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INVESTIGATION INTO THE MECHANISM OF ACTION OF ANTINEOPLASTIC NITROSOUREAS

by

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A thesis submitted for the degree of

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in the

UNIVERSITY OF ASTON IN BIRMINGHAM

September 1982

Department of Pharmacy

University of Aston in Birmingham
Birmingham B4 7ET

The best laid schemes o' mice an' men

Gang aft a - gley

Robert Burns 1759 - 1796

Investigation into the mechanism of action of antineoplastic nitrosoureas

by

Neil Walker Gibson

Submitted for the degree of Doctor of Philosophy, 1982 in the University of Aston in Birmingham.

The history and development of the antitumour haloalkylnitrosoureas is reviewed. Their reaction with DNA and the role that isocyanates may play in their *in vitro* cytotoxicity is discussed, along with other hypotheses concerning their mechanism of action.

The *in vitro* cytotoxicity of BCNU |1,3-bis(2-chloroethyl)-1-nitrosourea| and CCNU |1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea| was measured utilising an *in vitro*-*in vivo* bioassay. TLX5 lymphoma cell lines with resistance induced *in vivo* to either BCNU or a dimethyltriazene |5-(3,3-dimethyl-1-triazeno)-4-carboxyethyl-2-phenylimidazole| were found to be resistant *in vitro* to both the nitrosoureas and to the isocyanates formed upon their breakdown (chloroethylisocyanate and cyclohexylisocyanate respectively). An L1210 leukaemia line made resistant to BCNU *in vivo* was found to be resistant *in vitro* to the haloalkylnitrosoureas but not cross-resistant with cyclohexylisocyanate and only marginally resistant to chloroethylisocyanate.

The results suggest the TLX5 lymphoma may be sensitive to the haloalkylnitrosoureas as a result of the release of isocyanates. This was supported by the finding that the TLX5 lymphoma made resistant to a dimethyltriazene was not cross-resistant to other electrophilic compounds (formaldehyde, nitrogen mustard). Also there was no differences in either the transport of CCNU into sensitive and resistant TLX5 lymphomas or their non-protein thiol content. However, chlorozotocin |1-(2-chloroethyl)-2-D-glucopyranosyl-1-nitrosourea| a haloalkylnitrosourea with low carbamoylating potential had marginal activity against the TLX5 and L1210 cell lines *in vivo* yet remained selectively cytotoxic to the TLX5 lymphoma *in vitro*.

Azolastone |8-carbamoyl-3-(2-chloroethyl)imidazo|5,1-d||1,2,3,5|tetrazin-4(3H)-one| a novel heterocyclic antitumour agent was found to have excellent antitumour activity against both the TLX5 lymphoma and L1210 leukaemia *in vivo* Reaction of azolastone, CNU (1-(2-chloroethyl)-1-nitrosourea) and chloroethylisocyanate with DNA of L1210 leukaemia was assessed by the technique of alkaline elution. The DNA interstrand cross-link index, a measure of DNA-DNA interstrand cross linking activity, was calculated, both at zero and six hours post treatment, to be 0.004 and 0.032 for azolastone, 0.034 and 0.093 for CNU. Chloroethylisocyanate caused large quantities of DNA strand breaks, significantly more than 300R X-rays.

Keywords:

Haloalkylnitrosoureas, isocyanates, cytotoxicity, Azolastone, alkaline elution.

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ABBREVIATIONS

MNNG	1-methyl-3-nitro-1-nitrosoguanidine
MNU	1-methyl-1-nitrosourea
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
MeCCNU	1-(2-chloroethyl)-3-(4methylcyclohexyl)-1-nitrosourea
Chlorozotocin	1-(2-chloroethyl)-2-D-glycopyranosyl-1-nitrosourea
CNU	1-(2-chloreothyl)-1-nitrosourea
CENA	1-(N (2-chloroethyl)-N-nitroso)acetamide
BFNU	1,3-bis(2-fluoroethyl)-1-nitrosourea
FCNU	1-(2-fluroethyl)-3-cyclohexyl-1-nitrosourea
DCyNU	1,3-dicyclohexyl-1-nitrosourea
DCyU	1,3-dicyclohexylurea
BCPD	1-(N-butylcarbamoyloxy)-pyrrolidin-2,5-dione
CCI	1-(N-(2-chloroethyl)-carbamoyl)-imidazole
CF_3CNU	1-(3,3,3-trifluoropropyl)-3-cyclohexyl-1-nitrosourea
Azolastone	8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d][1,2,3,5] tetrazin-4(3H)-one
Methazolastone	8-carbamoyl-3-methylimidazo[5,1-d][1,2,3,5] tetrazin -4(3H)-one
MCTIC	5-[3-(2-chloreothyl)triazen-1-yl]imidazo-4-carboxamide
BCTIC	5-[3,3-bis(2-chloreothyl)triazen-1-yl]-4-carboxamide
Diazo-I.C.	4-diazo-imidazole-5-carboxamide
i.p.	intraperitoneally
s.c.	subcutaneously
p.o.	orally

i.c.	intracereberally
PEG	polyethyleneglycol
DNA	deoxyribonucleic acid
M.D.D.	Mean day of death
% I.S.T.	Percentage increase in survival time
R.P.M.I.	Rosewall Park Memorial Institute
E.D.T.A.	ethylenediaminetetracetic acid

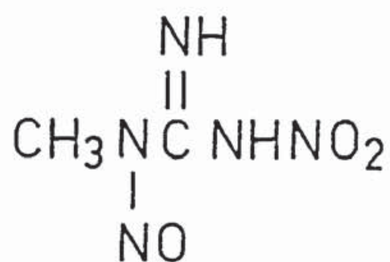
SECTION 1

INTRODUCTION

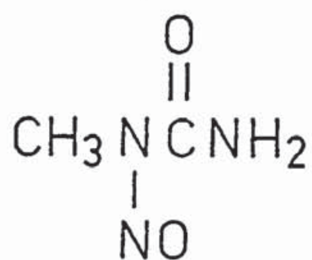
1.1 The development, synthesis and antitumour activity of the nitrosoureas

In 1956 1-methyl-3-nitro-1-nitroso guanidine (MNNG) (Figure 1) was entered into the National Cancer Institutes' screening programme. MNNG was found to have limited but reproducible activity against L1210 leukaemia (Leiter and Schneiderman, 1959). This observation prompted an investigation of structurally related compounds of MNNG such as 1-methyl-1-nitrosourea (MNU)(Figure 1). MNU was found to be active against intraperitoneally (i.p.) innoculated L1210 leukaemia when administered either subcutaneously (s.c.), i.p., or orally (p.o.) (Skipper et al, 1961). In contrast to the antitumour agents used at that time, such as methotrexate, 6-mercaptopurine, and cyclophosphamide, MNU was found to be active against L1210 leukaemia which had been innoculated intracerebrerally (i.c.)(Skipper et al, 1961). This observation suggested that MNU was able to cross the blood brain barrier and was suggested to be due to its lipophilicity (Skipper et al, 1961). The implication of these observations was that MNU would be of use in the treatment of brain tumours which until that time had been resistant to chemotherapy.

An active programme of synthesis and antitumour screening of nitrosourea analogues was then undertaken at the Southern Research Institute in Birmingham, USA. Early studies showed that N,N^1 disubstituted-N-nitrosoureas were the most active compounds against L1210 leukaemia (Schabel et al, 1963). Subsequent studies showed that greater activity was obtained when the substituent on the N-1 nitrogen was a 2-haloethyl group and the substituent on the N-3 nitrogen was



MNNG



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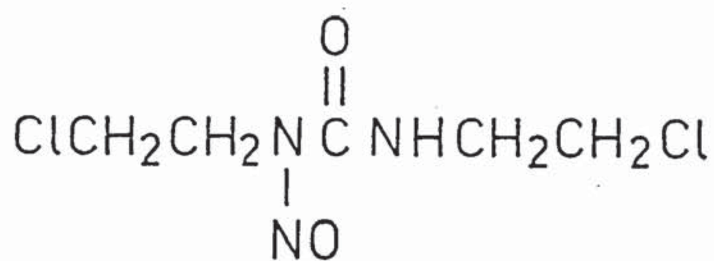

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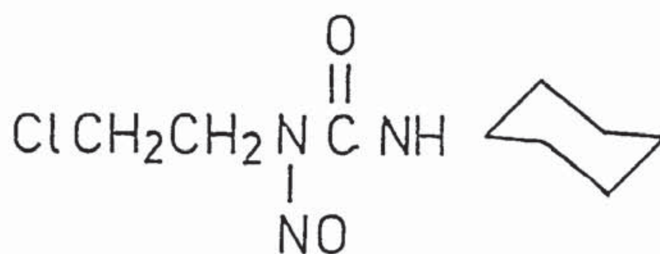
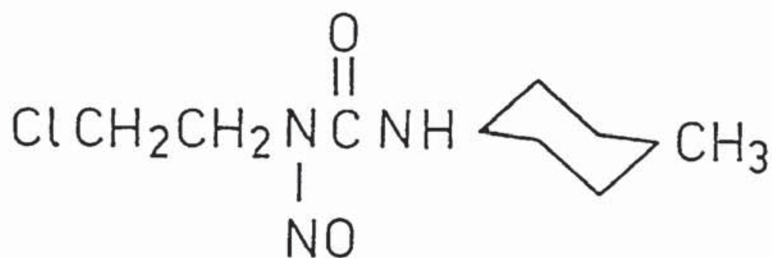


CCNU



MeCCNU

FIGURE 1 The structures of MNNG, MNU, BCNU, CCNU and MeCCNU

either a 2-haloethyl, cycloaliphatic or heteroalicyclic group (Johnston et al, 1966, 1971). The three most active nitrosoureas were found to be 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclchexyl-1-nitrosourea(CCNU) and 1-(2-chloroethyl)-3-(4 methyl cyclohexyl)-1-nitrosourea (MeCCNU)(Figure 1).

All three nitrosoureas were found to be active against B16 melanoma and Lewis Lung carcinoma, the most active of which was found to be MeCCNU (Carter et al, 1972). At that time MeCCNU was the most active antitumour agent ever tested against the Lewis Lung carcinoma. The growth kinetics of the Lewis Lung carcinoma was considered to be closely related to that of human solid tumours, and as such was a model which was considered to be able to predict those drugs which may be useful in the treatment of human solid tumours (Venditti, 1975).

BCNU, CCNU and MeCCNU were also highly active in a mouse ependymoblastoma system which was suggested to be a good model for brain tumours (Geran et al, 1974). This supported and strengthened the view that nitrosoureas may be of use in the treatment of brain tumours.

The nitrosoureas were found to be active against a wide variety of murine models such as L1210 leukaemia, sarcoma 180, P388 leukaemia and Yoshida sarcoma (for review see Schabel, 1976). Indeed the nitrosoureas were found to have the widest spectrum of experimental antitumour activity against murine systems when compared with the existing antitumour agents. These observations suggested that the nitrosoureas would result in a vast improvement in the treatment of human cancer.

In contrast to their activity in animal tumours the full promise of

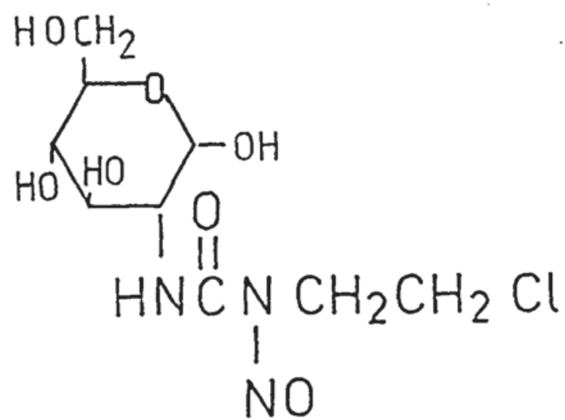
the nitrosoureas has yet to be realised in the treatment of human cancer. BCNU, CCNU and MeCCNU have been suggested to be of use in the treatment of brain tumours (especially gliomas), small cell carcinomas of the lung, gastrointestinal cancer, melanoma, and lymphoproliferative diseases (Table 1) (for review see Wasserman et al, 1975). The response rates shown in Table 1 include both complete and partial responses. A complete response was defined as the disappearance of all tumour masses and other evidence of disease. A partial response was defined as $> 50\%$ decrease in tumour mass without reappearance of new disease (Wasserman et al, 1974). The minimum duration before a response was considered valid was one month. This seems a weak set of criteria with which to evaluate a potentially lethal disease.

A major clinical problem associated with nitrosourea therapy is that they exhibit delayed bone marrow toxicity (De Vita et al, 1965) and this limits their use in chemotherapy. Reduced myelosuppression in animals was observed when the methylnitrosoureido group of MNU was attached at the 2-carbon position to glucose (Schein et al, 1974). This observation led to the synthesis of 1-(2-chloroethyl)-2-D-glucopyranosyl-1-nitrosourea (chlorozotocin)(Figure 2) in which the chloroethyl-nitrosoureido group of BCNU was linked to a glucose carrier (Johnston et al, 1975a). Chlorozotocin has similar antitumour activity in L1210 leukaemia when compared with BCNU and has the added advantage in that it is claimed to be non-myelosuppressive in mice (Anderson et al, 1975).

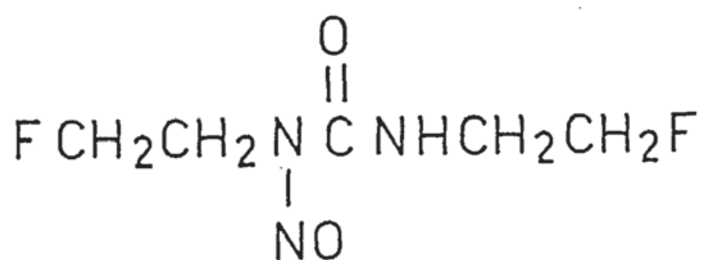
Chlorozotocin has undergone clinical trials in man and has been evaluated for its usefulness in melanoma, lung, breast and renal cell cancer. Only in melanoma was there evidence of activity and the activity observed was no greater than that of other chloroethyl nitrosoureas.

Table 1 Summary of results of clinical trials with nitrosoureas
(Taken from Wasserman et al, 1975)

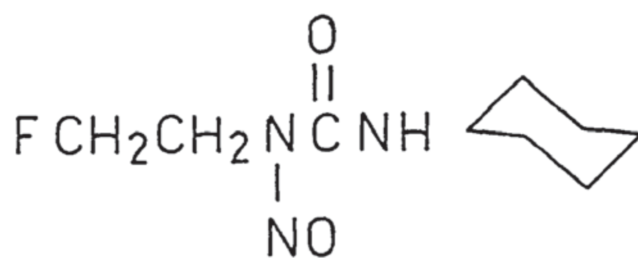
Tumour Type	Total Response Rate (%)		
	BCNU	CCNU	MeCCNU
Brain	47	41	22
Hodgkins Disease	44	48	26
Non Hodgkins Lymphoma	28	25	18
Melanoma	18	13	12
Lung	11	16	10
Breast	21	12	4



Chlorozotocin



BFNU



FCNU

FIGURE 2 The structures of chlorozotocin, BFNU and FCNU

Furthermore chlorozotocin has demonstrated all of the major known toxicities of other nitrosoureas in humans (for review see Hoth and Duque-Hammershaimb, 1981).

