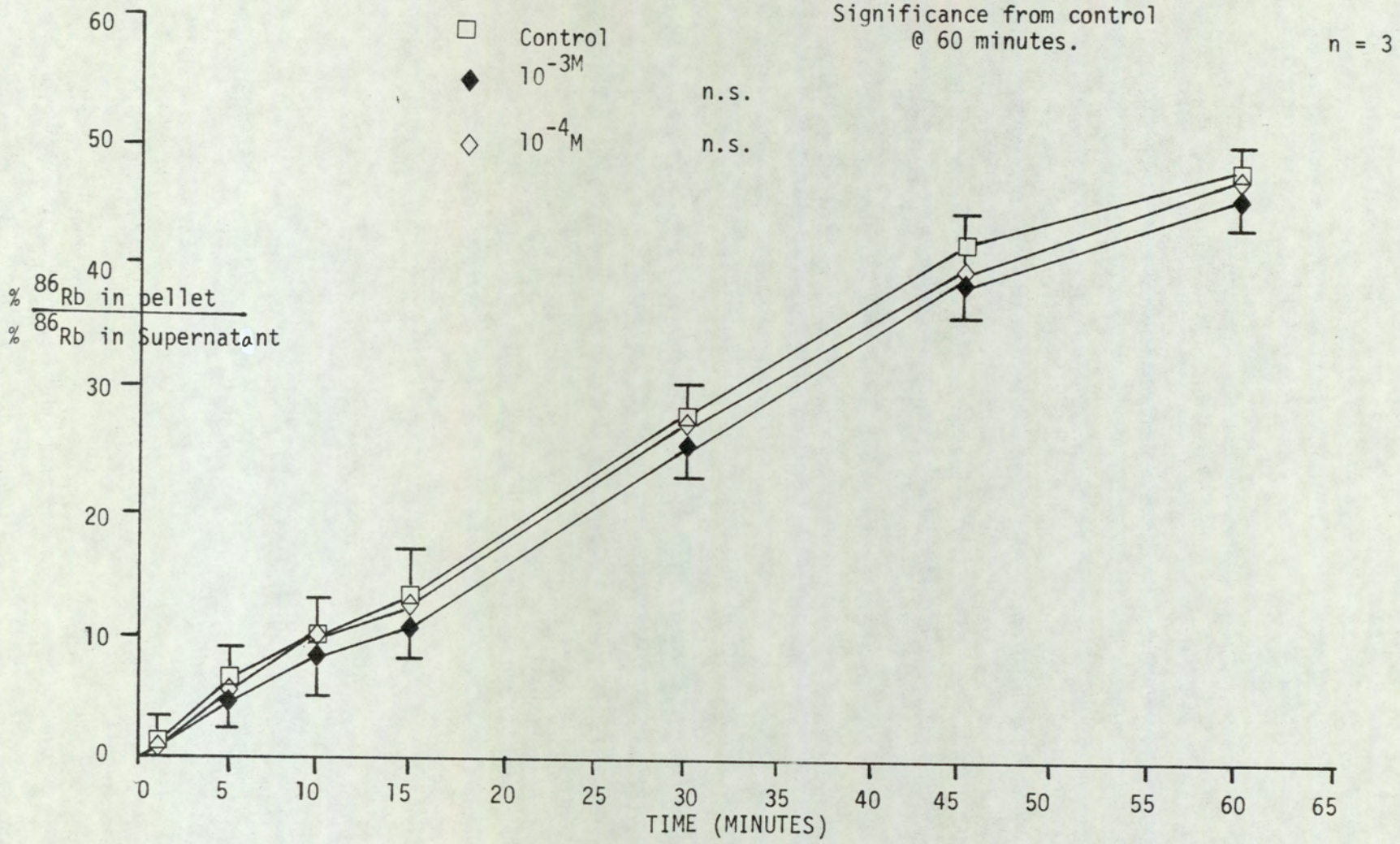


FIGURE 67 THE EFFECT OF THE ADDITION OF HYDROLYSED NITROGEN MUSTARD ONE HOUR PRIOR TO THE ADDITION OF RUBIDIUM ON THE RUBIDIUM INFLUX INTO PC6A CELLS.

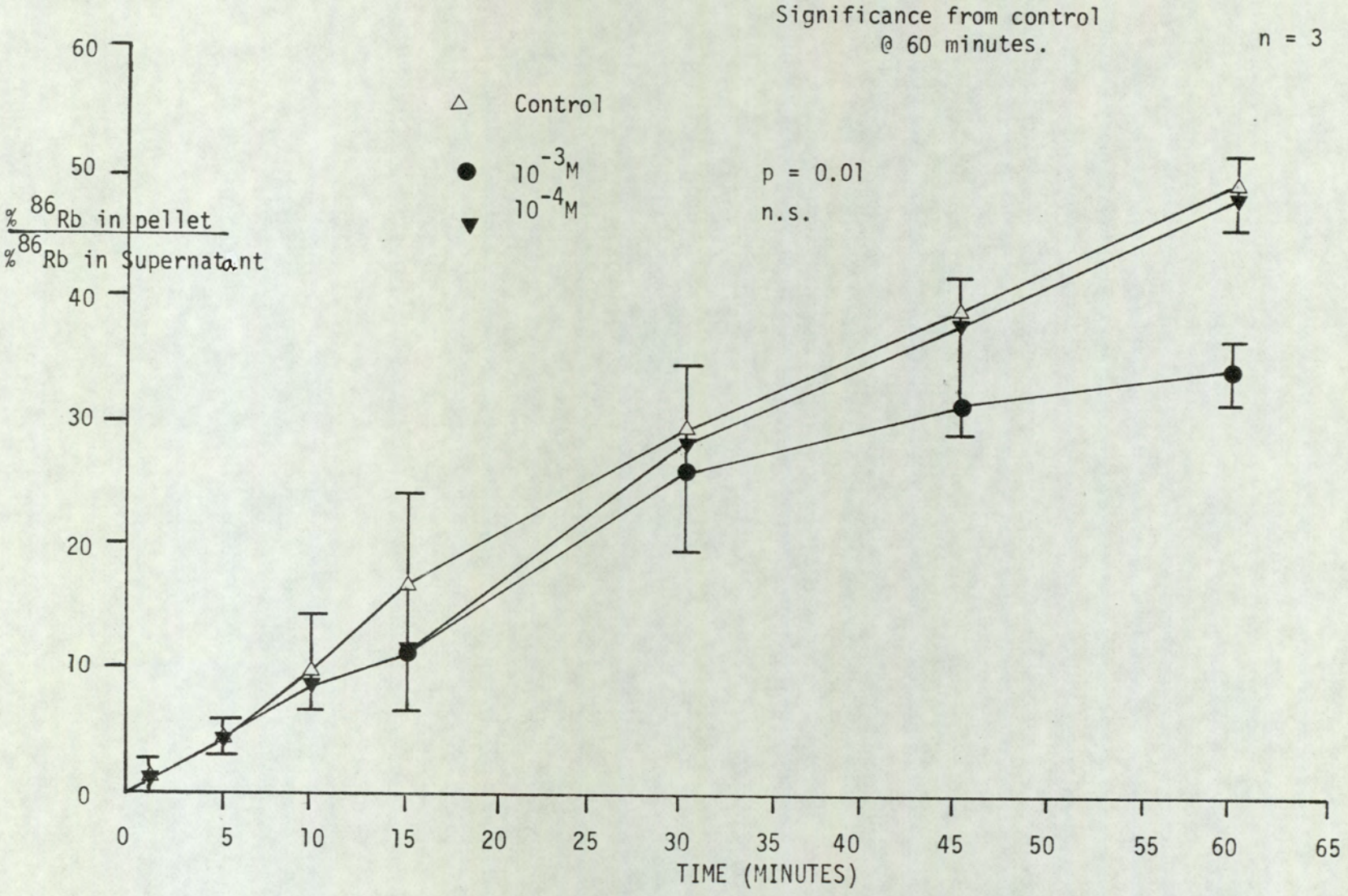


FIGURE 68

THE EFFECT OF THE ADDITION OF HYDROLYSED NITROGEN MUSTARD TWO HOURS PRIOR TO THE ADDITION OF RUBIDIUM ON RUBIDIUM INFLUX INTO PC6A CELLS