The nitrosoureas BCNU and CCNU are potent antitumour agents in murine models such as the L1210 leukaemia but they have achieved only limited success in man. An understanding of this disparity in activity, so that new agents may be designed depends upon understanding their mechanism of action. The object of this present study was to investigate aspects of the mechanism of action of the nitrosoureas. Before reviewing the mechanism of action of these agents, their chemical decomposition, metabolic transformation and pharmacokinetic properties will be discussed. The effect of these parameters upon the antitumour activity of the nitrosoureas will be reviewed. This introduction will conclude with the origin and scope of the work presented in this thesis.

1.2 Factors which affect the decomposition of the nitrosoureas

1.2.1 The mechanism of chemical decomposition of the nitrosoureas

Most of the nitrosoureas with antitumour activity are N-1-(2-haloethyl)-N-nitroso compounds. One problem in understanding their mechanism of action is that they are unstable under physiological conditions and spontaneously decompose to produce a variety of reactive intermediates according to the conditions, each of which may be potentially involved in nitrosourea cytotoxicity (Chatterji et al, 1978; Weinkam and Lin, 1979).

At least four decomposition pathways have been suggested for the nitrosoureas (Scheme's I - IV). Scheme I presents a hydrogen ion catalysed hydrolysis of the nitroso group to yield nitrous acid and the corresponding urea (Chatterji et al, 1978). This scheme is predominant under acidic conditions ($pH < 2$) and as such its importance under physiological conditions is likely to be negligible.

Montgomery et al (1967) observed that at pH 4 acetaldehyde was the major product which arose from the 1-(2-chloroethyl)nitrosoureas. They proposed that a cyclic mechanism, which involved the attack of the negatively charged carbonyl oxygen, occurred to form an unstable oxazolidine intermediate which then decomposed to acetaldehyde (Scheme II). The significance of this pathway under physiological conditions would also appear minimal.

The major product which arose from the decomposition of MNU was methanol, and the rate of decomposition was found to increase with increasing pH, above pH4. This result led Garret and Goto (1973) to

SCHEME I - Acid catalysed decomposition of the haloalkylnitrosoureas (Taken from Chatterji et al, 1978)

SCHEME II - Decomposition of the haloalkylnitrosoureas in which the carbonyl oxygen is the attaching nucleophile (pH 4 - 8) (Taken from Lown et al, 1979)

propose that the alcohol was formed via the corresponding carbonium ion and Reed et al (1975) to propose the same mechanism for 1-(2-chloroethyl)nitrosoureas (Scheme III).

These proposals do not account for the production of acetaldehyde as it has been shown that the 5-[3-(2-chloroethyl)triazen -1-yl] imidazo-4-carboxamide, which is predicted to be an excellent precursor of the chloroethylcarbonium ion (Shealy et al, 1975) yields mostly 2-chloroethanol in water (Montgomery et al, 1975). This suggested that the 2-chloroethylcarbonium ion did not appear to be the source of acetaldehyde and led Chatterji et al.(1978) to suggest, from their kinetic data, that under physiological conditions the cyclic mechanism (Scheme II) as proposed by Montgomery et al (1967, 1975) did occur, except that the nitroso group rather than the carbonyl group is considered to be the cyclizing nucleophile (Scheme IV). Chatterji et al (1978) argued that, on the basis of product analysis, either of the two oxygens could act as the nucleophile in displacing the chloride ion. They further argued that the polarity of the carbonyl oxygen is less pronounced than that in the corresponding urea, as the N-1 nitrogen lone pair of electrons will be strongly resonating with the nitroso group and thus be unavailable for amide resonance with the carbonyl group. This assumption was supported by the data from infra red spectroscopy (Chatterji et al, 1978) which suggested that the highly polarised N-nitroso group may be the nucleophile involved in the production of the oxadiazole intermediate (Scheme IV).

Lown et al (1979) proposed that the chloroethyldiazohydroxide formed in Scheme IV, in the case of BCNU decomposition at physiological

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SCHEME III – Proposed mechanism of decomposition of the haloalkylnitrosoureas at physiological pH (7.4) (Taken from Lown et al, 1979)

SCHEME IV – Decomposition of the haloalkylnitrosoureas in which the nitroso oxygen is the attacking nucleophile (pH 4 – 8) (Taken from Lown et al, 1979)

pH, undergoes rearrangement concerted with the loss of nitrogen to the cyclic chloronium ion and hence to acetaldehyde or the chloroethylcarbonium ion. These suggestions were supported by the findings of Brundrett et al (1976) in a study of the decomposition of deuterated BCNU under physiological conditions. Lown et al (1979) also provided supportive evidence for the formation of the cyclic oxadiazole intermediate as shown in Scheme IV. Brundrett (1980) has shown that the oxadiazole formation (Scheme IV) is a major pathway in the decomposition of nitrosoureas at pH 5.0. However at pH 7.4 this pathway was of minor significance (Brundrett, 1980).

Lown and Chauhan (1981) have recently shown that CCNU reacts in buffered solutions at physiological temperature and pH to yield a variety of products that are consistent with two pathways of decomposition (Scheme II and III). Decomposition occurred in one pathway through cyclisation to 2-(cyclohexylimino)-3-nitrosooxazolidines (Scheme II). Under physiological conditions this pathway is considered to be minor although its significance is unknown (Lown and Chauhan, 1981). The major pathway recognised by Lown and Chauhan (1981) which has previously been examined by other workers (Montgomery et al, 1967, 1975; Reed et al, 1975; Chatterji et al, 1978; Weinkam and Lin, 1979) is through formation of the 2-haloethyldiazohydroxide and isocyanate intermediates (Scheme III).

Chloroethylamine, 1,3-bis(2-chloroethyl)urea (Montgomery et al, 1967, 1975), and 2-[(2-chloroethyl)amino]-2-oxazoline (Montgomery et al, 1967; Weinkam and Lin, 1979) have been identified as products of BCNU decomposition in phosphate buffer. These results suggest that the isocyanate released reacts with water to form the carbamic acid which

then decarboxylates to the corresponding amine. This amine can then react with any isocyanate which remains to produce a urea (Scheme V).

In summary, substantial evidence has now accumulated to suggest that under physiological conditions the major pathway of decomposition of the nitrosoureas is via the formation of the alkyldiazohydroxide and isocyanate intermediates as shown in Scheme III (Montgomery et al, 1967, 1975; Reed et al, 1975; Chatterji et al, 1978; Weinkam and Lin, 1979; Lown and Chauhan, 1981). Although the importance of the minor pathways (Scheme II and IV) under physiological conditions cannot be neglected. The relevance of the alkyldiazohydroxide and isocyanate fragments in the mechanism of action of the nitrosoureas will be discussed in a later section of this introduction.

1.2.2 Alteration of the rate of decomposition by serum factors

The chemical half lives of the individual nitrosoureas in 0.1M sodium phosphate buffer (pH 7.4) varies from five minutes to as long as two hours, with BCNU and CCNU being forty three minutes and fifty three minutes respectively (Wheeler et al, 1974). The rate of nitrosourea decomposition in serum is much more rapid than the rate observed in aqueous buffer (Weinkam et al, 1980a). When incubated in human serum the half life of BCNU was reduced from fifty minutes to fifteen minutes, CCNU from sixty minutes to thrity minutes, and MeCCNU from fifty seven minutes to twenty nine minutes (Levin et al, 1979). The half live of BCNU in serum ultrafiltrate from which compounds with a molecular weight greater than 25,000 Daltons have been excluded is forty two minutes, which is approximately the same as in aqueous buffer (Levin et al, 1978a). Weinkam et al (1980) have suggested that BCNU

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SCHEME V - Decomposition of the isocyanate intermediate to the corresponding amine and urea
(Taken from Weinkam and Lin, 1979)

disappearance is catalysed by rather non specific interactions between BCNU and serum proteins, particularly albumin. Serum catalysed reactions are important because they alter the rate of conversion of the parent nitrosourea to its reactive intermediates. As pharmacokinetic data has shown that 50% of BCNU is accounted for in human plasma (Levin et al, 1978b) then serum reactions may thus be a major determinant of the in vivo biodispostion of the nitrosoureas in man.

The rates of BCNU decomposition in serum which contained high concentrations of triglyceride were found to be lower than the rates in normal serum (Levin et al, 1978a). Similarly, phosphatidylcholine and other lipids have been found to stabilise CCNU suspensions in aqueous buffer and cell culture media (Maker et al, 1978). Addition of lipoproteins to serum was found to stabilise nitrosourea degradation and a model was presented that correlated serum decomposition rates to lipoprotein concentrations (Weinkam et al, 1980b). Serum lipoproteins may therefore play a significant role in the biodistribution of the nitrosoureas. High lipid concentrations in vivo may also affect the antitumour activity or toxicity of these agents.

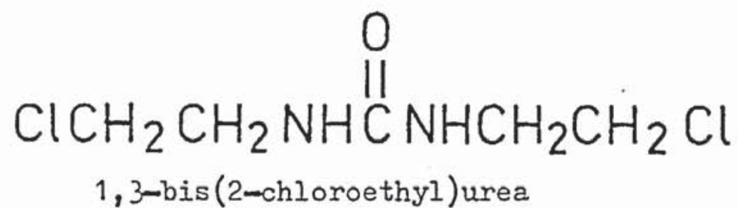
In conclusion variations in the concentration of serum in cell culture media in in vitro studies may affect the rate of decomposition of the nitrosoureas and this may alter the cytotoxicity of these compounds. Also variations in serum lipoprotein concentrations as well as serum protein concentrations in vivo may alter the pharmacokinetic parameters and possibly affect the biological response to the nitrosoureas.

1.3 The importance of metabolism of the nitrosoureas to their *in vivo* activity

In addition to their chemical decomposition the nitrosoureas are known to be metabolised by the liver microsomal mixed function oxidase system (May et al, 1974, 1975, 1979; Hill et al, 1975; Hilton and Walker, 1975; Lin and Weinkam, 1981). Hill et al (1975) observed that BCNU is converted to 1,3-bis(2-chloroethyl)urea (Figure 3) by a NADPH dependant microsomal mixed function oxidases. Some evidence has been presented for the formation of a glutathione conjugate of BCNU or 1,3-bis(2-chloroethyl)urea (Hill, 1976). Lin and Weinkam (1981) have recently confirmed the identification of 1,3-bis(2-chloroethyl)urea as a metabolite of BCNU and they indicated that this metabolite arose through an enzymatic denitrosation.

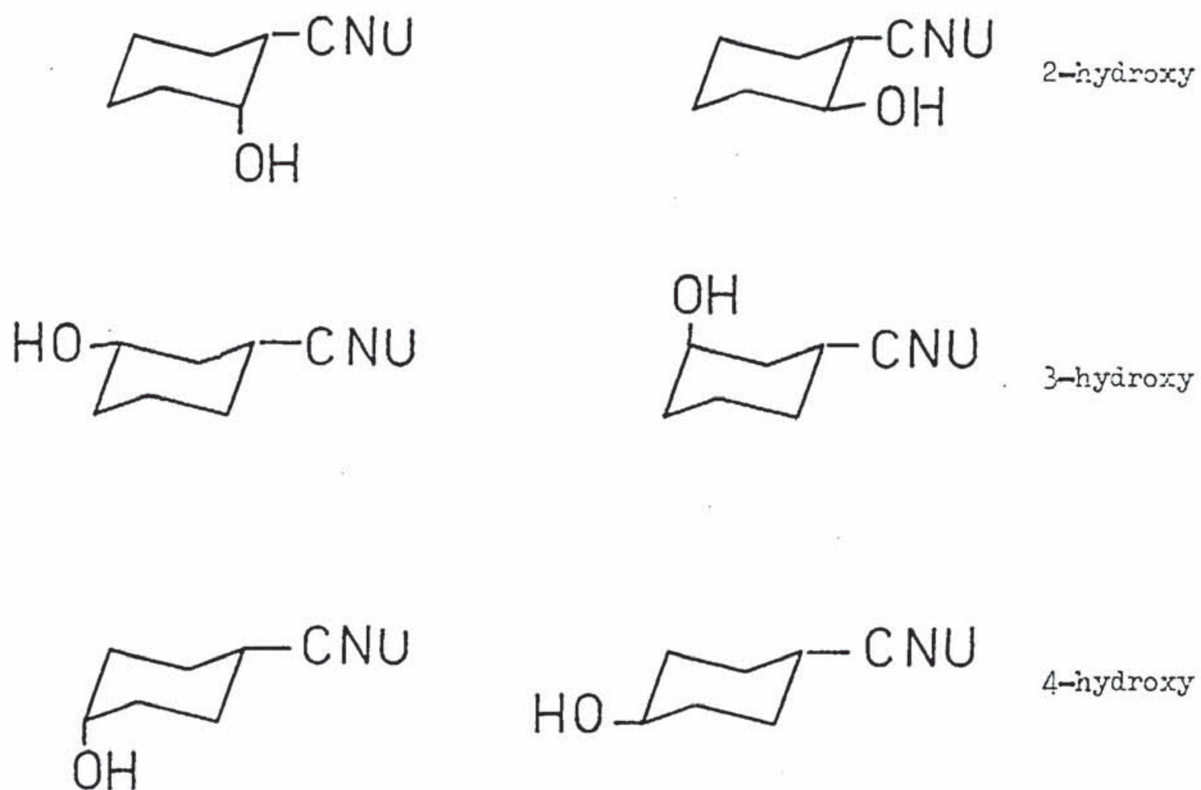
Evidence has been presented that hydroxylation of the cyclohexyl ring occurs very rapidly when CCNU is incubated with rat liver microsomes in the presence of NADPH and oxygen (Hill et al, 1975 ; May et al, 1974, 1975). Reed and May (1975) have demonstrated that CCNU undergoes rapid monoxygenation *in vivo* to yield the *cis* -3-, *trans* -3-, *cis* -4-, *trans* -4-, and *cis* -2-hydroxy metabolites (Figure 3). Hilton and Walker (1975) have independantly identified the two pairs of 3- and 4- isomers and the *trans* -2- hydroxymetabolite *in vitro*. They have also identified the same metabolites in the plasma of rats (Hilton and Walker, 1975), however, only the *cis* -4- and *trans* -4- hydroxy metabolites were identified in human plasma (Walker and Hilton, 1976).

May et al (1979) have shown that the metabolism of MeCCNU by



1,3-bis(2-chloroethyl)urea

cis TRANS



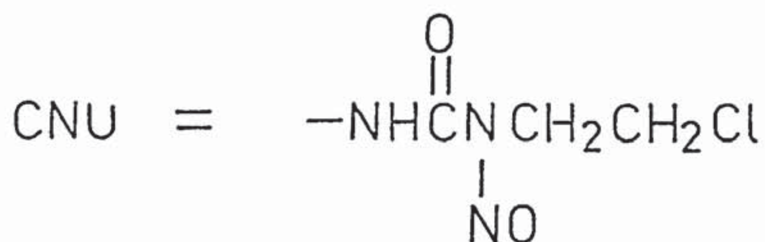


FIGURE 3 The structures of the metabolites of BCNU and CCNU

rat and mouse liver microsomes yields at least seven metabolites, three of which are ring hydroxylated products, a trans -4- hydroxymethyl derivàtive, an ∝ hydroxylation product of the 2-chloroethyl side chain, some denitrosated parent urea, and a yet unidentified metabolite. As with CCNU (May et al, 1974, 1975) formation of all hydroxylated metabolites were inhibited by carbon monoxide and May et al (1979) suggested that their formation was cytochrome P-450 dependant.

When animals were pretreated with phenobarbital an increase in the rate of formation of hydroxylated metabolites of CCNU and MeCCNU was observed as measured both in vitro (May et al, 1974, 1979) and in vivo (May et al, 1975; Hilton and Walker, 1975). Lin and Weinkam (1981) have also shown that microsomes prepared from phenobarbital pretreated rats increased thė formation of the 1,3-bis (2-chloroethyl)urea metabolite of BCNU. Addition of BCNU to an incubation mixture, which contains CCNU, at a concentration ten times greater than CCNU significantly slows the rate of CCNU metabolism. This indicates that both BCNU and CCNU may bind to the cytochrome P-450 active site (Lin and Weinkam, 1981).

The importance of metabolism on the antitumour activity and toxicity of the nitrosoureas has recently been investigated (Levin et al, 1979; Klubes et al, 1979). The hydroxylated metabolites of CCNU have been shown to have similar activity as CCNU (Johnston et al, 1975 b). This observation was supported by Levin et al (1979) who found that administration of phenobarbital to rats innoculated intracereberally with 9L glioma only slightly reduced the antitumour activity of CCNU. In contrast, however, the antitumour activity of BCNU, in the same system, was completely abolished by the pretreatment with phenobarbital

(Levin et al, 1979). Generally it would appear that metabolism of BCNU and MeCCNU reduced the antitumour activity and toxicity of these agents (Levin et al, 1979; Klubes et al, 1979) whereas the antitumour activity of CCNU is not diminished by metabolic transformation. (Johnston et al, 1975b; Wheeler et al, 1977).

In summary it would appear that alterations in both the rate of metabolism and relative formation of individual metabolites of the nitrosoureas could modify their toxicity and antitumour activity. Furthermore, as discussed previously serum lipoproteins and serum proteins are known to alter the chemical transformation of the nitrosoureas. Thus the antitumour activity of the nitrosoureas may be influenced by such factors as metabolism, serum lipoproteins, and serum proteins.

1.4 Pharmacokinetics of nitrosoureas

There have been few studies of the *in vivo* pharmacokinetics of the nitrosoureas, however, it would appear that many factors may influence nitrosourea pharmacokinetics. They include pH dependant hydrolysis, lipid stabilisation, enzymatic denitrosation and enzymatic hydroxylation (Section 1.2, 1.3).

The half live of BCNU was 15 minutes or less in the mouse (De Vita et al, 1967), the plasma of the dog (Loo et al, 1966; De Vita et al, 1967), the monkey and human (De Vita et al, 1967). Oliverio et al (1970) observed that the degradation of CCNU in the plasma of the mouse and in the plasma and cerebrospinal fluid of the dog occurred in two exponential phases, the first which indicated a half live of 5 minutes and the second a half live of more than 60 minutes.

After the administration of (^{14}C)-chloroethyl labelled BCNU or CCNU to mice, dogs, monkeys and humans most of the radioactivity was excreted in the urine as decomposition products or metabolites (De Vita et al, 1967; Oliverio et al, 1970). Radioactivity from (^{14}C) - chloroethyl labelled CCNU was widely distributed among tissues, including brain, liver, kidney, muscle and blood when administered to rats (Castronovo et al, 1980). Uptake of (^{14}C)- chloroethyl labelled CCNU was observed in rat brain glioma in the order of fluid $>$ capsule $>$ necrotic tissue. Tumour to muscle and tumour to blood ratios were maximised after 4 hours and decreased rapidly thereafter (Castronovo et al, 1980).

The distribution of CCNU and BCNU in kidney, fat and tumour

tissues was found to differ (Levin et al, 1978b) and this was suggested to be as a result of the different lipophilicities of BCNU and CCNU. The greater extent of BCNU than CCNU binding to nucleic acids in intracereberally innoculated 9L glioma (Levin et al, 1978b) supported a previous hypothesis that the relative in vivo nucleic acid binding affinities of BCNU and CCNU was related to their antitumour activities (Levin and Kabra, 1974).

In a study of BCNU pharmacokinetics in a sample of 20 humans Levin et al (1978a), using a two compartment open model, found a mean volume of distribution of 3.25 litres/kg, a mean clearance of 56mls/minute and a mean elimination constant of 0.0324 min^{-1}. The elimination constant observed by Levin et al (1978a) was similar to the decomposition rate determined for BCNU in serum (Weinkam et al, 1980a).

To summarise, the pharmacokinetic studies of the nitrosoureas indicate a very rapid absorption, distribution, and metabolism which is mediated in part by liver microsomal enzymes. Peak plasma levels of a combination of metabolites and degradation products appear within 1-6 hours. The products of degradation and metabolism are primarily excreted by the kidney (De Vita et al, 1967; Oliverio et al, 1970; Sponzo et al, 1973; Levin et al, 1978a). The pharmacokinetics of each nitrosourea can be affected by metabolism, serum proteins and lipoproteins and their chemical degradation. Each of these processes may thus also affect the antitumour activity of the nitrosoureas.

1.5 Mechanism of action of nitrosoureas

The mechanism of action of the nitrosoureas has yet to be clearly established. One reason for the failure to understand their mechanism of action is that as outlined previously (Section 1.2) nitrosoureas decompose spontaneously at physiological pH to produce two major reactive intermediates. An alkyldiazohdroxide species which is capable of alkylation reactions and an isocyanate which is capable of carbamoylation reactions are those species which are thought to be the two most important species.

Wheeler et al (1974) showed that the nitrosoureas reacted with 4-p-nitrobenzlpyridine an indication that the nitrosoureas were capable of alkylation. A hamster plasmacytoma that was resistant to cyclophosphamide was found to be cross resistant to BCNU (Wheeler and Bowden, 1965). These initial observations suggested that the nitrosoureas were similar to the bifunctional alkylating agents in their mechanism of action.

However, there is evidence to suggest that the nitrosoureas do not function solely as alkylating agents. Hodgkins disease which is resistant to alkylating agents has been shown to be sensitive to nitrosoureas (Selawry and Hansen, 1972). A TLX5 lymphoma which was naturally resistant to cyclophosphamide was highly sensitive to BCNU (Audette et al, 1973). An L1210 leukaemia resistant to BCNU was sensitive to cyclophosphamide and also an L1210 leukaemia resistant to cyclophosphamide was sensitive to BCNU (Wheeler et al, 1980). Recently Tew and Wang (1982) have shown that a Walker carcinoma cell line resistant to chlorambucil was sensitive to CCNU. Although these observations would seem to indicate that the nitrosoureas and alkylating

agents act by a different mechanism Schmid et al (1980) has shown that many cell lines with induced resistance to one particular alkylating agent are not cross resistant to other alkylating agents of the same type.

The main chemical difference between the nitrosoureas and the bifunctional alkylating agents is that only the nitrosoureas are capable of carbamoylation reactions, whereas both are capable of alkylation reactions although possibly of a different type. Structure activity investigations into the mechanism of action of the nitrosoureas will now be discussed.

1.5.1 Structure activity studies on the nitrosoureas

In 1972, Hansch et al proposed that the carbamoylating activity of the nitrosoureas contributed significantly to their toxicity in mice. However, Wheeler et al (1974) suggested that the lipophilicity is the dominant single factor in determining the relative toxicity of 1-(2-haloethyl)-1-nitrosoureas to mice. It was also suggested by these workers that nitrosoureas with optimal antitumour activity against L1210 leukaemia in mice would be 1-(2-haloethyl)-3-substituted nitrosoureas with low carbamoylating activity.

Colvin et al (1976) observed that 1-(2-chloroethyl)-1-nitrosourea (CNU) had equivalent cytotoxicity to L1210 leukaemia as BCNU and CCNU both _in vitro_ and _in vivo_. CNU upon decomposition produces the cyanate ion, a species that is incapable of inhibiting RNA processing, whereas this process has been shown to be inhibited by the isocyanates produced by BCNU and CCNU (Kann et al, 1974b). This suggests that the inhibition of RNA processing is of negligible importance to the cytotoxicity of the nitrosoureas. The lack of reactivity of the cyanate ion produced from CNU, an active antitumour agent, led Colvin

et al (1976) to suggest that the isocyanates are unimportant in the cytotoxicity of the nitrosoureas.

The carbamoylating activity of the nitrosoureas is measured by the extent of their reaction with radiolabelled lysine in vitro after a 6 hour incubation in 0.1M phosphate buffer pH 7.4. The extent of carbamoylation is defined as the percentage of the total radioactivity which cannot be attributed to radiolabelled lysine after thin layer chromatography (Wheeler et al, 1974). In a comparison of the carbamoylating activity of the nitrosoureas BCNU, CCNU and CNU, CNU was found to modify lysine to the greatest extent (Panasci et al, 1977a). This is a somewhat surprising result in view of the data of Kann et al (1974b) who showed that the cyanate ion was less reactive than the isocyanates derived from BCNU and CCNU, as measured by their ability to inhibit RNA processing. These results suggest that the isocyanates produced upon the decomposition of the nitrosoureas will have different degrees of reactivity, which may be dependant upon the nucleophilic environment.

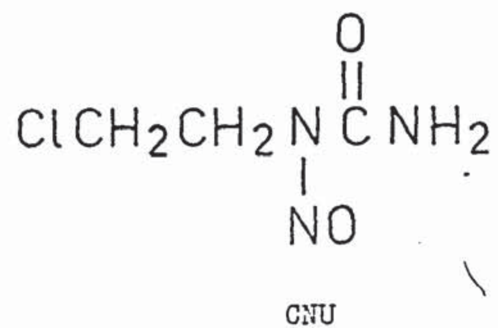
No significant correlation was found when carbamoylating activity was compared with lethal toxicity, granulocyte suppression or anti-tumour activity (Panasci et al, 1977a). In this study the alkylating activity, as measured by reaction with 4-p-nitrobenzylpyridine in vitro, of the nitrosoureas was correlated with whole animal toxicity and not antitumour activity. In a later study of the structure activity relationship of methylnitrosoureas Panasci et al (1977b) confirmed his previous finding that carbamoylating activity did not correlate with either antitumour activity against L1210 leukaemia of whole animal toxicity.

However, in this case they found that the _in vitro_ alkylating activity did correlate with antitumour activity against the L1210 leukaemia.

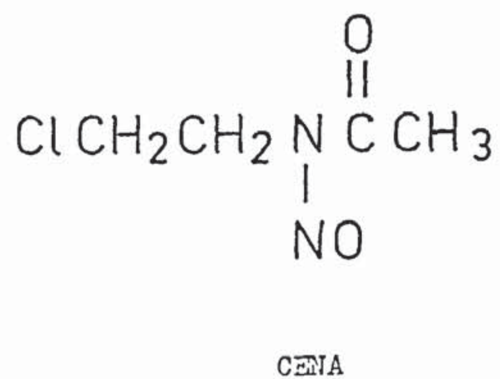
More recently Heal et al (1979) found no correlation between carbamoylating activity of a group of water soluble chloroethylnitrosoureas and their toxicity, but that a significant inverse correlation between _in vitro_ alkylating activity and whole animal toxicity existed.

A comparison of the antitumour activity, mutagenicity and _in vitro_ cytotoxicity of the chemically similar nitrosoureas and nitrosoamides has recently been studied (Brundrett et al, 1979). CENA and CNU (Figure 4) are both mutagenic and have _in vitro_ cytotoxicity to L1210 cells. Each compound was also toxic _in vivo_ to mice, however, only CNU had antitumour activity against L1210 leukaemia _in vivo_ (Brundrett et al, 1979). Both these classes of compound are thought to decompose to provide a chloroethyldiazohydroxide intermediate (Hecht and Kozarid, 1973; Douglas et al, 1978) and hence both are capable of alkylation. Only CNU however, is capable of producing a species that can carbamoylate. Brundrett et al (1979) also found that the _in vitro_ alkylating activity of the compounds was correlated with whole animal toxicity and not with antitumour activity against the L1210 leukaemia _in vivo_. This study would seem to indicate that the isocyanates, as a result of carbamoylation reactions, play a far greater role in the biological effects of the nitrosoureas than suggested by other workers.

In summary the structure activity relationships have suggested that neither the alkyldiazohydroxide or isocyanate species produced



CNU



CENA

FIGURE 4 The structures of CNU and CENA

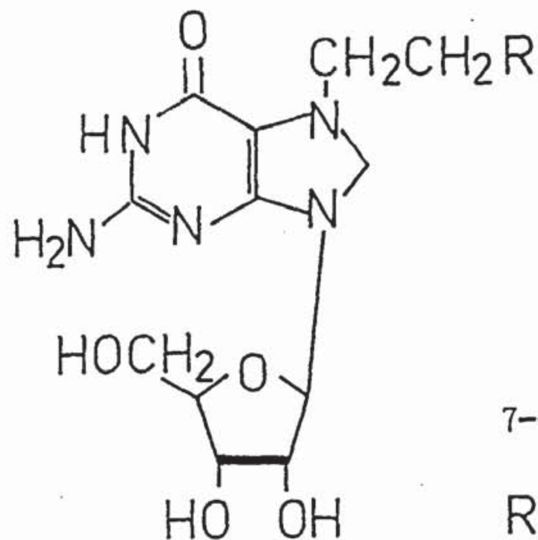
upon nitrosourea decomposition correlates well with antitumour activity against L1210 leukaemia (Panasci et al,1977a ; Heal et al,1979; Brundrett et al,1979). In contrast to these studies other investigations have suggested that alkylation is of greater importance than carbamoylation in the antitumour activity of the nitrosoureas against L1210 leukaemia (Wheeler et al, 1974; Colvin et al, 1976).

The alkylating and carbamoylating ability of each individual species will now be discussed. The modifications that may arise and a possible role of each modification in drug toxicity will also be considered.

1.5.2 Alkylation of nucleic acids by haloalkylnitrosoureas

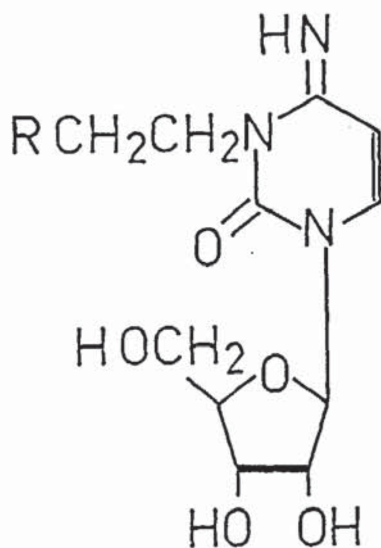
In 1972, Cheng et al found that CCNU labelled in the ethylene carbons reacted covalently with nucleic acids as well as proteins, although the type of modifications produced were not elucidated. Nucleic acid modifications were first studied by Kramer et al (1974) and Ludlum et al (1975) who identified three modified nucleosides in hydrolysates of synthetic polynucleotides reacted with BCNU (Figure 5); 3-B-hydroxyethylcytidine, 7-B-hydroxyethylguanosine and 3,N^4-ethanocytidine. At that time the significance of these reactions was not known.