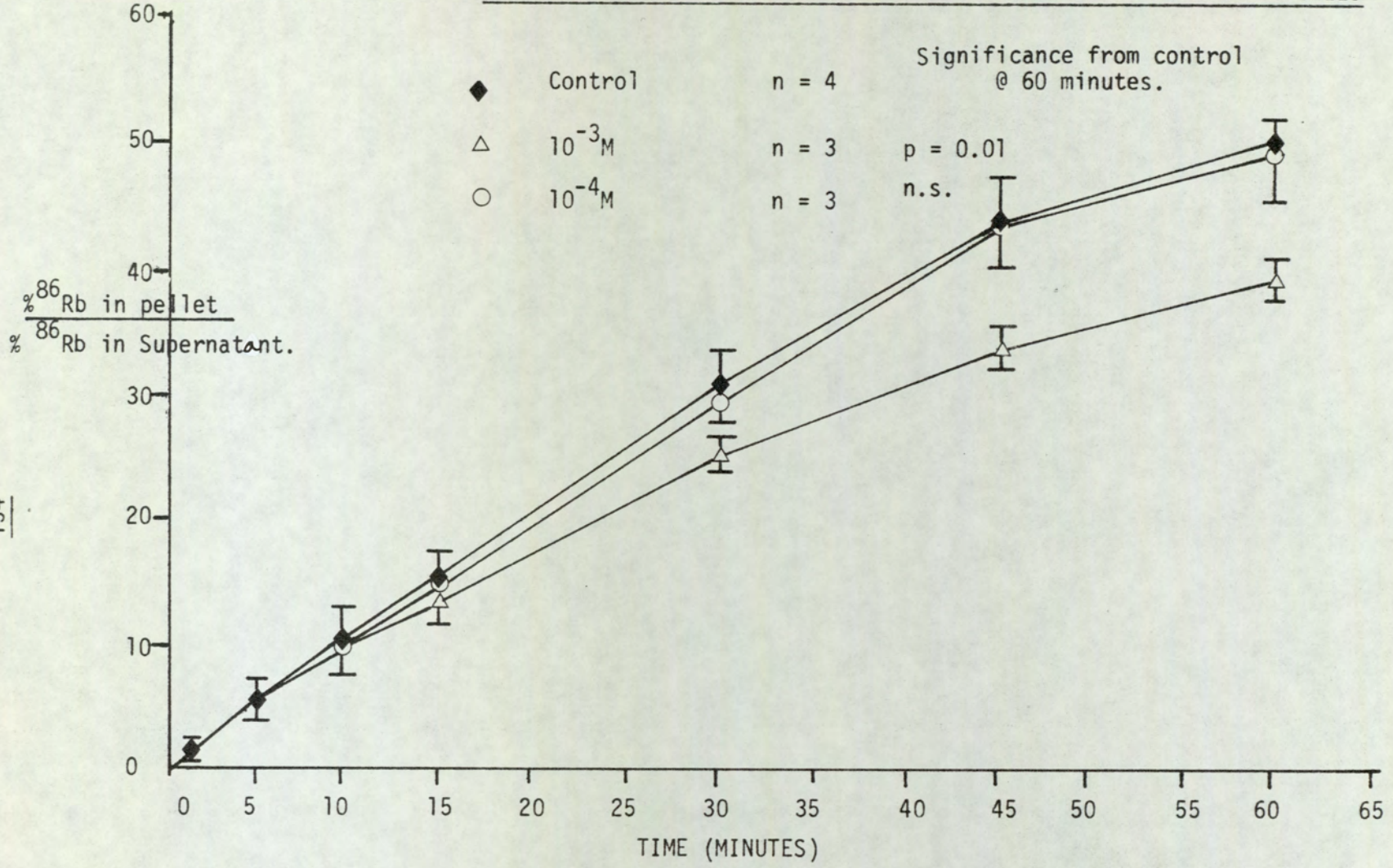


FIGURE 69

THE EFFECT OF THE ADDITION OF HYDROLYSED NITROGEN MUSTARD FOUR HOURS PRIOR TO THE ADDITION OF RUBIDIUM ON THE INFLUX OF RUBIDIUM INTO PC6A CELLS

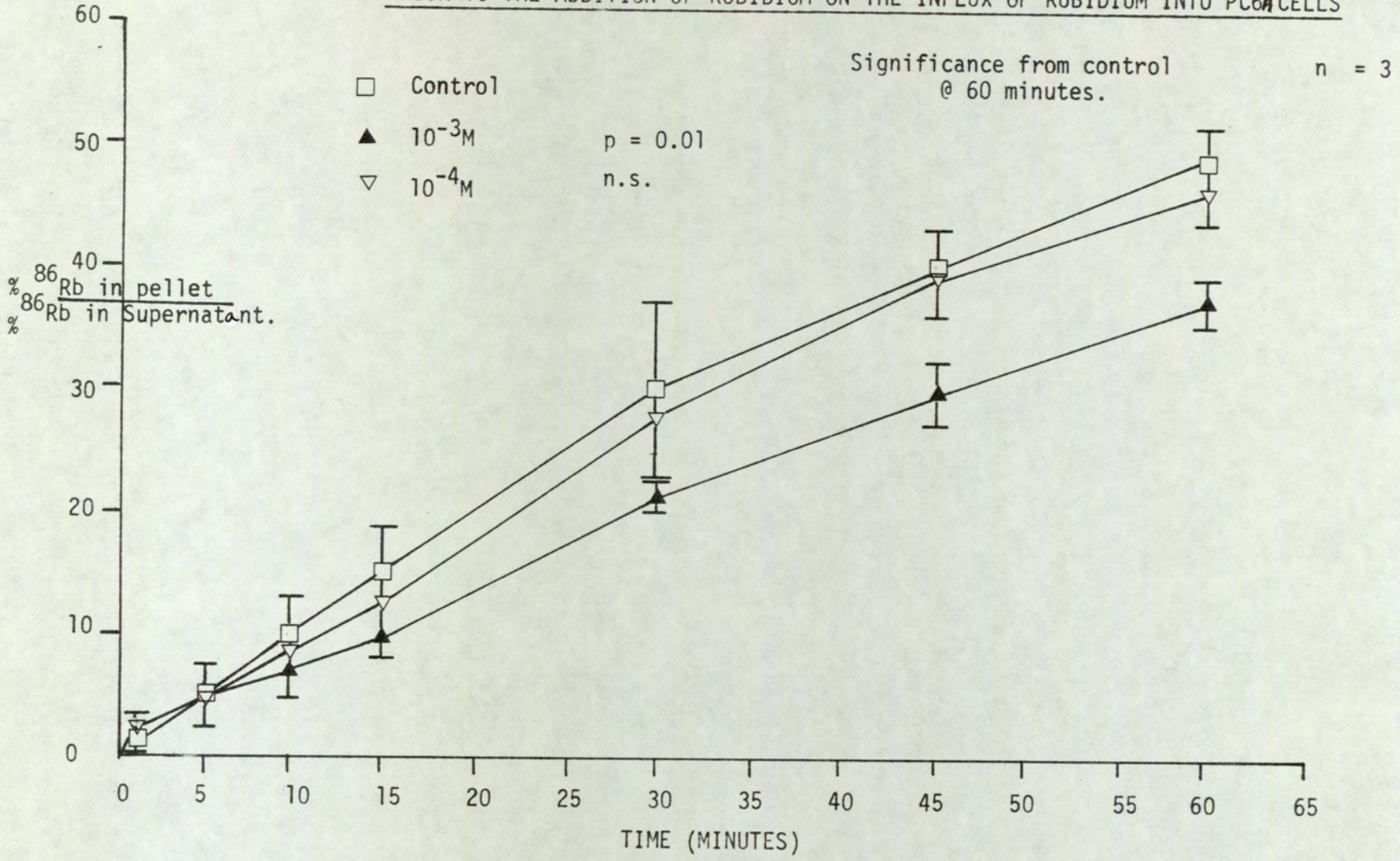


FIGURE 70

THE EFFECT OF THE ADDITION OF HYDROLYSED NITROGEN MUSTARD SIX HOURS PRIOR TO THE ADDITION OF RUBIDIUM, ON THE UPTAKE OF RUBIDIUM INTO PC6A CELLS