The fluoroethylcarbonium ion produced in the decomposition of BFNU (Figure 2) has been shown to react with nucleic acids (Tong and Ludlum, 1978, 1979; Ludlum and Tong, 1978). 3-fluoroethylcytidine (Tong and Ludlum, 1978), 7-fluoroethylguanosine (Ludlum and Tong,



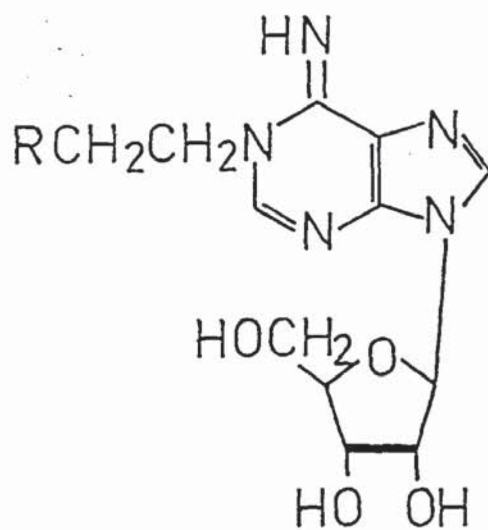
7-ethylguanosine

R = Cl , OH , F



3-ethylcytidine

R = Cl , OH , F



1-ethyladenosine

R = Cl , OH , F

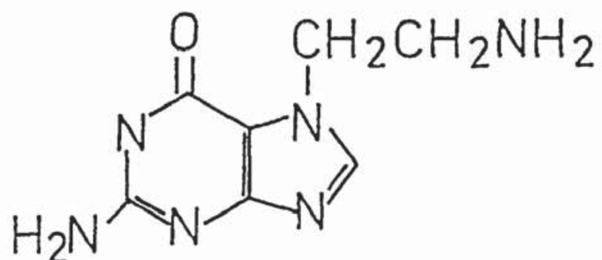
FIGURE 5 The structures of the modified nucleosides produced by reaction with nitrosoureas

1978), and 1-fluoroethylguanosine (Tong and Ludlum, 1979)(Figure 5) have all been isolated by reaction with BFNU and polynucleotides. These workers have also shown that 3-flouroethylcytidine and 1-flouroethyladenosine can cyclise by an intrastrand mechanism to produce 3,N^4 ethanocytidine and 1,N^6 ethanoadenosine. These products have an intramolecular bridge involving two base pairing positions thus the informational content of a nucleic acid which contained this modification might well be altered.

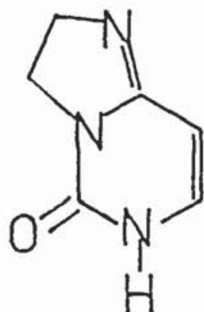
7-Aminoethylguanine (Figure 6) was identified in the hydrolysate of DNA treated with BCNU but not CCNU (Gombar et al, 1980). These workers found that 7-aminoethylguanine was also a product in the reaction between DNA and chloroethylisocyanate or chloroethylamine. This product is not thought to be involved in the antitumour activity of the nitrosoureas as CCNU is incapable of producing it (Gombar et al, 1980).

Hydroxyethylderivatives of nucleosides have been identified in all studies in which haloethylnucleosides are formed (Tong and Ludlum 1978, 1979; Lown et al, 1978). These derivatives are not formed by the hydrolysis of the haloethylnucleoside and it has been suggested that they arise by a direct attack of the nucleoside on an oxadiazoline intermediate (Tong and Ludlum, 1979; Lown et al, 1978). The oxadiazoline intermediate is suspected to be formed upon the decomposition of the haloethylnitrosoureas (see Section 1.2).

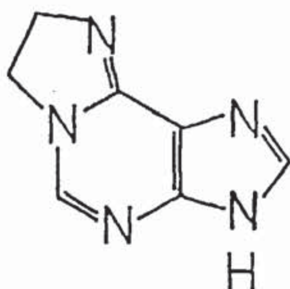
1,2-Di-(guanin-7-yl)ethane (Figure 6) was isolated as a product between guanosine and BCNU (Gombar et al, 1979) and also in DNA treated



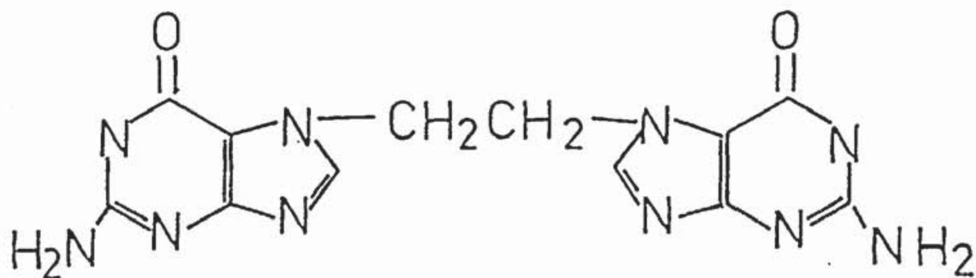
7-aminoethylguanine



3,N^4-ethanocytosine



1,N^6-ethanoadenine



1,2 -Di - (guanin-7-yl)ethane

FIGURE 6 The structures of bases modified by the nitrosoureas

with BCNU (Tong and Ludlum, 1981). The presence of this derivative in DNA indicates that BCNU can cross link nucleosides through a two carbon bridge. More recently evidence has been presented for the existence of guanylethyldeoxycytidine and a 6-substituted derivative of guanine (Tong and Ludlum, 1981). These results may help elucidate the mechanism of a possible cross link formation between guanine and deoxycytidine and lead to a greater understanding of the molecular events responsible for cytotoxicity.

1.5.3 Proposed mechanism for interstrand cross links of DNA by haloalkylnitrosoureas

The mechanism by which modifications of bases produces cytotoxicity has yet to be established although interstrand cross linking of DNA has been suggested to be the most likely cause (Lown et al, 1978; Ewig and Kohn 1978). Interstrand DNA cross links may prevent the DNA strand separation that must occur during normal DNA replication and transcription. However, the possibility that other base modifications may be cytotoxic cannot be ruled out.

Both Lown et al (1978) and Ewig and Kohn (1978) have demonstrated that chloroethylnitrosoureas when reacted with purified DNA have the ability to form interstrand cross links (Figure 7). The mechanism of cross linking suggested by Lown et al (1978) involves chloroethylation of a cytosine amino group (Lown and Maclaughlin, 1979a) followed by a second alkylation by the carbon bearing the chlorine at a nucleophilic site on the opposite strand of DNA. Kohn (1977) suggested that the initial chloroethylation occurs at the guanine-O^6 position and that the cross link is completed by a reaction with the cytosine amino group

Aston University

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FIGURE 7 Proposed mechanism of DNA interstrand crosslinking by nitrosoureas. X and Y are nucleophilic sites located on opposite DNA strands (Taken from Kohn, 1977).

to which the guanine $-O^6$ atom is normally hydrogen bonded. The second step in both proposed mechanisms, which converts monoadducts to interstrand cross links, is a slow chloroethylation and requires several hours for completion (Kohn, 1977; Ewig and Kohn, 1978; Lown et al, 1978).

The apparent inconsistency between the suggestions of Lown et al (1978) and Kohn (1977) as to which base is initially alkylated has not yet been resolved. It may even be possible that both the proposed mechanisms are correct.

1.5.4 Investigation of the modification of nucleic acids by haloalkylnitrosoureas by the technique of alkaline elution

Physical studies such as ethidium fluorescance (Lown et al, 1978) and alkaline elution (Ewig and Kohn, 1978) have been used to investigate drug induced DNA damage. Several types of DNA lesion can be measured: single strand breaks, DNA interstrand cross links and DNA protein cross links (Figure 8). As the technique of alkaline elution was used in this present study a short description of it follows.

Alkaline elution is a recently developed technique which is capable of measuring DNA macromolecular damage. This technique utilises filters to estimate DNA single strand length in mammalian cells (Kohn, 1979; Kohn et al, 1976, 1981). Two basic phenomena appear to control the ability of DNA single strands to penetrate filters under alkaline conditions. Firstly the filter retards the passage of long DNA strands in a size dependant manner, and secondly linkage of DNA to protein

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FIGURE 8

The types of DNA damage that can be measured by alkaline elution. (Taken from Kohn, 1979). Parallel lines represent double helix. Wavy lines represent random coils.

retards the passage of DNA through the filter apparently as a result of protein adsorption to the filter.

The measurement of the various types of lesions is of interest not only because of their possible role in drug toxicity but because they can now be measured at pharmacologically meaningful concentrations.

1.5.5 The importance of interstrand cross links of DNA in relation to the cytotoxicity of nitrosoureas

As previously mentioned, certain nitrosoureas have the potential to form interstrand cross links of DNA (1.5.3). Johnston et al (1966) have shown that modifications of the structures of the nitrosoureas results in a decrease or loss of antitumour activity against L1210 leukaemia *in vivo*. Lown et al (1978) have shown that the same structural modifications decrease the ability of these nitrosoureas to cross link DNA.

The relationship between DNA interstrand cross linking and cytotoxicity was measured in two human colon carcinoma cell lines, which were either sensitive or resistant to MeCCNU *in vitro* and *in vivo* (Thomas et al, 1978; Erickson et al 1978a). Resistance in the *in vitro* system was associated with the ability of the cells to remove drug induced DNA cross links (Erickson et al, 1978a). Thus it would appear from these observations that DNA interstrand cross links are an important facet of nitrosourea cytotoxicity. However, the proposed link between DNA interstrand cross link formation and cytotoxicity has been weakened by the results of

other investigations.

FCNU (Figure 2) produces a fluoroethyldiazohydroxide alkylating species which upon initial alkylation is less susceptible to nucleophilic attack by the opposite strand of DNA. Fluoroethyl alkylating agents are known to be much less reactive than chloroethyl-alkylating agents. FCNU has been shown to have negligible cross linking activity (Kohn, 1977; Lown et al, 1978) but intriguingly retains its antitumour activity (Johnston et al, 1966). This finding argues against the suggestion by Lown et al (1978) that the ability of a compound to show activity in the L1210 system is dependant upon its ability to cross link DNA. Furthermore, Sharkey et al (1982) have suggested that the cytotoxicity of FCNU may not be related to interstrand cross link formation. Their results suggest that monoadduct alkylation of DNA be sufficient to cause cytotoxicity to L1210 cells and that the ability of nitrosoureas to cross link DNA is not as important as previously thought.

Erickson et al (1978b) suggested that in V79 Chinese hamster cells the ability to cross link DNA confers increased cytotoxicity to the nitrosoureas. MNU, was found not to cross link DNA and a 40 fold increase in the drug concentration was required to produce toxicity equal to that of the haloethylnitrosoureas (Erickson et al 1978b). However, in Hela cells MNU has been shown to preferentially alkylate chromatin susceptible to the enzyme micrococcal nuclease (Sudhaker et al, 1979) whereas, haloethylnitrosoureas such as CCNU preferentially alkylate chromatin susceptible to DNase I (Tew et al, 1978; Sudhaker et al, 1979). These results may provide an alternate

explanation for the differential cytotoxicity observed by Erickson et al (1978b). Monoadduct formation, rather than interstrand cross linking, of DNA would appear a more consistent explanation for the cytotoxicity of the nitrosoureas. This may be the case particularly as the site of DNA lesion may be as important as the type of DNA lesion (Tew, 1981).

Differences in DNA interstrand cross linking associated with differences in cytotoxicity were observed in a pair of human embryo cell lines (Erickson et al, 1980a). Interstrand cross linking did not however correlate quantitatively with cytotoxicity. A normal cell strain, IMR 90, showed negligible cross linking when compared to an SV40 transformed VA13 cell line, even when the two cell types were compared with equitoxic concentrations of CCNU. Erickson et al (1980a) concluded that interstrand cross linking is not the only factor which determines cytotoxicity. Again it may be suggested that alkylation of one strand of DNA has the potential to be cytotoxic in this system.

The differential cytotoxicty observed between these two cell lines might be explained by their observed difference in repair of an O^6-chloroethylguanine adduct (Erickson et al, 1980b). The interstrand cross linking of DNA and <u>in vitro</u> cytotoxicity of 13 human cell lines were determined by Erickson et al (1980b). These cell lines were previously classified as Mer^+ or Mer^- dependant upon their ability to repair O^6- methylguanine lesions in DNA (Day and Ziolkowski, 1979; Day et al, 1980). Erickson et al (1980b) hypothesised that the enzyme that repairs an O^6-methylguanine

lesion may also repair an O^6-chloroethylguanine lesion. In their study the correlation between interstrand cross linking of DNA and <u>in vitro</u> cytotoxicity was suggested to be excellent (Erickson et al, 1980b). However, the initial O^6-chloroethylguanine lesion may itself be the cytotoxic lesion. The fact that the cells that are sensitive have cross links and those that are resistant do not can simply be explained by the fact that the sensitive cells do not repair the monoadduct lesion whereas the resistant cells do.

In summary it would appear that despite numerous investigations there is no conclusive proof that the major cytotoxic lesion of the nitrosoureas is a cross link of DNA. Indeed it would appear that monoadduct alkylation of DNA may be more consistent,in terms of cytotoxicty, with the observed data.

1.5.6 The specificity of nitrosourea alkylations within the cell nucleus

The last few sections of this introduction have concerned themselves with the ability of the nitrosoureas to cause a particular type of lesion. It has recently been suggested that the location and repair of a lesion within the nucleus as well as its type and quantity may be related to cell survival (Tew, 1981).

The different sites of alkylation of chromatin by CCNU and chlorozotocin support this concept. Chromatin has two distinct regions of DNA. DNA can either be associated with a double tetrameric core of histones (H2A, H2B, H3 and H4) and is called core DNA or

else it can be associated with histone (H1) and non-histone proteins and is called linker DNA. The non-myelotoxic chlorozotocin binds preferentially with linker DNA in bone marrow and core DNA in L1210 leukaemia whereas the myelotoxic CCNU binds to core DNA in both bone marrow and L1210 leukaemia (Tew et al, 1978). It was observed that the reduced myelotoxicity observed with chlorozotocin when compared with CCNU may be related to the differences in the location of DNA alkylation.

Certain regions of chromatin are more transcriptionally active than others and it has been shown that these particular areas are more susceptible to nitrosourea interaction (Tew et al, 1978). Transcriptional activity can be stimulated by sodium butyrate and it was found that this stimulation caused a two fold increase in the amount of nitrosourea bound to chromatin, and that this increase was unique to the transcriptionally active area of chromatin (Tew et al, 1978).

Pretreatment of Hela cells with hydrocortisone has been shown to increase the alkylation of extended chromatin by approximately two fold (Tew, 1981). This region of chromatin is presumed to be transcriptionally active. The increase in alkylation was specific to the region of extended chromatin as there was no increase in the total nuclear alkylation. The importance of this finding in terms of cytotoxicity has recently been shown (Tew et al, 1982). These workers showed that pretreatment of Hela cells with hydrocortisone enhanced the cytotoxicity of nitrosoureas. Thus modification of the nuclear structure by sodium butyrate or hydrocortisone results in an increased interaction of the nitrosoureas with transcriptionally

active chromatin.

The nuclear matrix which is responsible for many aspects of nuclear structure and function has been shown to be another possible site for nitrosourea interaction (Tew,1981). Chromatin which is actively replicating has been shown to be attached to the nuclear matrix (Pardoll et al, 1980). In view of this it has been suggested that DNA synthesis is facilitated by specific DNA-protein attachment sites (Tew,1981). Thus the ability of nitrosoureas to alkylate or carbamoylate the specific sites on DNA or on the protein may interfere with DNA synthesis. This in turn may result in cytotoxicity.

In summary both transcriptional chromatin (Tew et al, 1978, 1980) and the nuclear matrix (Tew, 1981) are preferential targets for nitrosourea alkylation and carbamoylation. Hence not only may alkylation of nuclear macromolecules result in cytotoxicity but there is the potential for the carbamoylation reactions of the nitrosoureas to exhibit cytotoxicity.

1.5.7 Covalent modifications of the histone proteins by the nitrosourea derived isocyanates

Nitrosoureas decompose under physiological conditions to produce two major reactive intermediates (see Section 1.2). The alkylation reactions of these agents has been discussed (Section 1.5.2 – 1.5.6). The second reactive intermediate produced is an isocyanate

(Section 1.2, scheme III) which is capable of carbamoylating nucleophilic centres. This reaction involves formation of a covalent bond between the isocyanate and nucleophilic centre and is termed a carbamoylation reaction.

Cheng et al (1972) showed that the cyclohexyl labelled degradation products of (^{14}C) – cyclohexyl CCNU were extensively bound to the cellular proteins of L1210 leukaemia both _in vivo_ and _in vitro_. N^6– cyclohexylcarbamolyllysine was isolated from hydrolysates of polylysine, albumin, histone and of protein of L1210 cells treated with CCNU _in vitro_ (Schmall et al, 1973). This observation was in agreement with that of Bowdon and Wheeler (1971) who isolated N^6–(2–chloroethylcarbamoyl)lysine from the hydrolysate of histone incubated with BCNU _in vitro_.

Wheeler et al (1975) showed that the carbamoylation of both the ε amino group of lysine and the α amino groups of amino acids, peptides, and proteins by nitrosoureas _in vitro_. Woolley et al (1976) have shown that the interaction of the decomposition products of CCNU with nuclear proteins of L1210 cells results primarly in reaction of the cyclohexyl group with histones, particularly the lysine rich HI histone. In Hela cells the non–histone proteins were carbamoylated to a greater extent than histone proteins (Tew et al, 1978) although the significance of these findings is not known.

Using Triton–acetic acid gel electrophoresis to separate the reaction products Pinsky et al (1979) found that HI histone and H2B histone of L1210 cells were carbamoylated to the greatest extent by

MNU. In contrast when SDS gel electrophoresis was used to separate the reaction products, H2B and H3 histones of L1210 cells were preferentially carbamoylated by MNU (Pinsky et al, 1979). The interpretation of such results is not clear. Sudhaker et al (1979) showed that MNU carbamoylated the H2B histone in the Hela cells to the greatest extent whereas CCNU was found to carbamoylate the non-histone proteins and histone-like high mobility group proteins preferentially.

The importance of the carbamoylation of histone proteins in relation to cytotoxicity remains obscure. Histones are known to undergo five types of natural modification within the cell: acetylation, phosphorylation, methylation, poly (ADP) ribosylation, and the formation of protein A24. It has been suggested that phosphorylation of histones might act as a trigger for mitosis(Bradbury et al,1974). Methylation of histones would appear to be important for DNA-histone stability (Byvoet and Baxter, 1975). Poly (ADP) ribosylation of chromatin histones is thought to be an important event associated with the repair of DNA lesions (Durkacz et al, 1980). Protein A24 disappears from cells that enter mitosis (Matsui et al, 1979).

Thus if nitrosoureas carbamoylate histones and prevent a natural modification from occurring and if this modification is important to cell growth and division then the nitrosoureas - induced modification may result in cytotoxicity. Indeed the enzymatic cleavage of protein A24 in Ehrlich ascites is inhibited by the carbamoylation reactions of the nitrosoureas (Dornish and Smith-Kielland, 1981), although whether this results in toxicity was not shown.

1.5.8 Covalent modifications of the non-histone proteins by the nitrosourea derived isocyanates

Wheeler and Bowden (1968) have reported that BCNU inhibits DNA nucleotidyltransferase activity in L1210 cells. They also demonstrated that this efffect was mediated by the chloroethylisocyanate produced upon the decomposition of BCNU. Baril et al (1975) have shown that DNA polymerase II but not DNA polymerase I was inhibited by the alkylisocyanates produced from BCNU and CCNU. BCNU and chloroethylisocyanate are known to inhibit the repair of irradiation produced strand breaks in DNA (Kann et al, 1974a). More recently Kann et al (1980a, 1980b) have suggested that nitrosoureas are strong inhibitors of repair of L1210 DNA strand breaks. These workers found that alkylating activity was unrelated to repair inhibition and that carbamoylating activity was the essential requirement for this inhibition.

Certain alkylisocyantes have been shown to be active-site-directed inactivators of chymotrypsin (Babson et al, 1977; Brown and Wold 1973a), alcohol dehydrogenase (Twu and Wold, 1973), elastase (Brown and Wold, 1973b) and transglutaminase (Gross et al, 1975). The inactivation of chymotrypsin by CCNU has been shown to be due to the cyclohexylisocyanate produced upon the decomposition of CCNU (Babson et al, 1977).

Babson and Reed (1978) have reported that isocyanates derived from the chloroethynitrosoureas inactivate glutathione reductase.

The *in vivo* inactivation of human erythrocyte glutathione reductase by BCNU has been observed (Frisher and Ahmed, 1977) and suggests that this highly specific inactivation may have important consequences *in vivo*. Loos et al (1976) have shown that lack of erthrocyte glutathione reductase was compensated for by increased glutathione biosynthesis. However, an oxidative challenge resulted in severe haemolysis, and this resembles the myelosuppression observed after the administration of certain chloroethylnitrosoureas. Babson and Reed (1978) showed that the chloroethylnitrosoureas which did not inactivate glutathione reductase did not exhibit myelosuppresive activity. Thus it would appear that the inhibition of glutathione reductase is implicated in the myelotoxicity of the nitrosoureas.

Brodie et al (1980) have shown that several nitrosoureas which degrade to form isocyanates inhibit the polymerisation of brain tubulin. Inhibition of tubulin may prevent microtubule assembly and this in turn may result in an inhibition of cell division. This has previously been shown with colchicine and the vinca alkaloids (Wilson, 1975). BCNU, CCNU and McCCNU caused cell cycle related difference in the human T1 lymphoma cell line (Drewinko et al, 1976). The increased sensitivity to BCNU and MeCCNU in G2 and to CCNU and MeCCNU in early S phase of the cell cycle could reflect, in part, disturbance by the drugs of the synthesis or polymerisation and degradation of tubulin which occurs at these two times respectively (Forrest and Klevecz 1972). However, nitrosoureas which have negligible carbamoylating activity when compared with BCNU or CCNU fail to inhibit the polymerisation of purified brain tubulin (Brodie et al, 1980). As these agents invariably retain antitumour activity the importance of the inhibition of tubulin in cytotoxicity is not clear.

The observations of Gross et al (1975) who showed that isocyanates inhibit transglutaminase were confirmed by those of Laki et al (1978) who reported that BCNU and its isocyanate moiety, chloroethylisocyanate are potent inhibitors of transglutaminase. This was also shown for CCNU and its cyclohexylisocyanate intermediate (Tarantino et al, 1979). These findings are especially relevant as recent observations have indicated the involvement of tissue transglutaminase in the proliferation of malignant tissue (Laki et al, 1977).

Fesus and Laki (1976) observed that the surface of YPC-1 tumour cells became coated with fibrin or fibrinogen after the cells were incubated in the presence of transglutaminase, fibrin or fibrinogen. Furthermore fibrin or fibrinogen can induce neoplastic cells to clump together and it has been shown that these cells are more resistant to attack by immunocompetent cells *in vitro* (Fidler, 1974). Hunyadi et al (1981) showed that YPC-1 tumour cells were more susceptible to lysis by immune T-cells after treatment with nitrosoureas. Inactivation of transglutaminase is thought to prevent the coating of tumour cells with fibrin or fibrinogen, this allows the recognition of these tumour cells by the immune T-cells (Hunyadi et al, 1981). These workers suggested that as both the nitrosoureas and isocyanates are known to inactiviate transglutaminase (Laki et al, 1978) then the carbamoylating activity of the nitrosoureas might be responsible for this effect. Therefore, nitrosoureas were considered to be drugs with an immunochemotherapeutic effect, a fact that has been recognised only to a limited extent.

In summary it would appear that nitrosourea interaction with histone or non-histone proteins may induce cytotoxicity by a variety of mechanisms. These include: alteration of the nuclear structure

alterations of the various DNA - protein interactions within the cell; inactivation of specific enzymes or receptors which includes a reduction in the activity of repair enzymes. These modifications may result in an inhibition of transcription or replication of DNA and eventually result in cell death. In addition, the inactivation of glutathione reductase, the inhibition of the polymerisation of tubulin, and the inactivation of transglutaminase may also be involved in the cytotoxicity of nitrosoureas.

1.5.9 The role of the isocyanates in the cytotoxicity of nitrosoureas

A similar but not identical isocyanate concentration to that produced upon the nitrosourea degradation was achieved by the intermittent administration of an isocyanate solution. Exposure of L1210 or Hela cells either to the parent nitrosourea or to the isocyanate by this method showed no deficiency in the repair of gamma irradiation damage (Hilton et al, 1978). This is in direct contrast to the work of Kann et al (1980a, 1980b) who have shown that nitrosoureas are strong inhibitors of repair of L1210 DNA strand breaks produced upon irradiation. This novel approach which was also used in a comparison of the *in vitro* cytotoxicity of L1210 cells exposed to either the parent nitrosourea or the isocyanate, suggested that the isocyanates play a minor role in the cytotoxicity of the nitrosoureas (Hilton et al, 1978). These conclusions of Hilton et al (1978) may be questioned in view of the fact that nitrosoureas are latentiated form of isocyanates which are transported intact into tumour cells (Begleiter et al, 1977) and the addition of extracellular isocyanates at these concentrations may not

be equivalent to the addition of an intact nitrosourea.

Tew and Wang (1982) have recently shown that the resistance of Walker carcinoma cells to bifunctional alkylating agents of the nitrogen mustard type was overcome by the simultaneous addition of N,N^1 - bis(4-hydroxycyclohexyl)-N-nitrosourea. This compound is water soluble and has the potential to undergo carbamoylation reactions. The mechanism by which the potentiation of cytotoxicity occurs is not known. However, in view of the many possible targets for carbamoylation (Section 1.5.7 and 1.5.8) various suggestions can be proposed. Inactivation of specific enzymes such as the enzymes involved in the repair of alkylations or the modification of nuclear structure may provide two separate explanations. Further evidence in support of the hypothesis that carbamoylation may be important in the cytotoxicity of nitrosoureas to Walker carcinoma cells was provided in that chlorozotocin a weak carbamoylator, was the least cytotoxic nitrosourea tested (Tew and Wang,1982).

1.6 The aim and scope of the present work

The aim of the present study was to investigate the mechanism of action of the nitrosoureas. As outlined previously (Section 1.2) the nitrosoureas decompose to produce two major reactive species, either of which may be responsible for the mechanisom of action of the parent compound (Section 1.5). However, the antitumour nitrosoureas, such as BCNU and CCNU have generally been considered to exert their cytotoxicity via their ability to alkylate DNA. The role of the isocyanates in the cytotoxicity of nitrosoureas remains largely speculative (see Section 1.5.9) but is considered to be of minor importance since nitrosoureas which have been designed to have low carbamoylating activity still retain their antitumour effects (Panasci et al, 1977a).

The original stimulus for this study was the observation that the TLX5 lymphoma was naturally resistant to alkylating agents of the 2-chloroethylamine type such as cyclophosphamide but was highly sensitive to the nitrosourea BCNU (Audette et al, 1973). This led to the hypothesis that the mechanism of action of the nitrosoureas against the TLX5 lymphoma was unrelated to their ability to undergo alkylation reactions. As the majority of studies investigating the mechanism of action of the nitrosoureas have utilised only the L1210 leukaemia it was considered important in this study to compare the L1210 leukaemia with the TLX5 lymphoma.

Previous studies on the mechanism of action of other classes of antitumour agents which similarly cleave to release a number of

reactive species, each capable of exerting a cytotoxic effect, have chosen to compare the *in vitro* cytotoxicity of each isolated species to cell lines which are either sensitive or resistant to the progenitor compound *in vivo* (Gescher et al, 1981). This approach has been adopted in this study and both an L1210 leukaemia, and a TLX5 lymphoma with induced resistance to BCNU *in vivo* as well as their sensitive counterparts have been used. Of additional interest was the finding that a TLX5 lymphoma with induced resistance to a dimethyltriazene *in vivo* was cross resistant to the nitrosoureas *in vivo* (Connors and Hare, 1975). It was thus considered that an *in vitro* analysis of the cytotoxic effects of the isolated cytotoxic fragments generated from a nitrosourea, particularly the isocyanates, to this triazene resistant tumour would be interesting since it had not been exposed to either the alkylating or carbamoylating fragments of nitrosoureas *in vivo* as resistance was induced.

The object of the experiments performed in this study was to dissect the overall cytotoxicity of the nitrosoureas into elements of selective and non-selective cytotoxicity. This was considered possible as it has previously been argued that, if a cytotoxic species is observed to preferentially kill cells of a sensitive line *in vitro* in comparison to those which are resistant then this species is likely to be responsible for the elements of selective toxicity, or antitumour activity, observed *in vivo*. On the other hand, when a species has equivalent *in vitro* cytotoxicity to both sensitive and resistant cell lines it was argued that this represents a non-selective cytotoxicity (Gescher et al, 1981).

In this study the results of experiements which were aimed

at assessing the contribution which chloroethylisocyanate and cyclohexylisocyanate, derived from the breakdown of BCNU and CCNU respectively, may make towards the selective toxicity which these nitrosoureas demonstrate in vivo to the TLX5 lymphoma and L1210 leukaemia are reported.

It was hoped that the results obtained in this study would suggest which of the two reactive species produced upon the decomposition of the nitrosoureas is responsible for their observed antitumour activity to both the L1210 leukaemia and TLX5 lymphoma. This in turn should enable the rational synthesis of new agents with an improved therapeutic index in man.

S E C T I O N 2

M A T E R I A L S

2.1 Chemicals and Reagents

2.1.1 Purchased

Cyclohexylisocyanate, Alldrich Chemicals Limited (Gillingham, U.K.). Chloroethylisocyanate, Eastman-Kodak Limited (Kirby, U.K.). Cyclohexylamine, trichloroacetic acid, Bovine serum albumin, 5,5^{1} dithiobis-(2-nitrobenzoic acid), formic acid, calcium nitrate, magnesium sulphate, potassium chloride, di-sodium hydrogen phosphate, sodium chloride, sodium bicarbonate and glucose were purchased from BDH Chemicals (Poole, Dorset)Ltd.

All amino acids were purchased from Sigma.

Dow Corning Silicon oil 550 was obtained from Hopkins and Williams (Essex, England) and Mazola corn oil was purchased from Asda's (Birmingham).

2.1.2 Gifts

BCNU, BFNU, BCTIC, CCNU, CNU, (see Abbreviations) were obtained as gifts from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Maryland.

Chlorozotocin was provided by Dr. K.D. Tew, Georgetown University, Washington D.C.

FCNU was provided by Dr. T.P. Johnston, Southern Research Institute, Birmingham, Alabama.

Nitrogen mustard was the gift of Boots Company, Nottingham, U.K.