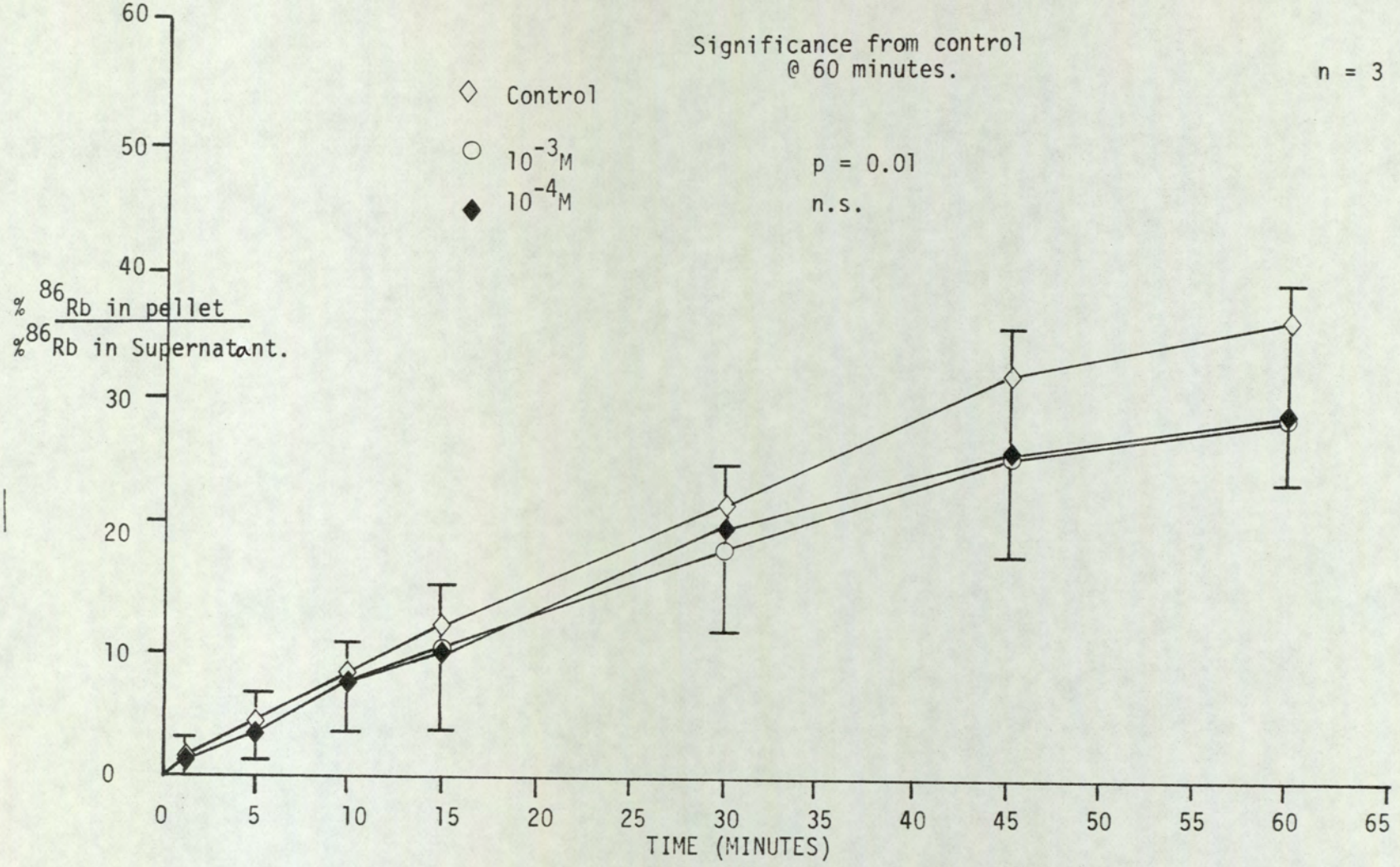


FIGURE 71

THE EFFECT OF INCUBATION TIME WITH HYDROLYSED NITROGEN MUSTARD ON THE PERCENTAGE ACTIVITY AS MEASURED BY RUBIDIUM INFLUX IN COMPARISON TO THE CONTROL

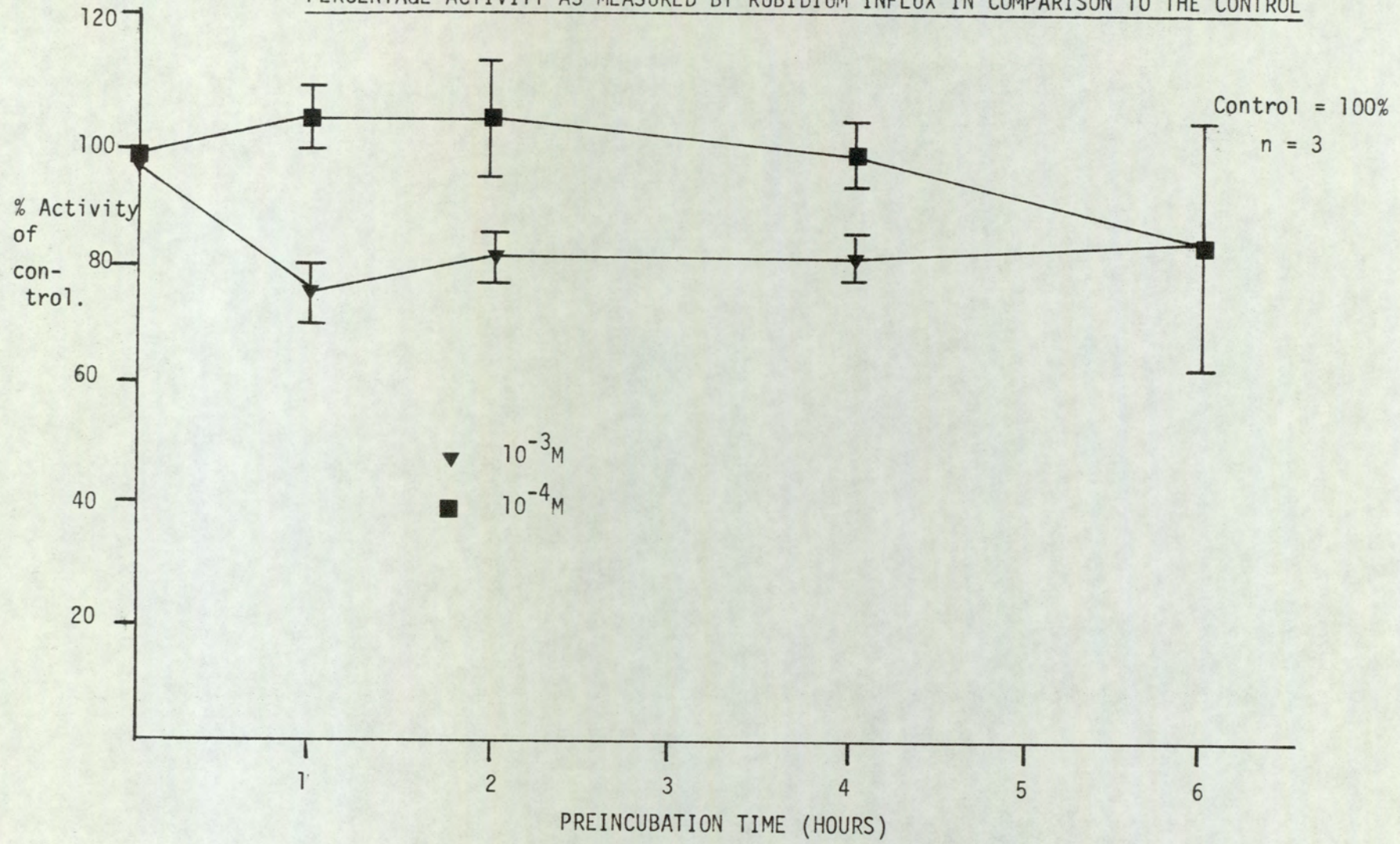


FIGURE 72

A COMPARISON OF THE EFFECT OF A FOUR HOUR INCUBATION WITH NITROGEN MUSTARD AND TWO ANALOGUES ON THE INFLUX OF RUBIDIUM INTO PC6A CELLS.