CF_3CNU was the gift of Dr. P Coe, Birmingham University, Birmingham, England.

CENA was provided by Professor J.W. Lown, University of Alberta, Edmonton, Canada.

2.1.3 Synthesised

Azolastone and methazolastone were synthesised by Dr. R. Stone (1981), in this department as were DCyNU and DCyU (Abbreviations, page xvii) using the method of Johnstone et al (1981).

MCTIC was synthesised by Professor M.F.G. Stevens using the method of Sheally et al (1975).

2.2 Radiochemicals

[^{14}C] - cyclohexyl CCNU (43.3×10^7 Bq/mmol) was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Maryland.

[methyl - ^3H] thymidine (163×10^{10} Bq/mmol), [2 - ^{14}C] thymidine (2×10^9 Bq/mmol), n-[1 - ^{14}C] hexadecane (33.3×10^4 Bq/ml), n-[1,2 - ^3H] hexadecane (25.9×10^4 Bq/ml) and [1,2 - ^3H] polyethyleneglycol (4.4×10^7 Bq/gm) were all purchased from Amersham International Ltd., Amersham, U.K.

[methyl - ^{14}C] toluene (74×10^7 Bq/mmol), [4 - ^3H] toluene (148×10^{10} Bq/mmol), [methyl - ^3H] thymidine (74×10^{10} Bq/mmol) and

$[2-{}^{14}C]$ thymidine (207×10^7 Bq/mmol) were all purchased from New England Nuclear, Boston, Massachusetts.

2.3 Scintillation Fluids

NEN 260, Nuclear Enterprises Ltd, Edinburgh

Aquassure, New England Nuclear, Boston, Massachusetts

Aquassure, New England Nuclear, Southampton, U.K.

2.4 Cell Culture

2.4.1 Media

2.4.1.1 Purchased

R.P.M.I. 1640 with 25mM Hepes, Gibco, Glasgow

R.P.M.I. 1630, Flow laboratories, Rockville, Md.

Horse serum, Gibco, Glasgow

Fetal Calf Serum, Gibco, Glasgow

Newborn Calf serum, Gibco, Glasgow

Fetal Calf serum, Flow laboratories, Rockville, Md.

Penicillin/Streptomycin (5000 units/ml) Gibco, Glasgow

2.4.1.2 Prepared

2.4.1.2.1 Cell lysis media pH 7.2 (Boyle, 1968)

Ammonium chloride 14.94gms

Tris (hydroxymethyl) amino methane base 4.12gms

Glass distilled water to 2 litres

pH was adjusted to 7.2 by the dropwise addition of IN hydrochloric acid.

2.4.1.2.2 R.P.M.I. colourless media (see Appendix 1) R.P.M.I. colourless media was prepared as described in the appendix

2.4.1.2.3 Sulphanilamide reagent

Sulphanilamide	5gms
2N Hydrochloric Acid to	1 litre

2.4.1.2.4 Bratton Marshall reagent

N-(napthylethylenediamine)dihydrochloride	0.3gm
Glass distilled water to	100mls

2.4.2 Agar

Noble Agar and Bacto Agar were purchased from Difco Laboratories, Detroit, Michigan.

2.4.3 Equipment

25cm^3 tissue culture flasks were obtained from Falcon, Oxnard, California.

40mm^3 petri dishes were obtained from Gibco, Glasgow.

10% CO_2 in air gas cylinders were purchased from the British Oxygen Company, Wolverhampton.

2.5 Estimation of DNA damage by Alkaline Elution

2.5.1 Equipment

60ml polyethylene leur lock syringes, 25mm swinnex type polyethylene filter holders, silicone rubber gaskets, 2μm

polyvinyl chloride filters (25mm diameter), were all purchased from Millipore (U.K.Ltd., Middlesex).

0.8 um polycarbonate filters (25mm diameter) were purchased from Nucleopore Limited (Pleasanton, California). Silicone pump tubing (I.D.- 0.034") was purchased from Elkay products (London) and Intramedic non radiopaque polyethylene tubing was purchased from Clay Adams (A. Horwell Limited, London). 21G leur conical hypodermic needles were obtained from Rocket (London).

2.5.2 Reagent Solutions

All chemicals unless otherwise stated were reagent grade and obtained from B.D.H. Chemicals (Poole, Dorset)Limited.

2.5.2.1 Phosphate buffer saline pH 7.4

Sodium chloride 150mM

Potassium dihydrogen phosphate 4.3mM

Di-potassium hydrogen phosphate 0.7mM

2.5.2.2 EDTA solution pH10

Disodium EDTA 20mM

Sodium hydroxide 40mM

pH was adjusted to 10 by the dropwise addition of 12N hydrochloric acid.

2.5.2.3 Sodium Lauryl Sulphate lysis solution pH10

Glycine 100mM

Sodium Lauryl Sulphate 69mM

Disodium EDTA 25mM

pH was adjusted to 10 by the dropwise addition of 5N sodium hydroxide.

2.5.2.4 Alkaline elution solutions (high pH)

2.5.2.4.1 Tetrapropylammonium hydroxide solution pH >13

EDTA 2.9gms

10% Tetrapropylammonium hydroxide to 500ml

2.5.2.4.2 Tetraethylammonium hydroxide solution pH > 13

EDTA 2.9gms

10% Tetraethylammonium hydroxide to 500ml

2.5.2.5 Alkaline elution solution

2.5.2.5.1 Tetrapropylammonium hydroxide solution pH 12.1

EDTA 5.8gms

25% Tetrapropylammonium hydroxide 70gms

Glass distilled water to 1 litre

pH was adjusted to 12.1 by the dropwise addition of a high pH alkaline elution solution (2.5.2.4.1)

2.5.2.5.2 Tetraethylammonium hydroxide solution pH 12.1

EDTA 5.8gms

25% Tetraethylammonium hydroxide 70gms

Glass distilled water to 1 litre

pH was adjusted to 12.1 by the dropwise addition of a

high pH alkaline elution solution (2.5.2.4.2)

2.5.2.6 0.4M phosphate buffer pH 8.0

A solution of 0.4M potassium dihydrogen phosphate (5.5mls) was made up to 100mls with 0.4M di-sodium hydrogen phosphate solution. This final mixture was 0.4M phosphate buffer and was found to have a pH of 8.0.

2.5.3 Chemicals

Proteinase K was purchased from either E.M. Laboratories Inc (Elmsford, New York) or from B.D.H. Chemicals (Poole, Dorset).

2.6 Animals

CBA/CA male and female mice (18-22g) were supplied by Bantim and Kingman (Hull). BDF1 male and female mice (18-22g) were also supplied by Bantim and Kingman (Hull).

Animals were kept for at least one week before they were used for experiments. They were fed on a 41B modified breeding diet (Pilsbury, Birmingham) and water ad libitum.

2.7 Cell lines (discussed in detail in 3.1)

2.7.1 TLX5 lymphomas

The TLX5S, sensitive to nitrosoureas (Audette et al, 1973), the

TLX5RB, with induced resistance to BCNU (Connors and Hare, 1975) and the TLX5RT, with induced resistance to a dimethyltriazene (Audette et al, 1973), were all obtained from the Institute of Cancer Research, London. The TLX5Cy lymphoma with induced resistance to cyclohexylisocynate was developed in this laboratory (3.1.4).

2.7.2 L1210 Leukaemia

2.7.2.1 <u>In vivo</u>

L1210S leukaemia which is sensitive to nitrosoureas, was obtained from Dr G Atassi, Institute Jules Bordet, Brussels.

L1210R leukaemia with induced resistance to BCNU, was obtained from Dr A.E. Bogden, Mason Research Institute, Worcester, Massachusetts.

2.7.2.2 <u>In vitro</u>

L1210 cells previously established in culture were obtained from Flow Laboratories (Teddington, Middlesex).

SECTION 3

METHODS

3.1 Development of cell lines utilised in this study

3.1.1 TLX5S lymphoma

The TLX5S lymphoma is a fast growing invasive murine tumour which was originally induced in the thymus of CBA mice by X irradiation (Connors and Jones, 1970).

3.1.2 TLX5RB lymphoma

A TLX5 lymphoma resistant to BCNU was originally developed by Connors and Hare (1975). CBA mice were innoculated i.p. with TLX5S lymphoma cells and the animals treated with a subcurative dose of BCNU (5mg/kg), also administered i.p. Tumour cells which survive this treatment were harvested and transplanted back into mice and subsequently treated with an increased dose of BCNU. This procedure was repeated until the survival of mice treated with the maximum tolerated dose of BCNU (40 mg/kg) was no greater than that of the untreated control mice.

3.1.3 TLX5RT lymphoma

A TLX5 lymphoma cell line resistant to 5 – (3,3 – dimethyl – 1 – triazeno) – 4 – carboxyethyl – 2 – phenylimidazole was originally developed by Audette et al (1973). CBA mice were innoculated i.p. with TLX5S lymphoma cells and the animals treated with a subcurative dose of the dimethyltriazene (12.5 mg/kg), also administered i.p. Tumour cells which survive this treatment were harvested and tranplanted back into mice and subsequently treated with an increased

dose of the dimethyltriazene. This procedure was repeated until the survival of mice treated with the maximum tolerated dose of this dimethyltriazeme (50 mg/kg) was no greater than that of the untreated control mice.

3.1.4 TLX5Cy lymphoma

TLX5S lymphoma cells were obtained from a routine passage and suspended in R.P.M.I. 1640 media (6 parts) and horse serum (4 parts) at a cell concentration of 2×10^6 cells/ml. The suspension was treated with a sublethal concentration of cyclohexylisocyanate (10–40 μg 1ml) and then incubated at 37°C for 2 hours. A 0.1ml aliquot (2×10^5 cells) was then injected i.p, into female CBA/CA mice (18–22g). The cells which survived this treatment were harvested from the animals after 7 – 10 days. This procedure was repeated and the cells were treated with an increasing concentration of cyclohexylisocyanate until resistance was observed. These cells were then used for antitumour tests *in vivo*.

3.1.5 L1210S leukaemia

L1210S leukaemia cells originally appeared in DBA/2 after exposure to 3-methylcholanthrene and was first isolated by Law et al (1949). This cell line was found to be sensitive to BCNU by Schabel et al (1963).

3.1.6 L1210R leukaemia

An L1210 leukaemia with resistance to BCNU was developed by Schabel et al (1978). BDF1 mice were innoculated i.p. with L1210S leukaemia cells and the animals treated i.p. with BCNU (8mg/kg). Tumour cells which survive this treatment were harvested and transplanted back into mice and subsequently treated with an increased dose of BCNU. This procedure was repeated until the survival of mice treated with the maximum tolerated dose of BCNU (25mg/kg) was no greater than that of the untreated control mice.

3.2 Routine Passage

The TLX5 lymphoma cell lines were maintained by weekly passage under asceptic conditions into CBA/CA male mice (18–22g). Ascitic fluid (0.1ml) was withdrawn from the peritoneal cavity of a donor mouse and diluted with 10ml of sterile isotonic saline (0.9% w/v). The cell suspension (0.1ml) of approximately 2×10^5 cells was injected i.p. into recipient mice.

The L1210 leukaemia were passaged likewise in BDF1 mice (18–22g).

3.3 Antitumour test

Tumours were obtained by subcutaneous implantation of a TLX5 or L1210 cell suspension in the inguinal region of CBA/CA or BDF1 female mice (18–22g) respectively. Compounds under test were injected i.p. on the third day after tumour implantation as a single dose or as five daily doses from Day 3 – Day 7 where Day 0 is the day of tumour implantation. Injections were prepared by dissolution in DMSO and addition to arachis oil to give a 10% DMSO arachis oil vehicle. The effectiveness of a compound was assessed by its ability to extend the lifespan of the animals, which were in groups of five, compared with controls which received vehicle alone. Results were expressed as a percentage increase in survival time using the following formula:

$$\%\ \text{I.S.T.} = \frac{T - C}{C} \times 100$$

where % I.S.T. is the percentage increase in survival time, T is the mean day of death of treated animals and C is the mean day of death of the untreated control animals.

3.4 In vitro - In vivo Assays

3.4.1 Relationship between cell number innoculated and mean day of death of recipient

A TLX5 lymphoma or L1210 leukaemia cell suspension (2×10^6 cells/ml) harvested from a routine passage was diluted 1 : 10 with R.P.M.I. 1640 media (6 parts) and horse serum (4 parts) with 1% penicillin/streptomycin solution (5,000 units/ml) to give a cell suspension of 2×10^5 cells/ml. This procedure was repeated to give a range of cell suspensions of 2×10^6 cells/ml - 2×10^1 cells/ml. After a 2 hour incubation at $37^{\circ}C$, 0.1ml aliquots (2×10^5 cells/ml - 2×10^0 cells/ml) were injected i.p. into female mice (18–22g) in groups of five. The mean day of death of each group of animals was then compared with the cell number of the injected suspension.

3.4.2 In vitro - In vivo cytotoxicity assay

Various concentrations of the drugs studied dissolved in DMSO were added to a suspension (2×10^6 cells/ml) of TLX5 lymphoma cells or L1210 leukaemia cells, in RPMI 1640 (6 parts) and horse serum (4 parts) with 1% penicillin/streptomycin solution (5000 units/ml). After a 2 hour incubation at $37^{\circ}C$, 0.1 ml aliquots (2×10^5 cells) of the suspension were injected i.p. into female mice (18–22g). The cytotoxic effect of the drug was assessed by comparing the life span of these mice with those which had been innoculated with known cell numbers (3.4.1). In experiments where there was sufficient data the results were

expressed as an estimated log cell kill. A direct comparison of the percentage increase in survival time of the sensitive and resistant cell lines assumes that when an equivalent cell number of untreated sensitive or resistant cells is innoculated into mice an indentical survival time will result. This was found not to be the case. An estimated logarithmic cell kill produced by each compound <u>in vitro</u> (before injecting the cells into the animals) was calculated by reference to assays of the survival time of animals given from 2×10^5 to 2×10^0 cells of each cell line (3.4.1). This was considered possible in view of the fact that the life span of BDF1 mice was directly related to the number of L1210 cells injected i.p. (Skipper et al, 1964). Also the life span of CBA mice was directly related to the number of TLX5 cells injected i.p. (Connors and Hare, 1975).

Otherwise the results were expressed as a percentage increase in survival time which was calculated using the following formula.

$$\% \text{ I.S.T.} = \frac{T - C}{C} \times 100$$

where %I.S.T. is the percentage increase in survival time, T is the mean day of death of the treated animals and C is the mean day of death of the untreated control animals.

3.5 *In vitro* cell culture methods

3.5.1 Routine culture of the TLX5 lymphoma cell lines

Ascitic fluid was withdrawn from the peritoneal cavity of male CBA/CA mice bearing a routine passage of either the TLX5S or TLX5RT lymphomas. This fluid was added to saline and mixed well. The cells were spun down on a Minor bench centrifuge (MSE, Sussex) at approximately 500g and resuspended in R.P.M.I. 1640 media supplemented with 20% horse serum to give a cell concentration of 5 - 10 x 10^4 cells/ml. Cells were gassed with 10% CO_2 in air and incubated at 37°C. Stock cultures were maintained in exponential phase at a density of 0.05 - 3 x 10^6cells/ml.

3.5.2 Routine culture of L1210 leukaemia

L1210 leukaemia cells were either grown in spinner culture in R.P.M.I. 1630 media supplemented with 20% heat inactivated (56°C, 30 mins) fetal calf serum and incubated at 37°C, or, grown in R.P.M.I. 1640 media supplemented with 10% fetal calf serum. Cells were gassed with 10% CO_2 in air and incubated at 37°C. Stock cultures were maintained in exponential phase at a density of 0.05 - 3 x 10^6 cells/ml.

3.5.3 Colony forming assay's

3.5.3.1 TLX5 Colony forming assay

Various methods were employed in an attempt to establish a consistent and reliable method which optimised the colony forming ability of the TLX5S and TLX5RT lymphoma cells. In all methods log

phase cultivated TLX5S and TLX5RT lymphoma cells were used.

Method 1

The first method used was that of Hamburger and Salmon (1977) in which 5% noble agar (1ml) was added to RPMI 1640 media (9mls) supplemented with 20% horse serum and 1% penicillin/streptomycin solution (5000 units/ml). This mixture was added to sterile $40mm^3$ petri dishes and allowed to stand for 15 mins at room temperature to solidify.

3% noble agar (1ml) was added to RPMI 1640 media (8mls) supplemented with 20% horse serum and 1% penicillin/streptomycin solution (5000 units/ml). This mixture was allowed to cool to about $40^{\circ}C$ before 1ml of either 10^6 cells/ml, 10^5 cells/ml or 10^4 cells/ml was added. This gave a range of final cell concentrations of $10^5 - 10^3$ cells/ml. An aliquot of this cell suspension (1ml) was then plated out on top of the 0.5% noble agar mixture (1ml) and was left to stand for 15 minutes at room temperature.

The petri dishes were then covered and placed in air tight containers under an atmosphere of 10% CO_2 in air, which was replenished every 24 hours, and incubated at $37^{\circ}C$. The appearance of colonies was estimated after 10 - 14 days incubation.

Method 2

The second method adopted was that of Tatsumi et al (1979) in which a range of cell suspensions in R.P.M.I. 1640 media (2mls) supplemented with 10% horse serum was added to 2.4% bacto agar (0.4mls) to give final cell concentrations of $10^5 - 10^3$ cells/ml. An aliquot of each cell suspension (2mls) was plated out into a $40mm^3$ sterile petri dish and was left to stand for 15 minutes at room temperature.

The petri dishes were then covered and placed in air tight containers under an atmosphere of 10% CO_2 in air, which was replenished every 24 hours, and incubated at 37^oC. The appearance of colonies was estimated after 10 - 14 days incubation.

Method 3

The third method employed was that of Chu and Fischer (1968). In this case a cell suspension ($10^6 - 10^4$ cells/ml) in R.P.M.I. 1640 media supplemented with 20% horse serum was seeded out in 0.1% noble agar to give a range of cell numbers from 10^5 to 10^3 cells/ml. After a period of a 10 - 14 day incubation at 37^oC in 6ml colony tubes the number of colonies was estimated.

3.5.3.2 L1210 Colony forming assay

For L1210 colony forming assay the method of Chu and Fischer (1968) was adopted. L1210 cells were exposed to drugs for 2 hours at 37°C and then centrifuged at 500g for 5 minutes, washed and resuspended in R.P.M.I. 1630 media supplemented with 20% heat inactivated fetal calf serum. Cells were seeded out in 0.1% noble agar in the above media, ranging from 10^5 to 10^3 cells/ml. Colony forming ability of the cells was determined by counting colonies ($>$32 cells) after a period of 10–14 days incubation at 37°C. Drug treated cells were then compared with untreated control cells. The plating efficiency of the control L1210 cells was always $>$70%.

3.5.4 Cell Counting

A coulter counter, model ZBI (Coulter Electronics, Harpenden, U.K.) was used to count TLX5 lymphoma and L1210 leukaemia cells during routine culture. An aliquot of tumour cell suspension was diluted with "isoton" (modified Eagles' medium; Coulter Electronics) and counted at the predetermined settings of Amplification 4; Aperture Current, ½; Lower Threshold, 13; Upper Threshold 85; and Aperture diameter 100 μ. These settings excluded the counting of erythrocytes.

3.6 Non protein thiol assay

Ascitic tumour cells were harvested from a routine passage and washed with cell lysis media. (2.4.1.2.1). After centrifugation at 500g for 5 minutes the tumour cell pellet was homegenised in 5% trichloroacetic acid (5mls per gm cells). The preparations were centrifuged at approximately 300g for 10 minutes and an aliquot of the supernatant (o.5ml) was added to 0.4M phosphate buffer (3.2mls)(2.5.2.6) before the addition of 0.01M 5,5'-dithiobis(2-nitrobenzoic acid)(0.3ml) in phosphate buffer (Ellman, 1959). Duplicate samples were incubated at room temperature for 30 minutes and the absorbance measured at 412nm using a Cecil CE 5095 spectrophotometer. Standards prepared with reduced glutathione were assayed concurrently and protein content determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

3.7 Studies of nitrosourea transport

TLX5 lymphoma cells (10^7cells/ml) were incubated in R.P.M.I. 1640 media for 15 minutes before the addition of 3.25×10^4Bq/ml of (^{14}C)-cyclohexyl CCNU (43.3×10^7 Bq/mmol). L1210 leukaemia cells (10^7 cells/ml) were incubated likewise before the addition of 1.625×10^4 Bq/ml of (^{14}C)-cyclohexyl CCNU (43.3×10^7Bq/mmol). 10^6 cells were removed at time periods between 0 and 120 minutes and were placed into tubes in which 100 µl of a mixture of Dow Corning silicon oil 550 and corn oil (10 parts to 3) was layered above 50 µl of 98% formic acid. The tubes were cetrifuged at 9000g for 30 seconds using a Beckman microfuge B, frozen in liquid nitrogen and then cut across the oil layer. Each part was placed into a scintillation vial, thawed, and 10ml of NEN 260 was added as a scintillant. Samples were counted using a Packard Tricarb 2606 liquid scintillation counter.

3.8 Determiniation of the chemical half lives

Various concentration of nitrosoureas were dissolved in colourless R.P.M.I. media (2mls) to which sulphanilamide reagent (1ml) was added. These solutions were incubated at 50°C for 45 minutes before Bratton Marshall reagent (0.2ml) was added at 0°C. After 10 minutes at room temperature the absorbance was measured at 540nm using a Cecil CE 5095 spectrophotometer. These absorbances were then plotted against concentration and a calibration graph obtained.

To determine the half life of the nitrosourea, 10 μcg/ml in colourless R.P.M.I. media was incubated at 37°C for various time intervals before the procedure outlined above was followed. The rate of reaction and the half life of the nitrosourea was obtained by comparing the absorbances at set time intervals with those of the calibration graphs. The logarithm of the concentrations obtained was then plotted with respect to time.

3.9 Estimation of DNA damage by alkaline elution

L1210 leukaemia cells were labelled with 370 - 740 Bq/ml of [2 - ^{14}C] thymidine or with 1850 - 3700 Bq/ml of [methyl-^{3}H] thymidine (see Section 2.2). After a 24 hour incubation period these cells were resuspended in fresh media in the absence of label and incubated for a further 12 hours at 37°C.

2 x 10^6 cells/ml, labelled with [2 - ^{14}C] thymidine were treated with various concentrations of drugs. After a 2 hour incubation at 37°C, 2-5 x 10^5 drug treated [2 - ^{14}C] thymidine labelled cells were mixed in ice cold phosphate buffered saline (2.5.2.1) and were either left unirradiated at 0°C or were irradiated with 3 Gy of Xrays at 0°C. The Xrays were delivered by 2 vertically opposed Philips RT - 250 Xray tubes operating at 250 keV, 15ma, equipped with 0.55mm aluminium and 0.25mm copper filters.

5 x 10^5 cells, labelled with [methyl-^{3}H] thymidine and which had been irradiated with 3Gy of Xray at 0°C were then added to both the unirradiated and irradiated cells labelled with [2 - ^{14}C] thymidine. (The [methyl-^{3}H] thymidine labelled cells serve as an internal standard). Cell suspensions were kept at 0°C to inhibit the activity of any DNA repair enzyme systems that may be present.

The cells were layered, using mild suction, onto a 25mm, 2 μm polyethylene chloride filter which was held within a 25mm polyethylene, swinnex type, filter holder, attached to a 60ml polyethylene leur lock

syringe. The cells were immediately lysed with a sodium lauryl sulphate lysis solution (2.5.2.3) and following lysis, the filter and lysate were washed with an EDTA solution pH 10 (2.5.2.2).

An alkaline elution solution (2.5.2.5) was pumped, by a Gilson Minipuls II (Anachem Limited, Luton) pump, through the filter at 0.035ml/min via 0.035" inside diameter silicone manifold pump tubing. The solution was delivered from the filter holder to the fraction collector via 0.030" inside diameter polyethylene tubing. Fractions (3 hours) were collected over an 18 hour period by an ISCO Model 2111 (MSE Scientific Instruments, Sussex) fraction collector which was adapted to accomodate 80 scintillation vials.

After 18 hours the filter was removed and placed in a scintillation vial to which 0.4ml of 1N HCL was added. The vial was sealed and heated at 60°C for 1 hour to depurinate the DNA. After 1 hour 2.5mls of 0.4N NaOH was added to the vial which was shaken vigourously and allowed to stand for 1 hour at room temperature. The NaOH treatment converted apurinic sites to strand breaks and fragmented the DNA. The filter holder and the pump tubing were washed with 10mls of 0.4N NaOH and a 2.5ml aliquot of this solution was counted. A 2ml aliquot of the lysis solution was also taken for counting. Water was added to all fractions to give a final volume of 6mls. The fractions were then mixed with 10ml of Aquassure liquid scintillation counting reagent, to which 0.7% glacial acetic acid had been added to prevent chemilluminescance in the alkaline solution.

Samples were counted on a Packard 2450B liquid scintillation counter using internal standards or on a Packard Tricarb 2660 liquid scintillation counter with quench correction using external standards.

Using the above procedure single strand breaks were measured by comparing the elution profiles of control, untreated unirradiated cells with drug treated unirradiated cells. Total DNA cross linking (DNA-DNA interstrand cross linking and DNA-protein cross linking) was measured by comparing the elution profiles of untreated irradiated cells with drug treated irradiated cells.

3.9.1 DNA-DNA interstrand cross linking assay

DNA-DNA interstrand cross linking was measured by minimising DNA-protein cross linking. This was achieved using the modifications suggested by Kohn et al (1981) to the general procedure.

0.8 μm pore size polycarbonate filters were used in order to minimise protein adsorption to the filter. Following lysis, a lysis solution of sodium lauryl sulphate containing proteinase K (0.5 mg/ml) was pumped through the filter for approximately 1 hour. The proteinase enzyme was included in the lysis solution in order to remove protein that had been cross linked to the DNA. The procedure as outlined previously (3.9) was now followed except that 0.1% sodium dodecyl sulphate was added to the alkaline elution solution.

DNA interstrand cross linking was expressed as a cross link

index which is defined as

$$\text{Cross link index} = \sqrt{\frac{1 - ro}{1 - r}} - 1$$

where r and ro are the fractions of ^{14}C – labelled DNA from treated and untreated control cells remaining on the filter when 25% of the ^{3}H– labelled DNA is still retained on the filter (Kohn et al, 1981).

Since ^{3}H – labelled DNA internal standard elution has been shown to be nearly first order with regard to time (Kohn et al, 1981) the fraction of ^{14}C – labelled DNA retained on the filter was plotted on a double logarithmic scale against the fraction of ^{3}H – labelled DNA retained on the filter. In this way both r and ro were determined and when substituted into the equation allowed the calculation of a cross link index.

3.9.2 DNA – protein cross linking assay

Cells labelled with $[2 - ^{14}C]$ thymidine and treated as before and cells labelled with $[\text{methyl} - ^{3}H]$ thymidine were irradiated with 30Gy of X-rays at $0^{o}C$. X rays were delivered by 2 vertically opposed Philips R-250 X ray units operating at 200keV with 0.25mm copper and 0.55mm aluminium filters. The mixture of cells was then subjected to alkaline elution using polyvinylchloride filters and without the use of proteinase K as previously described (3.9).

DNA-protein cross link frequency was estimated using the

formula derived by Kohn and Ewig (1979).

$$\sqrt{1 - r} = 1 + \frac{Pr}{b} + \frac{PD}{b}$$

where r is the fraction of slow eluting DNA extrapolated to zero time, Pr and PD are DNA - protein cross link frequencies due to X rays and drug respectively, and b is the single strand break frequency due to X rays. At a 30Gy Xray dose drug induced strand breaks are negligible (Kohn and Ewig, 1979). DNA-protein cross link frequency was calculated as follows

$$PD = (\sqrt{1 - r} - \sqrt{1 - r^1}) - (\sqrt{1 - ro} - \sqrt{1 - ro^1})b$$

where r and ro are the fraction of slow eluting DNA for drug treated and untreated control cells, respectively, and r^1 and ro^1 are the corresponding values for the ^3H-labelled cells assayed together with drug treated or untreated control cells, respectively. The fraction of slow eluting DNA was estimated graphically by extrapolating the elution curves to zero time. The single strand break frequency, b, due to 30Gy was assumed to be 2.7 per 10^6 nucleotides (Kohn et al, 1976).

SECTION 4

RESULTS

4.1 *In vivo* antitumour activity of the haloalkylnitrosoureas

4.1.1 Activity of the haloalkylnitrosoureas and related compounds against the TLX5S, TLX5RT and TLX5RB lymphomas

The antitumour activity of the haloalkylnitrosoureas BCNU, CCNU, BFNU and FCNU against the TLX5S, TLX5RT and TLX5RB lymphomas is shown on Tables 2 - 5. The percentage increase in survival times (% I.S.T.) of these four haloalkylnitrosoureas against the TLX5S lymphoma were consistently higher than those against the TLX5RT and TLX5RB lymphomas. In this study the antitumour activity of a compound is significant when the % I.S.T. ≥ 20% as this is considered to be greater than a 2 log cell kill. On this basis BCNU, CCNU, BFNU and FCNU were observed to have significant antitumour activity at 10, 20 and 40 mg/kg.

The antitumour activity of chlorozotocin against the TLX5S and TLX5RT lymphomas is shown on Table 6. Chlorozotocin has significant antitumour activity only at 20mg/kg and compared with BCNU, CCNU, BFNU and FCNU has reduced activity against the TLX5S lymphoma.

Cyclophosphamide and chlorambucil are both inactive against the TLX5S lymphoma (Table 7 and 8). Chlorambucil has also no *in vivo* antitumour activity against the TLX5RT lymphoma (Table 8). These results are in agreement with those of Audette et al (1973) and show that the TLX5S lymphoma is naturally insensitive to the alkylating agents of the 2-chloroethylamine type. Of interest is the fact that cyclophosphamide was found to have marginal antitumour activity against the TLX5RT lymphoma (Table 8). These results are in agreement with those of Audette et al (1973) and show that the TLX5S lymphoma is

naturally insensitive to the alkylating agents of the 2-chloroethylamine type. Of interest is the fact that cyclophosphamide was found to have marginal antitumour activity against the TLX5RB lymphoma (Connors and Hare, 1975). It would thus appear that a collateral sensitivity to alkylating agents had developed when the TLX5RB lymphoma was made resistant to BCNU (Connors and Hare, 1975).