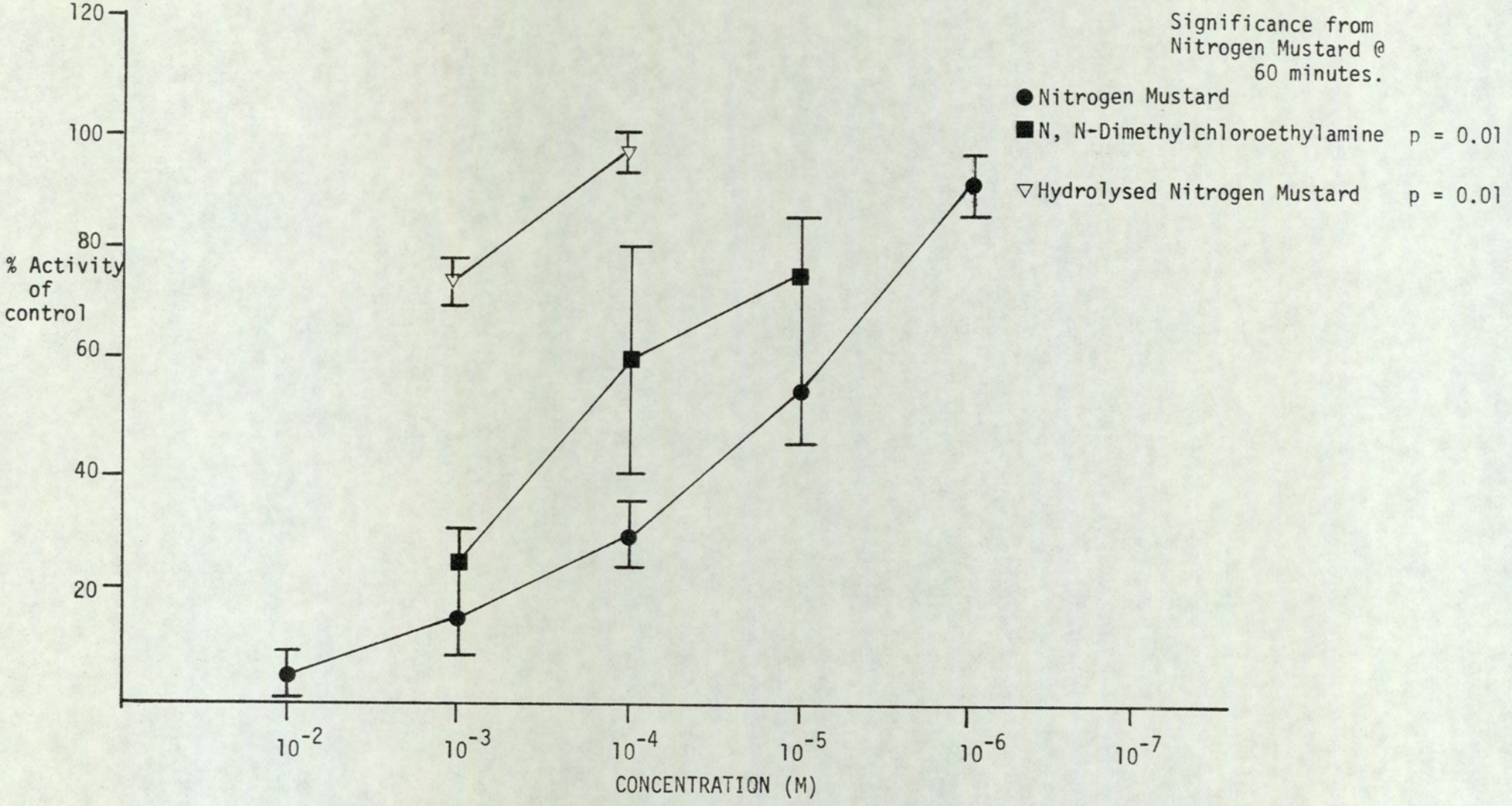


FIGURE 73 THE EFFECT OF THE ADDITION OF CYCLOPHOSPHAMIDE TWO HOURS PRIOR TO THE ADDITION OF RUBIDIUM ON RUBIDIUM INFLUX INTO PC6ACELLS.

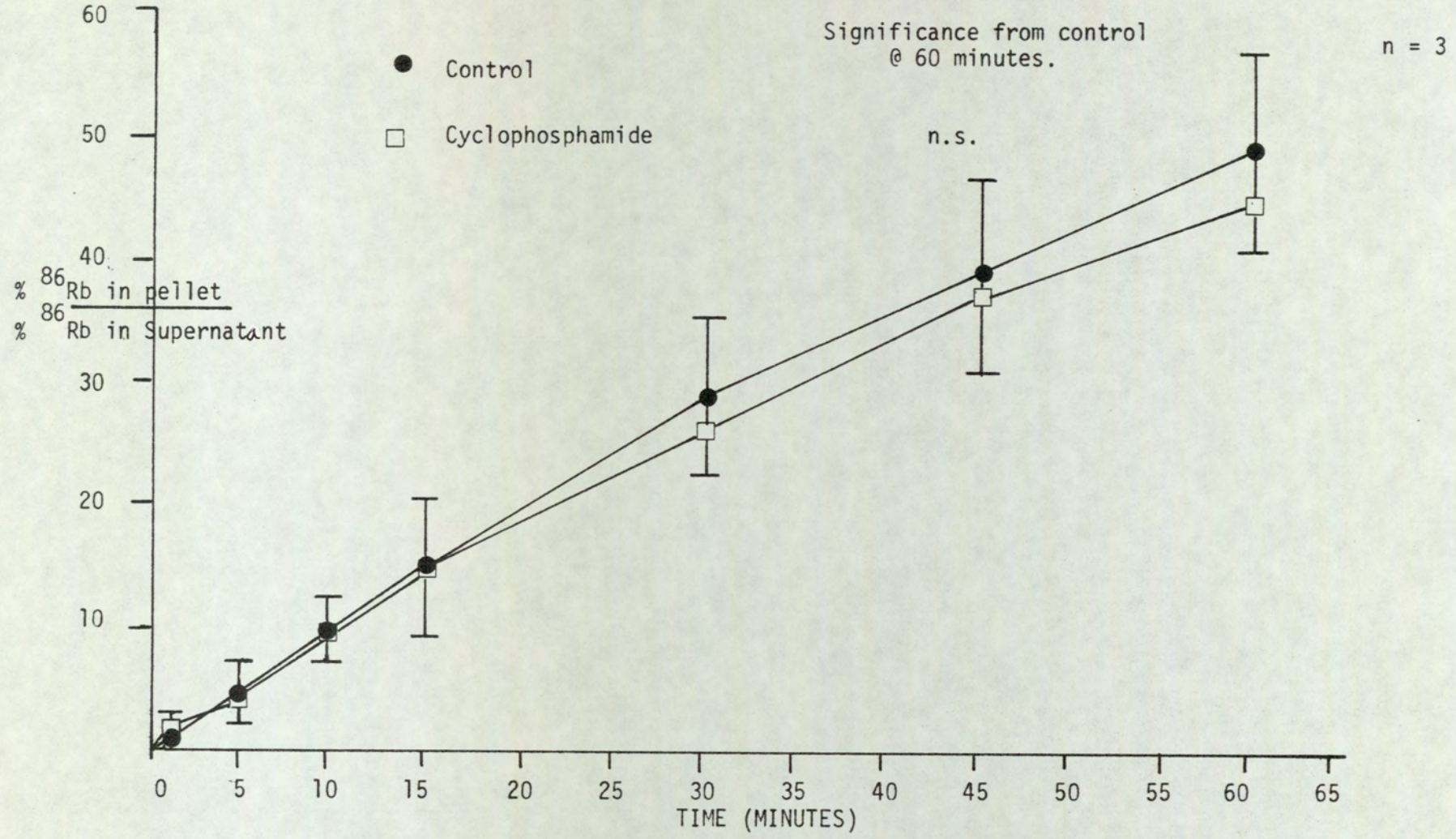
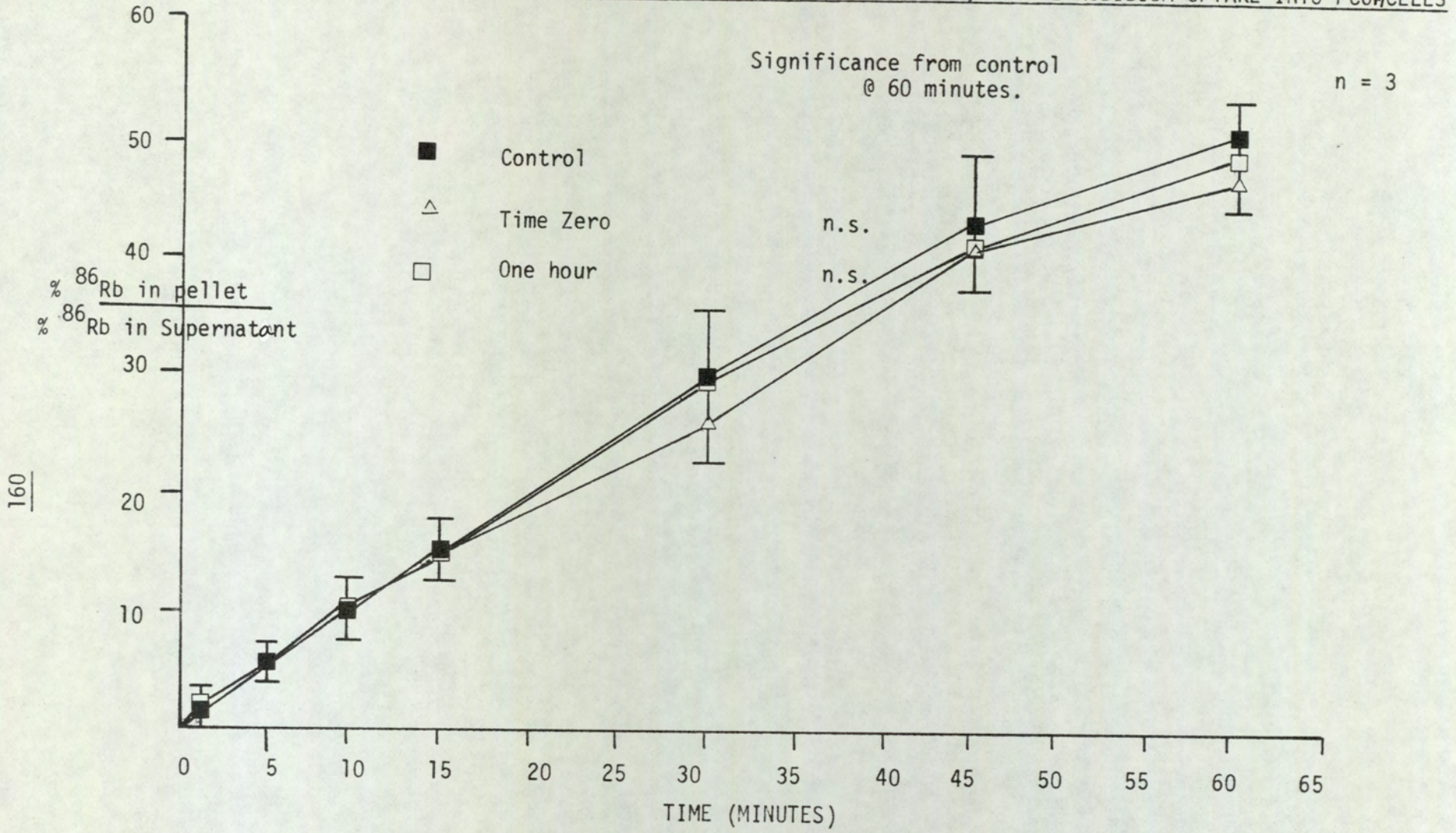


FIGURE 74 THE EFFECT OF THE ADDITION OF 10^{-6}M DIBUTRYL CYCLIC AMP AT TIME ZERO, AND AT ONE HOUR PRIOR TO THE ADDITION OF RUBIDIUM, ON THE RUBIDIUM UPTAKE INTO PC6A CELLS



DISCUSSION

The mechanism of the cytotoxic action of the nitrogen mustards is generally considered to be related to their ability to cross-link DNA. However, this may not fully explain the cytotoxic activity of these agents, and for this reason, other targets have been postulated. (see Introduction).

The basis of the present investigation concerned the hypothesis that nitrogen mustard may inhibit the cell membrane enzyme, NaKATPase. It was postulated that since this enzyme was known to be sensitive to alkylating agents such as iodoacetic acid, inhibition of this enzyme by the nitrogen mustards might affect the intracellular cationic content of cells and thus effect consequent cellular metabolism and replication processes known to be controlled by these cations. Hence, the NaKATPase enzyme might be a vital target which may explain the cytotoxicity of the nitrogen mustards.

The cell line chosen in the study was the ADJ/PC6A plasmacytoma, a line which has previously been shown to be highly sensitive to nitrogen mustard type alkylating agents (e.g. Connors and Hare 1975). In addition to the studies using a sensitive cell line, it was proposed that the formation of a PC6A cell line resistant to alkylating agents might allow a comparison to be made of the enzyme activity on alkylating agent sensitive and resistant cell lines.

The assay systems used were designed to study the ability of the simplest of the antitumour β chloroethylamine alkylating agents, nitrogen mustard, to inhibit the enzyme NaKATPase *in vitro* in both a broken cell preparation and in whole cells. Prior to the study of the effects of nitrogen mustard on a crude cell membrane system from broken PC6A cells, the properties of the enzyme were compared with the known properties of NaKATPase from other sources.

Three techniques of cell lysis were used to prepare a crude cell membrane preparation. All yielded comparable Total ATPase activities (Table 6) and with the exception of the hypotonic lysis method the techniques gave preparations that were similar in Total NaKATPase and ouabain sensitive NaKATPase activity. Common to all three

techniques was the high degree of variation in the enzyme activities, and the reasons for this are discussed below. The storage for long periods of time of the broken cell preparation was found to effect the activity of the NaKATPase enzyme, a result also found by Monteil et al (1974) who used a membrane bound NaKATPase isolated from Proteus $P_{18}L$ forms. Due to this, the preparation was used either immediately, or within 18 hours if stored at 4^{o}, after which time little change in activity had occurred (Table 5).

It was found that a large excess of either K^{+} or Na^{+} diminished the activity of the PC6*A*ATPase enzyme in a crude cell membrane preparation in a manner that was suggestive of a competition between the two cations for their respective sites in a manner possibly similar to that described by Karlish et al (1978). Further, Skou (1960, 1973) has demonstrated that Na^{+}:K^{+} ratios of between 5 and 10 to 1 are required for maximal activity. The results in Figure 9 also show that an increased Na^{+} concentration at a constant Na^{+}:K^{+} ratio of 1:1 caused inhibition of the enzyme as the cationic content was increased. This phenomenon has previously been observed for an enzyme preparation from guinea pig kidney medulla by Gutman and Katzper-Shamir (1971).

A study was made of the ionic composition of the media required for maximal ATPase activity. It was found (Figure 10) that Na^{+},K^{+} and Mg^{2+} all had to be present for maximum enzyme activity, a result found originally by Skou (1957). Skou (1973b) has also shown that the enzyme has a high specificity for ATP compared to other nucleoside triphosphates. This was confirmed by Monteil et al (1974) with a bacterial NaKATPase. The Km value for NaKATPase from PC6*A*cells (Table 7) was found to be approximately a factor of 5 greater than that obtained by Monteil et al (1974) from Proteus P_{18} cells and twice as high as that obtained by Robinson (1967), from a microsomal preparation of rat brain tissue.

In addition to a study of the optimal ionic composition of the media and the specificity of the NaKATPase for ATP, previous workers had suggested that there was an optimal ratio of Mg^{2+} and ATP for maximal enzyme activity. All the assay procedures used here were based on that of Taylor (1962) who used a Mg^{2+} concentration

of 5mM together with an ATP concentration of 2.5mM. However, Monteil et al (1974) demonstrated that a Mg^{2+}:ATP ratio of 1:2 gave optimal activity whilst Glick (1972 p5) in his review showed that a ratio of 1:1 and 2:1 had also been reported to give maximal hydrolysis.

The results shown in Figure 11 show that for maximal NaKATPase activity the broken PC6A cell preparation had to be incubated at 37^{o}. The fact that the enzyme system worked below normal physiological temperature has been shown before and is thought to explain why an animal in hibernation with a physiological temperature below 10^{o} can maintain ionic gradients across its cell membranes (Russell and Chambers 1976).

The overall results thus suggested that the NaKATPase from PC6A cells was generally comparable to that from other sources and that the assay was acceptable for further studies. It was thought possible that the sensitivity of the PC6 cell line to alkylating agents might be due to a high level of NaKATPase activity as has been reported for some transformed cells in comparison to their normal counterparts (Kasarov and Friedman 1974, Kimelburg and Mayhew 1975). However, Elligsen et al (1974) and Tisdale and Phillips (1976) stated that the difference in ATPase activity between the malignant and normal cells used in their study was a reflection of growth rate rather than innate differences between them. Comparison of ouabain sensitive NaKATPase activity of PC6A cells with the activity from other cell sources (Tables 8, 9), showed that there was no significant difference between it and the enzyme activities from homogenates of heart, gut or TLX5 lymphoma cells, the latter being resistant to alkylating agents.

The working hypothesis outlined above suggested that a cytotoxic action of the antitumour alkylating agents may be via the inhibition of the NaKATPase enzyme. Prior to the study of the effect of nitrogen mustard in the NaKATPase enzyme, a study of the inhibition of the enzyme by known inhibitors was undertaken. Iodoacetic acid has been found to be a weak inhibitor of NaKATPase obtained from eukaryotic cells (Sullivan et al 1974, Glick 1972 p24). The inhibition of ouabain sensitive PC6A NaKATPase by this agent (Table 11) may confirm the specific requirement for SH group(s) for

NaKATPase activity. The low potency of this agent could be a reflection of its hydrophilicity, since the vital SH group involved in the binding of ATP is hydrophobic (Schoot et al 1978).

N-ethylmaleimide has been reported as a possible antimitotic agent when used in cancer chemotherapy (Mercx Index No.3762 1977). The results in Table 11 show that the agent was under these assay conditions a more potent inhibitor than reported by Sullivan et al (1974) who found 100% inhibition at 10^{-3}M and Matsui and Schwartz (1966) who reported 50% inhibition at 6×10^{-4}M. p-Chloromercuribenzoic acid was the most potent of the three "classical" sulphydryl reagents tested. Sullivan et al (1974) found 100% inhibition of NaKATPase from purified plasma membranes of the marine diatom Nitzchia alba at 10^{-4}M, whilst Matsui and Schwartz (1966) demonstrated 50% inhibition at 5×10^{-6}M on a purified enzyme preparation from calf cardiac tissue. Fahn et al (1966) found 100% and 0% inhibitions at concentrations very similar to those shown in Table 11 on the microsomal preparation from Electrophorus electric organ.

In the assay procedure used here the sulphydryl agents were incubated with the broken cell preparation for 15 minutes prior to the addition of ouabain (Methods 6:11:5).

The determination of residual ouabain sensitive NaKATPase by the addition of ouabain after treatment of the preparation by sulphydryl reagents was considered valid, and is based on the work of Erdmann and Schoner (1977). They reported that the ouabain receptor was stable to sulphydryl reagent treatment and they concluded that the ouabain binding site and the nucleotide binding site were separate from each other.

These results with the "classical" sulphydryl reagents demonstrated that the NaKATPase enzyme from PC6A cells acted in a manner similar to that of enzymes from other sources reported in the literature.

It was found that the inhibition of Total ATPase activity by nitrogen mustard was time dependent (Figure 19) and required incubation of the membrane preparation and the agent for periods up to 60 minutes. The effect on Total ATPase activities of this long incubation time at 37^{o} prior to the addition of ATP, was

investigated (Figure 12) and it was found that there was a significant decrease in activity with incubation periods longer than 30 minutes.

Erdmann et al (1974) found a similar decrease in enzyme activity with incubation time using an enriched membrane preparation from rat and guinea pig skeletal muscle. They were unable to say exactly how this damage to the enzyme arose, but thought it was due either to effects of the media on the enzyme macromolecule, or effects on the membrane itself, which play an important role in the proper functioning of the enzyme. Therefore to account for the effect of incubation time on activity of the enzyme, both control and test systems were preincubated for the same period.

The delay in the inhibition of Total ATPase activity by nitrogen mustard has to be explained with reference to Table 8 which showed that the half life of nitrogen mustard in water at 37^{o} was not due to the time dependent generation of a cytotoxic product on hydrolysis of the mustard, since hydrolysed nitrogen mustard had no significant effect on NaKATPase activity up to 10^{-4} M (Table 11).

As discussed in Section 1:2, prior to the attack of nucleophilic centre bis-2-chloroethylamines undergo a S_N1 type cyclisation reaction to form an aziridium ion. The role of a polar solvent in the stabilisation of ions formed in such cyclisation reactions is most important. In non polar solvents such as ether, and presumably lipids, this activating cyclisation reaction does not occur (Price 1975 p2). Thus the delay in inhibition would be caused by the retardation of the reaction by solution in areas of high lipophilicity within the membrane. These areas may then act as a depot for the slow release of the drug.

In addition, certain nitrogen mustards may also reversibly bind to proteins without alkylation, presumably by van der Waals type binding (Linford 1961). Chlorambucil has been shown to be rapidly absorbed by serum proteins and at concentrations comparable to those prevailing in vivo, absorption was virtually complete. Thus the delay in inhibition of NaKATPase by nitrogen mustard may also be due to the reversible binding of the agent to proteins present in the assay system.

The concentrations of bifunctional alkylating agent required to inhibit the NaKATPase were far less than the concentrations of the "classical" monofunctional sulphydryl reagents and the monofunctional alkylating agents (Table 11). This difference in potency is possibly due to the cross-linkage of the NaKATPase subunits by the difunctional agents, so preventing the conformational changes required for activity.

Sweadner (1977) has reported the presence of two nucleophilic centres, one in each chain, in close proximity to each other, which after cross-linkage by imidoesters resulted in the inhibition of the enzyme.

The reduced potency both in this assay and with the study of rubidium uptake (see later) of the "classical" sulphydryl reagents and the monofunctional alkylating agents N, N-dimethylchloroethylamine and monofunctional chlorambucil could be due to the fact that inhibition of the enzyme by these agents was via a different mechanism than that with the difunctional agents. The sulphydryl reagents and the mono and difunctional alkylating agents are not structural analogues of the enzyme substrate or product. Thus the sulphydryl reagents and monofunctional alkylating agents possibly act by the random collision with, and alkylation of an SH group(s) at the active site of the enzyme.

In addition to the studies with nitrogen mustard on the NaKATPase system the effects of certain antitumour platinum complexes on NaKATPase activity were studied. It was considered that this might yield some insight into the cytotoxic action of these agents, and also might give some information on the geometry of the alkylated sites on the enzyme, due to the existence of cis and trans isomers of the platinum compound.

It has been shown that cis and trans isomers of Pt 11 $(NH_3)_2$ Cl_2 inhibit certain enzymes with reactive sulphydryl groups (Friedman and Teggins 1974, Hull et al 1979). The results obtained on the NaKATPase enzyme from PC6A cells showed that the cis isomer of Pt 11 $(NH_3)_2$ Cl_2 was a more potent inhibitor than the trans isomer (Figures 30-32).

Reactions that involve chloro-platinum complexes invariably involve the substitution of chloride rather than ammonia ligands (Thompson et al 1972 p18-28). Because of the differences in the halide-platinum-halide bond angles for the cis isomer (90^{o}) and the trans isomer (180^{o}), the steric requirements for cross-linkage of protein chains are vastly different for the two isomers. Hence the different potency of inhibition of NaKATPase by the cis isomer compared to the trans isomer indicates that geometrical considerations are important in the inhibition mechanism. The results (Figures 30-32) suggest that the cross-linkage of the two subunits of NaKATPase involves two nucleophilic centres at an enzyme-platinum-enzyme bond angle of 90^{o}. It is possible that the potent inhibition obtained by the trans isomer was due to the reaction of the agent with a sulphydryl group(s) at the active site, in a manner similar to the inactivation caused by sulphydryl reagents and monofunctional alkylating agents.

The results with the difunctional alkylating agent and the platinum complexes show that these agents are highly potent inhibitors of ouabain sensitive NaKATPase in this assay system. Similar inhibitions of high potency produced by various agents against NaKATPase have been noted elsewhere. p-Chloromercuribenzoic acid was found to cause 50% inhibition of the enzyme from a marine diatom at a concentration of 10^{-8}M (Sullivan et al 1974). Schwartz et al (1979) and Erdmann et al (1976) reported Ki values for ouabain of between $5x10^{-11}$M and $1.7x10^{-7}$M dependent on species. Gosalvez et al (1979) reported 50% inhibition of rabbit heart NaKATPase at approximately $5x10^{-11}$M adriamycin.

A possible factor on the high sensitivity of NaKATPase to alkylating agents could be the low protein contents used in the assay. Amino acids with nucleophilic centres, for example, cysteine are known to react with alkylating agents, and so an increased protein content could possibly reduce the potency of the agents. This hypothesis was tested by assaying nitrogen mustard against an ouabain sensitive NaKATPase enzyme from a total cell homogenate rather than a broken cell preparation. In the former preparation, cytoplasmic proteins were not removed, and the results (Figure 22) suggest that the alkylating agent may have reacted with cellular proteins, so reducing

the amount of agent available for reaction with the NaKATPase. Hence the values obtained for the inhibition of NaKATPase in the assay systems appear only to be valid in the protein concentration range used, that is 40-60 µg/ml (see 6:11:4).

In the presence of high concentrations of each inhibitor inhibition greater than that accounted for by the total inhibition of ouabain sensitive NaKATPase from PC6A cells was observed (Figures 17, 18, 23, 26 and 27). This questioned the specificity of the assay for inhibition of NaKATPase. Nitrogen mustard thus also inhibits other enzymes which utilise ATP and which may be involved in its cytotoxic action.

Further studies were performed on three such enzyme systems: MgATPase, p-nitrophenylphosphatase and an external CaMgATPase. N-ethylmaleimide, p-chloromercuribenzoic acid and nitrogen mustard all failed to give 100% inhibition of the MgATPase under the assay conditions used. Matsui and Schwartz (1966) demonstrated that the MgATPase of heart preparations was relatively insensitive to the sulphydryl reagents. N-ethylmaleimide has been shown to have little effect on the MgATPase of human red blood cell membranes, or of microsomes obtained from turtle bladder mucosal cells although in both cases the NaKATPase activity was sensitive to this inhibitor (Glick 1972 p20). Skou and Hillberg (1965) however found that MgATPase prepared from brain was inhibited by both N-ethylmaleimide and p-chloromercuribenzoate, and appeared to be more sensitive than the ouabain sensitive NaKATPase component. Thus, although sulphydryl reagents inhibit NaKATPase, they have been shown to have variable effects with regard to their action on MgATPase.

Iodoacetic acid was a more potent inhibitor of MgATPase than NaKATPase (Figure 28, Table 11). This was the only agent tested that gave 100% inhibition of MgATPase in the assay. It is possible that inhibition was due to alkylation of a nucleophilic group that was accessible to the agent by virtue of its hydrophilicity. It is also possible that high concentrations of the agent acted in a manner similar to that of sodium iodide by providing iodide ion. This agent is known to inhibit MgATPase and is used in the preparation of purified NaKATPase (Nakao et al 1965).

In 1975, Ronquist and Agren reported an ATPase enzyme present on the outer surface of the membrane in Ehrlich ascites tumour cells. This enzyme was here demonstrated to be present in PC6 cells and required Mg^{2+}, Na^{+}, K^{+} and Ca^{2+} for maximal activity. However, the activity was not due to the presence of NaKATPase, since the addition of ouabain had no significant effect on the activity of whole cells. (Figure 40). No significant inhibition of ATPase activity by nitrogen mustard occurred (Figure 41). Ronquist and Agren (1975) reported that N-ethylmaleimide produced a weak inhibition of the enzyme that never exceeded 10% up to a concentration of $5x10^{-4}$M. Senior (1973) and Monteil et al (1974) have both shown the presence of ATPase enzymes that are not inhibited by sulphydryl agents. It is possible that the external ATPase, along with the enzymes used by Senior and Monteil et al have a structure which involves the masking of nucleophilic groups essential for activity within the body of the enzyme.

Both MgATPase and the external CaMgATPase are discrete enzyme systems independent functionally from NaKATPase.

Under the conditions of the assay (Figure 35) the phosphatase activity from a broken cell preparation of PC6 cells was low. Coleman and Finean (1966) similarly reported low levels of the enzyme in liver plasma membranes, erythrocyte membrane and myelin when assayed under similar conditions. Of the four agents tested, only iodoacetic acid had any significant effect on the p-nitrophenylphosphatase activity. The lack of sensitivity of this enzyme to nitrogen mustard could be due to possibly three factors. Firstly, the enzyme could be insensitive *per se* to inhibition by nitrogen mustard. If the view is taken that this enzyme reflects the second half of the catalytic cycle of NaKATPase (see Introduction), this would suggest that nitrogen mustard inhibits the first half of the catalytic cycle of NaKATPase. Secondly, the enzyme could be insensitive to nitrogen mustard under the assay conditions used. Thirdly, the rapid hydrolysis of the nitrogen mustard in the alkaline media could significantly limit the amount of nitrogen mustard available to inhibit the enzyme. Further work is required at pH 7.4 before any conclusion on the susceptibility of the p-nitrophenylphosphatase derived from PC6A cells to sulphydryl reagents can be made.

Study of MgATPase, CaMgATPase, and the p-nitrophenylphosphatase enzymes show that in addition to the low levels of activity of those enzymes, especially p-nitrophenylphosphatase, all three enzymes were insensitive to inhibition by nitrogen mustard. This suggests that the inhibition of NaKATPase by nitrogen mustard is a specific effect, and the fact that the inhibition greater than that accounted for by the total inhibition of ouabain sensitive NaKATPase occurs, suggests that this was due to inhibition of ouabain resistant NaKATPase.

The use of a broken cell preparation from PC6A cells showed that the NaKATPase enzyme system was extremely sensitive to inhibition by nitrogen mustard. However, a major problem with this assay was the high degree in variation in the results. The reason for this variation is unknown since preparatory techniques were standardised. However, two factors were possibly involved. Zachowski et al (1977) demonstrated the presence of a protein from the inside of the membrane which affected ouabain sensitivity. Alteration in the content of this protein on preparation of the homogenate could affect the ouabain sensitive NaKATPase activity. Secondly, the PC6A cells were harvested 7 days after passage and it was assumed that the growth fraction of the tumour on the 7th day was constant. Johnson and Weber (1979) and Kaplan (1978) have shown that in replicating cells there was a 1.5 to 1.8 fold increase in ^{3}H ouabain binding, which is considered to be related to the number of pump sites, compared to density inhibited cells. The variation in the results from the assay of the broken cell preparation could be due to an individual variation in the growth fraction of the tumour from animal to animal. This would effect the number of pump sites per cell and hence the NaKATPase activity.

This large variation in results has been reported before. Limos and Cohn (1974) showed a large variation in MgATPase and NaKATPase activity in an enzyme preparation from canine mesenteric arteries which resulted in an NaKATPase activity between 4.0 and 7.8 μMP/mg protein/hr.

The fact that the NaKATPase enzyme from a broken cell preparation of PC6A cells is highly sensitive to nitrogen mustard does not necessarily mean that physiological concentrations of the agent inhibit the enzyme in vivo. To get a closer approximation to the

effect of the agent in vivo, it is necessary to study the effect of nitrogen mustard on intact cells. This was attempted by the use of an in vitro-in vivo technique to study the effect of nitrogen mustard on the growth of cells, and in vitro techniques to study the effect of nitrogen mustard on the NaKATPase of intact PC6 cells.

To correlate the inhibition of the enzyme in vitro with cell death in vivo requires a knowledge of the physiological concentration of agent required to kill the tumour in the animal. The estimation of this concentration was attempted by the use of an in vitro-in vivo technique.

Assuming that ouabain is a selective inhibitor of NaKATPase (but see Hammerstrom and Smith (1979), the cytotoxicity of this agent was estimated. It was found that 10^{-3}M ouabain incubated with the cells for 4 hours and then removed by washing prior to injection, had no effect on the cell number harvested 7 days later. Shank and Smith (1976) have shown that this agent is cytotoxic to mouse lymphoblasts in vitro, although continual exposure in excess of 10 hours was required to effect cell growth. The results in Table 13 suggest that ouabain at a concentration that is toxic to the animal in vivo is not significantly toxic to PC6Acells in vitro within a 4 hour period of incubation. It would appear from the results of Shank and Smith that periods of continual exposure to ouabain far longer than 4 hours are required to show if any significant toxic effect is observed against PC6Acells.

The difunctional nitrogen mustard, unlike its monofunctional analogue, reduced the number of cells harvested after a 7 day growth period (Figure 43, Table 13). A concentration of 0.238 ug/ml decreased the cell number by 90% in comparison to controls (Figure 43). By reference to the standard curve (Figure 42) this approximated an "apparent" 3 log cell kill, that is a 99.9% cell kill. These conflicting results illustrate a major problem in the interpretation of the results with this assay. The technique only allows the determination of total cell numbers harvested from an animal at one time point, that is 7 days after incubation. This growth period cannot be increased due to haemorrhaging and subsequent host death at approximately day 9. A reduction of 90% in total cell

numbers does not necessarily mean that 90% of the cells were killed. It is possible that the reduction in numbers was due to the temporary G_2 block observed by Brox et al (1980) for melphalan and subsequent retardation in growth. Brox et al (1980) reported that 36-48 hours after exposure to the melphalan the cells overcame this block and again started to divide. Hence, a reduction in cell numbers may be a reflection not only of cell kill, but also of blockade followed by repair. The ID_{90} value of 0.238 µg/ml obtained is a reflection of the effect of nitrogen mustard on the growth of PC6A cells over a 7 day period and great caution must be taken on correlating this result with other results obtained using different techniques. Using an *in vitro* technique similar to that used in the *in vitro-in vivo* assay with an ascitic PC6A tumour (see mthods 6:8), the inhibition of growth of a tumour which grew as a solid was investigated. The method differed from the assay involving the determination of cell numbers removed from the peritoneum as ascites, in that the cells were injected subcutaneously after an *in vitro* incubation of 2 hours with various agents. The tumour was excised after 28 days and its weight recorded. The advantage of this assay was that it was performed over a 28 day period, during which time any temporary blockade of the cell cycle as recorded by Brox et al (1980) would become insignificant. Therefore the ID_{90} value of 0.248 µg/ml obtained with the solid tumour assay is likely to be a truer reflection of cell kill than was the estimation of cell numbers removed from the peritoneum.

In a previous study which gave an ID_{90} for nitrogen mustard against PC6A tumour of 0.77 mg/kg (J.A.Hickman, personal communication) PC6A tumour was grown in the flank of a female Balb/C mouse. Twenty four days after implantation the animal was given a single dose of nitrogen mustard intraperitoneally. After a further period of 7 days the animal was killed and the tumour excised and weighed. As the cells were not treated *in vitro* prior to injection, it is probable that a larger dosage is required *in vivo* to obtain a cytotoxic concentration due to the pharmacokinetic behaviour of the agent in the animal.

The difficulty in the determination between a cytotoxic and cytostatic effect of an agent might be overcome by the use of a clonogenic assay. In this assay cells are seeded out on to agar

plates (Aidells et al 1979) and colony formation checked on a day to day basis. Hence it is possible to determine not only cytotoxic activity, but also cytostatic behaviour followed by repair and growth. However, the PC6A plasmacytoma does not readily grow for extended periods in vitro (J.A.Hickman, personal communication). The determination of sodium pump activity and the effect of various agents on its activity in whole cells was then studied using two systems. The first system was dependent on the role the sodium pump plays in the regulation of cell volume. The second system was based on following the movement of a radio-labelled monovalent cation known to be transported by the sodium pump.

Shank and Smith (1976) described a sensitive and rapid method for the determination of the ouabain inhibition of cell volume regulation of mouse lymphoblasts after hypotonic swelling. They suggested that such a technique might possibly prove useful in the rapid and sensitive screening of agents that could alter pump activity.

Studies using thePC6A cell showed that ouabain produced a time dependent inhibition of changes in cell volume, and hence by inference (Shank and Smith 1976) may affect the sodium pump (Figure 47). The fact that there was only a 50% inhibition of the pump, activity of PC6 cells was unlike the results of Shank and Smith who used mouse lymphoblasts. Here, 10^{-4}M ouabain completely inhibited changes in cell volume. This difference could be due to the sensitivity of the cell types to inhibition by ouabain.

Using nitrogen mustard it was found that 10^{-4}M of this agent produced an approximate 50% inhibition of cell volume regulation after a 60 minute incubation (Table 17). However, this result does not necessarily mean that 10^{-4}M nitrogen mustard inhibited the sodium pump per se. Gilman and Philips (1946) noted that nitrogen mustard inhibited the swelling response of avian erythrocytes, nucleated ghosts and of rabbit bone marrow and lung cells on the application of detergents. This inhibition of swelling was interpreted to result from a primary change in the properties of the cytoplasmic stroma. Wildenauer and Weger (1979) found that HN3 cross linked the membrane protein spectrin, which along with actin is thought to be important in the

maintenance of the cytoskeletal structure. Therefore the result obtained with nitrogen mustard using the hypotonic shock assay, together with the above observations, suggest that nitrogen mustard may not only affect cell volume regulation by a possible direct action on the sodium pump.

The determination of the activity of the sodium pump in intact cells has been shown to be possible by following the active flux of either radiolabelled sodium or radiolabelled potassium across the cell membrane. Due to the problems in the use of these two radioisotopes, radiolabelled rubidium, a congener for potassium (Beauge and Ortiz 1970) has often been used (Banerjee and Bosmann 1976, Bannerjee et al 1977, Gery et al 1979).

10^{-4}M Ouabain inhibited the rate of rubidium influx into PC6 cells by approximately 50% in comparison to controls(Figure 47). This result was similar to that observed by Cuff and Lichtman (1975a) who found a maximum inhibition of rubidium uptake of 70% in mouse lymphoblasts at 10^{-4}M ouabain. This inhibition of the uptake of rubidium by ouabain suggests that rubidium influx is via the sodium pump. Hence this technique may show whether an agent affects the NaKATPase enzyme system of intact cells. The initial hypothesis suggested that agents that alkylate and inhibit NaKATPase in vitro may inhibit this enzyme in vivo as part of their cytotoxic lesion. N-ethylmaleimide has been shown to inhibit the NaKATPase from a broken cell preparation of PC6A cells. With the use of radiolabelled rubidium it was found that 10^{-2}M N-ethylmaleimide had an immediate effect on rubidium influx (Figure 52). When the sodium pump is totally inhibited, the passive diffusion of the monovalent cations is not countered by the sodium pump dependent active transport of these ions against the concentration gradient. Hence, on total inhibition of the pump a fall in the intracellular K^+ concentration can be expected to occur. The fall in influx of rubidium observed within 20 minutes with 10^{-2}M N-ethylmaleimide and after 35 minutes with 10^{-3}M N-ethylmaleimide was possibly due to the passive back efflux of the ion along with the fall in intracellular K^+, when the pump was totally inhibited.

Concentrations of nitrogen mustard between 10^{-2}M and 10^{-5}M were found to inhibit the rate of influx of rubidium in a time dependent manner. The monofunctional analogue of nitrogen mustard also showed a time dependent inhibition of rubidium influx (Figures 60-65).

These results demonstrated three important points. Firstly, all the alkylating agents used here inhibited the sodium pump in intact cells. Secondly, the difunctional antitumour agent, nitrogen mustard was more potent than its monofunctional analogue. Thirdly, as the concentration of agent was decreased, the time lag before inhibition of influx was observed, increased.

The first point suggests that the nucleophilic groups of the enzyme alkylated by the agents in a broken cell preparation are accessible to the agents in intact cells. This raises the question whether these groups are on the inside of the membrane or on the outside, that is, is transport of the agent across the membrane a prerequisite for cytotoxicity? Vistica et al (1978) and Brox et al (1980) have demonstrated that the transport of melphalan across the cell membrane is essential for the toxicity of the agent. Their results would suggest that, if the NaKATPase enzyme was a primary target for activity, then the essential nucleophilic groups alkylated by the mustards would be accessible from the inside of the membrane. However, Grunicke et al (1979) have suggested that chlorambucil may not have to enter the cell to cause cytotoxicity. This would imply that the primary site of action of this agent was accessible from the outside of the membrane. Linford et al (1963) have suggested that chlorambucil might be an atypical alkylating agent in that its primary action might be the removal of a simple chemical component from the cell surface. Further work is required to see if Grunicke's result with chlorambucil is unique to that agent.

It was considered that the inhibition of rubidium influx by nitrogen mustard was not necessarily evidence for the direct inhibition of the sodium pump by the agent or for the role of the pump in the cytotoxic lesion. It was suggested that cyclophosphamide should be tested on the system. This alkylating agent is non-cytotoxic in the unmetabolised form, but is a highly potent antitumour agent after undergoing metabolism. If rubidium influx

was inhibited by the unmetabolised agent it would suggest that the fall in the rate of rubidium influx was not necessarily linked to the alkylation of the sodium pump or to the cytotoxic action of the antitumour alkylating agents. The results obtained (Figure 73) show that no inhibition of rubidium influx was found with this agent at the concentration and incubation time tested.

The second point demonstrated by the results with this assay was that the difunctional nitrogen mustard was a more potent inhibitor than the monofunctional analogue or N-ethylmaleimide. This difference in potency between mono and di-functional agents could be due to a different type of mechanism of action as discussed earlier (page166). The results also demonstrate a decrease in potency in the intact cell compared to the broken cell preparation. With a broken cell preparation, 100% inhibition of ouabain sensitive NaKATPase was obtained after 30 minutes incubation with 10^{-10}M nitrogen mustard (Table 11). This potency was reduced by a factor of 100 when a cell homogenate preparation was used, but in contrast, in intact cells rubidium influx was inhibited by only 60% with 10^{-4}M nitrogen mustard incubated for 60 minutes.

This difference in potency could be due to the reaction of the agents with the increased number of nucleophilic groups, due to the increased protein content and the presence of amino acids in the RPMI 1640 media used in the intact cell assay. In addition if transport across the cell membrane is a prerequisite for alkylation of the sodium pump, as discussed earlier, the reduction in potency might also be due to the reaction of the agents with non-essential proteins prior to, or during, the transport of the agent across the membrane.

The final point shown by the results of this assay, was the increase in time lag before inhibition of rubidium influx with the reduction in the concentration of the agent. These results were vital to the initial hypothesis as they quantify the concentrations of nitrogen mustard required to inhibit the sodium pump and the time periods necessary to obtain this inhibition.

The fact that there was a time lag before inhibition was observed

at the lower concentrations of nitrogen mustard tested suggested, as one possible explanation that the inhibitory species was being formed in vitro on hydrolysis (see page 165). This was found not to be the case as the addition of the hydrolysed mustard itself was found to have an inhibitory effect on rubidium influx only at 10^{-3}M. A result similar to that obtained in studies of the time dependence of the inhibition of the NaKATPase enzyme in the broken cell preparation. This inhibition of rubidium influx at high concentrations of the hydrolysis product might be due to the general toxicity of this agent.

It was further thought that the time lag before inhibition could be due to the decrease in rubidium influx being a secondary, not a primary, effect In 1969 Moszik reported a direct inhibition of NaKATPase from human gut mucosa by cyclic AMP and 5' AMP. It was considered possible that the alkylation and inhibition of the cyclic 3'5'-adenosinemonophosphatase phosphodiesterase enzyme by nitrogen mustard and the other agents might cause an elevation in intracellular cyclic AMP levels, as proposed by Tisdale (1974). This rise in intracellular cyclic AMP may then cause the inhibition of NaKATPase, which in turn would reduce rubidium influx.

The addition of 10^{-6}M dibutryl cyclic AMP, which is considered to be a maximum concentration cyclic AMP reaches in a rat thymic lymphocytes cell (MacManus and Whitfield 1969), had no effect on rubidium influx after 2 hours.

The results of the inhibition of the rubidium influx into PC6 cells by alkylating agents showed that the influx of this ion, and by implication, the sodium pump, was inhibited after the incubation of the cells in high, in comparison to physiological levels, concentrations of nitrogen mustard. If the sodium pump is a primary site for the toxicity of nitrogen mustard, then this inhibition must be observed as physiological concentrations of the agent.

Wheeler et al (1970) have shown that the ID_{90} of nitrogen mustard against H.Ep.No.2 cells was approximately 0.64 μg/ml after a 30 minute incubation with the agent in vitro. Wilkinson et al (1979) showed that physiological levels of chlorambucil induced

a large increase in nuclear protein phosphorylation within 2 hours of treatment. Brox et al (1980) showed that sublethal doses of melphalan (1 μg/ml) induced a temporary G_2 block and DNA cross-linkage within 6 hours of treatment. Grunicke et al (1975) noted that 10^{-6}M moles/kg of Trenimon (2,3,5-trisethyleneiminobenzoquinone) caused a complete inhibition of Ehrlich ascites tumour cell multiplication and depressed thymidine incorporation to less than 20% of the controls, although DNA synthesis proceeded normally. Grunicke et al (1979) have reported that the same concentration of Trenimon inhibited rubidium influx after 4 hours incubation in vivo in Ehrlich ascites tumour bearing animals. However, nitrogen mustard at approximately the same concentration that gave a 90% inhibition in tumour growth as measured by an in vitro-in vivo technique (0.248 μg/ml Figure 46) had no effect on the influx of rubidium, even after 6 hours incubation. Increasing the treatment period above 6 hours by the use of the in vivo technique used by Grunicke et al (1979) was not attempted. This was due to the fact that nitrogen mustard cannot be given subcutaneously and also to the problems of the calculation of the concentration of the agent which reaches the ascitic tumour, of which, the volume varies from animal to animal.

The results show, contrary to those of Grunicke et al (1979) with Trenimon, that nitrogen mustard at physiological concentrations does not effect rubidium influx, within a time that comparable concentrations of alkylating agents have been found by other workers to effect other targets (see introduction). This suggests that the inhibition of the sodium pump by nitrogen mustard does not play a role in the primary cytotoxic lesion, if it is assumed that the primary lesion occurs within 6 hours. This may not be the case. For example, the rate of thymidine incorporation has been shown to fall after alkylating agent treatment within this time period and for this reason DNA has been considered a primary target for alkylating agents (see introduction), despite the fact that Grunicke et al (1975) have shown that DNA synthesis is not affected by this fall in thymidine incorporation. Hence it is possible that a primary cytotoxic lesion could occur after a period in excess of 6 hours. Therefore, despite the fact that a physiological concentration of nitrogen mustard had no effect on rubidium influx after a 6 hour incubation, this does not rule

out the possibility that the sodium pump is an important target in the cytotoxic lesion.

The fact that 10^{-5}M nitrogen mustard did have a significant effect on rubidium influx within 6 hours suggests that the sodium pump may also be an important target in relation to the toxic effects of the agent observed at higher concentrations, that is, hyperglycaemia, loss of extracellular electrolytes and intracellular potassium and water (Gilman and Philips 1946).

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