The antimetabolite, methotrexate, was found to have significant antitumour activity against both the TLX5S and TLX5RT lymphomas as shown on Table 9. Methotrexate has also been shown to have significant activity against the TLX5RB lymphoma (Connors and Hare, 1975). It should be noted that methotrexate was administered as five daily doses from Day 3 - Day 7 whereas the haloalkylnitrosoureas and the known alkylating agents cyclophosphamide and chlorambucil were administered as a single dose on Day 3. These different treatment schedules are those previously used on this tumour model for antimetabolites and alkylating agents respectively (Connors and Hare, 1975).

Cyclohexylisocyanate, a known degradation product of CCNU was tested against the TLX5S lymphoma (Table 10) and was found to have no significant antitumour activity <u>in vivo</u>. However, it was found to be toxic to the host animal at the doses tested as indicated by the death of treated mice before the death of untreated control mice.

1-(N-(2-chloroethyl)-N-nitroso)acetamide (CENA) has been suggested to decompose to produce a 2-chloroethyldiazohydroxide species (Hecht and Kozarid, 1973). This species is also produced upon the decomposition of the chloroethylnitrosoureas BCNU and CCNU (Lown et al,1979).

CENA was found to have no significant *in vivo* antitumour activity against the TLX5S lymphoma (Table 10) and was found to be toxic to the host animal at the doses tested.

1,3-dicyclohexyl-1-nitrosourea (DCyNU), a compound which is known to produce cyclohexylisocyanate upon its decomposition (Wheeler 1976), had no significant *in vivo* antitumour activity against the TLX5S lymphoma when administered either as a single dose or as five daily injections from Day 3 - Day 7 (Table 11).

It was considered relevant to investigate the antitumour activity of compounds which are potent carbamoylators, as predicted by their ability to inhibit glutathione reductase (Eisenbrand, 1982). 1-(N-butylcarbamoyloxy)-pyrrolidin-2,5-dione(BCPD) and 1-(N-(2-chloroethyl)-carbamoyl)-imidazole (CCI) were tested against the TLX5S lymphoma and found to have no significant antitumour activity *in vivo*(Table 12).

4.1.2 Activity of the haloalkylnitrosoureas and cyclophosphamide against the L1210S and L1210R leukaemias

BCNU and CCNU were found to have excellent antitumour activity against the L1210S leukaemia *in vivo* (Tables 13 and 14). In this study the tumours were obtained by subcutaneous implantation in the inguinal region (Section 3.3) whereas in most studies reported in the literature on this tumour model tumours were obtained by i.p. implantation (Schabel, 1976). BCNU and CCNU were found to produce a %I.S.T. $>$ 20% against the L1210S leukaemia *in vivo* at 10, 20 and 40 mg/kg. Indeed long extensions in survival time were observed in mice treated with 40 mg/kg. However the L1210R leukaemia was found to be resistant to both BCNU and CCNU (Tables 13 and 14) and no extensions in survival

time were observed.

Chlorozotocin was found to have slight yet significant antitumour activity against the L1210S leukaemia (Table 15) but only at 20 mg/kg. In contrast to the results presented in this study chlorozotocin has previously been shown to produce a long extension in the survival time of mice innoculated i.p. with L1210S leukaemia (Montgomery, 1976). The difference in the results obtained may be explained by the different sites of tumour innoculation.

Cyclophosphamide was found to have significant antitumour activity against both the L1210S and L1210R leukaemias (Table 16). Long extensions in survival time were observed in mice innoculated with either L1210S or L1210R leukaemias and then treated with 200 or 400 mg/kg of cyclophosphamide as a single dose.

Table 2 The antitumour activity of BCNU against the TLX5S, TLX5RT, TLX5RB lymphomas

Dose Mg/kg	Days of Treatment	TLX5S				TLX5RT				TLX5RB	
		M.D.D.		%I.S.T.		M.D.D.		%I.S.T		M.D.D.	%I.S.T
		Experiment Number									
		1	2	1	2	1	2	1	2	1	1
Control	Day 3 only	10.0	9.6	–	–	10.4	8.8	–	–	10.3	–
5	"	11.1	12.6	11	31	11.0	–	5	–	10.8	5
10	"	14.3	14.0	43	46	11.6	9.0	12	2	10.0	–3
20	"	14.3	14.8	43	54	11.8	9.2	13	5	10.2	–1
40	"	16.6	15.4	66	60	9.2	9.4	–11	7	10.4	1
80	"	6.5	11.9	–35	24	7.0	10.8	–32	22	8.2	–20

M.D.D. – Mean day of death, % I.S.T. – Percentage increase in survival time

Table 3 The antitumour activity of CCNU against the TLX5S, TLX5RT and TLX5RB lymphomas

Dose Mg/kg	Days of Treatment	TLX5S M.D.D.		TLX5S %I.S.T.		TLX5RT M.D.D.		TLX5RT %I.S.T		TLX5RB M.D.D.	TLX5RB %I.S.T.
		Experiment Number									
		1	2	1	2	1	2	1	2	1	1
Control	Day 3 only	9.4	10.0	–	–	9.2	9.4	–	–	10.3	–
5	"	12.0	10.4	28	4	–	–	–	–	10.2	–1
10	"	14.4	12.4	53	24	10.0	9.8	9	5	10.2	–1
20	"	14.8	16.0	57	60	10.4	9.4	13	0	10.2	–1
40	"	14.0	15.2	49	52	10.4	10.8	13	15	10.8	5
80	"	9.2	9.0	–2	–10	9.0	8.6	–2	–9	9.0	–13

M.D.D. – Mean day of death, % I.S.T. – Percentage increase in survival time

Table 4 The antitumour activity of BFNU against the TLX5S and TLX5RT lymphomas

Dose mg/kg	Days of Treatment	TLX5S		TLX5RT	
		M.D.D.	% I.S.T.	M.D.D.	% I.S.T.
Control	Day 3 only	10.0	-	9.8	-
10	"	13.5	35	9.6	-2
20	"	14.3	43	9.4	-4
40	"	15.0	50	9.8	0
80	"	9.2	-8	9.8	0

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time.

Table 5 The antitumour activity of FCNU against the TLX5S and TLX5RT lymphomas

Dose mg/kg	Days of Treatment	TLX5S		TLX5RT	
		M.D.D.	% I.S.T.	M.D.D.	% I.S.T.
Control	Day 3 only	14.2	-	9.8	-
5	"	13.6	-4	9.4	-4
10	"	15.4	9	9.8	0
20	"	*18.0	*27	10.0	2
40	"	> 60	> 300	10.0	2
80	"	> 60	> 300	8.2	-16

* 3 survivors > Day 60 not included in results

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

Table 6 The antitumour activity of chlorozotocin against the TLX5S and TLX5RT lymphomas

Dose mg/kg	Days of Treatment	TLX5S M.D.D.		TLX5S %I.S.T.		TLX5RT M.D.D.	TLX5RT %I.S.T.
		Experiment Number					
		1	2	1	2	1	1
Control	Day 3 only	10.0	10.0	-	-	9.0	
5	"	9.6	10.0	-4	0	9.2	2
10	"	10.8	11.2	8	12	9.4	4
20	"	14.4	13.2	44	32	9.4	4
40	"	8.8	9.2	-12	-8	7.8	-13
80	"	6.2	7.6	-38	-24	6.6	-27

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

Table 7 The antitumour activity of cyclophosphamide against the TLX5S lymphomas

Dose mg/kg	Days of Treatment	TLX5S M.D.D.	TLX5S %I.S.T.
Control	Day 3 only	12.0	-
50	"	14.0	17
100	"	14.2	18
200	"	13.0	8
400	"	9.8	-18
800	"	4.0	-67

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

Table 8 The antitumour activity of chlorambucil against the TLX5S and TLX5RT lymphomas

Dose mg/kg	Days of Treatment	TLX5S		TLX5RT	
		M.D.D.	%I.S.T.	M.D.D.	%I.S.T.
Control	Day 3 only	10.8	-	10.0	-
5	"	10.6	-2	10.2	2
10	"	10.6	-2	11.0	10
20	"	10.6	-2	10.8	8
40	"	10.4	-4	8.2	-18
80	"	3.0	-72	3.0	-70

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

Table 9 The antitumour activity of methotrexate against the TLX5S and TLX5RT

Dose mg/kg	Days of Treatment	TLX5S			TLX5RT		
		M.D.D.	%I.S.T.	ΔW	M.D.D.	%I.S.T.	ΔW
Control	Day 3 - Day 7	10.4	-	+2.2	10.4	-	+0.8
0.3125	"	13.4	29	-	14.4	39	-
0.625	"	14.0	35	-	15.6	42	-
1.25	"	15.8	52	-	16.3	57	-
2.5	"	16.2	56	+0.3	18.2	75	+0.2
5.0	"	13.4	29	-1.6	10.6	2	-0.7

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time, ΔW - Weight change between Day 0 and Day treatment ends.

Table 10 The antitumour activity of cyclohexylisocyanate and CENA against the TLX5S lymphoma

Compound	Dose mg/kg	Days of Treatment	TLX5S	
			M.D.D.	%I.S.T.
Cylohexyl isocyanate	Control	Day 3 only	10.0	-
	5	"	10.4	4
	10	"	9.8	-2
	20	"	8.6	-14
	40	"	3.8	-62
	80	"	3.8	-62
CENA	Control	Day 3 only	10.8	-
	5	"	10.2	-6
	10	"	9.6	-11
	20	"	6.6	-40
	40	"	6.0	-44
	80	"	5.2	-52

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

Table 11 The antitumour activity of DCyNU against the TLX5S lymphoma

Dose mg/kg	Days of Treatment	TLX5S		Days of Treatment	TLX5S		
		M.D.D.	% I.S.T.		M.D.D.	% I.S.T.	ΔW
Control	Day 3 only	10.8	–	Day 3 – 7	10.1	–	+0.9
5	"	9.6	–11	"	9.8	–3	–
10	"	10.4	–4	"	10.0	–1	–
20	"	10.0	–7	"	9.6	–5	–
40	"	10.0	–7	"	10.0	–1	–
80	"	10.2	–5	"	9.4	–7	+0.3
160	"	–	–	"	9.3	–8	–1.8

M.D.D. – Mean day of death, % I.S.T. – Percentage increase in survival time, ΔW – Weight change between Day 0 and Day treatment ends

Table 12 The antitumour activity of BCPD and CCI against the TLX5S lymphoma

Compound	Dose mg/kg	Days of Treatment	TLX5S	
			M.D.D.	%I.S.T.
BCPD	Control	Day 3 only	10.8	-
	2.5	"	12.2	13
	5	"	12.0	11
	10	"	11.8	9
	20	"	11.6	7
	40	"	8.0	-26
CCI	Control	Day 3 only	10.8	-
	2.5	"	12.2	13
	5	"	11.4	6
	10	"	11.4	6
	20	"	11.2	4
	40	"	11.9	9
CCI	Control	Day 3 only	11.0	-
	50	"	10.8	-2
	60	"	11.0	0
	80	"	10.8	-2
	100	"	10.2	-8

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

Table 13 The antitumour activity of BCNU against the L1210S and L1210R leukaemias

Dose mg/kg	Days of Treatment	L1210S						L1210R			
		M.D.D.			% I.S.T.			M.D.D.		%I.S.T.	
		Experiment Number									
		1	2	3	1	2	3	1	2	1	2
Control	Day 3 only	9.2	11.1	11.9	–	–	–	9.4	8.6	–	–
5	"	10.2	11.4	12.4	11	3	4	9.2	9.4	–2	9
10	"	11.4	15.4	15.3	24	34	29	9.6	9.3	2	8
20	"	14.2	21.6	19.2	54	95	61	10.8	9.8	15	14
40	"	60	29.7	25.0*	500	168	110*	10.2	10.4	8	21
80	"	8.4	8.3	10.2	–9	–25	–14	7.4	8.6	–22	0

* 2 survivors > Day 60 not included in results

M.D.D. – Mean day of death, % I.S.T. – percentage increase in survival time

Table 14 The antitumour activity of CCNU against the L1210S and L1210R leukaemia

Dose mg/kg	Days of Treatment	L1210S M.D.D.			L1210S % I.S.T.			L1210R M.D.D.		L1210R %I.S.T.	
	Experiment Number	1	2	3	1	2	3	1	2	1	2
Control	Day 3 only	9.2	10.3	9.9	–	–	–	9.4	8.6	–	–
5	"	11.2	12.2	11.3	22	18	15	9.4	9.1	0	6
10	"	13.4	18.4	13.7	46	50	38	10.2	9.6	9	12
20	"	18.6	21.2	22.4	102	106	126	10.2	10.2	9	19
40	"	> 60	21.6	11.4*	> 500	110	15*	12.2	11.7	30	36
80	"	8.3	9.2	7.5	–10	–11	–24	8.6	7.4	–9	14

* 3 survivors   > Day 60 not included in results

M.D.D. – Mean day of death

% I.S.T. – Percentage increase in survival time

Table 15 The antitumour activity of chlorozotocin against the L1210S leukaemia

Dose mg/kg	Days of Treatment	L1210S	
		M.D.D.	% I.S.T.
Control	Day 3 only	12.4	-
10	"	13.8	11
20	"	16.4	32
40	"	8.4	-32
80	"	8.0	-36

M.D.D. - Mean day of death

%I.S.T. - Percentage increase in survival time

Table 16 The antitumour acitivity of cyclophosphamide against the L1210S and L1210R leukaemias

Dose mg/kg	Days of Treatment	L1210S		L1210R	
		M.D.D.	% I.S.T.	M.D.D.	% I.S.T.
Control	Day 3 only	12.4	-	9.8	-
50	"	16.8	35	13.0	33
100	"	18.0	45	15.2	55
200	"	> 60	> 400	18.5*	89*
400	"	> 60	> 400	23.6+	145+

* 1 survivor > Day 60 + 2 Survivors > Day 60 both not included in results

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

4.2 *In vitro - In vivo* cytotoxicity assays

4.2.1 The relationship between cell number of either TLX5 lymphoma or L1210 leukaemia cells innoculated and the mean day of death

Serial logarithmic dilutions of the sensitive and resistant lines of both the TLX5 lymphomas and the L1210 leukaemias and their injection into animals, after incubation *in vitro* at 37° for 2 hours (see Section 3.4.1), showed that a linear inverse relationship exists between the survival time of the host and the number of cells injected into the host (Figures 9 - 13). In this study the control animals received 2×10^5 untreated cells and the % I.S.T. for this number of cells is thus 0%. After calculation of the % I.S.T. produced by each drug concentration in the *in vitro - in vivo* cytotoxicity assay, the relationship between log cell kill and drug concentration may be estimated. Such estimates will be presented below.

4.2.2 *In vitro - In vivo* cytotoxicity assay of BCNU, CCNU, chloroethylisocyanate, and cyclohexylisocyanate against the TLX5S, TLX5RT and TLX5RB lymphomas

The estimated log cell kills of TLX5S, TLX5RT and TLX5RB lymphomas by BCNU, CCNU, chloroethylisocyanate and cyclohexylisocyanate are shown in Figures 14 - 17. The TLX5RT and TLX5RB lymphomas which were resistant *in vivo* to BCNU and CCNU (Tables 2 and 3) were also resistant *in vitro* (Figures 14 - 17). In addition the TLX5RT and TLX5RB lymphomas were resistant *in vitro* to both chloroethylisocyanate and cyclohexylisocyanate (Figures 14 - 17). The degree of resistance of the TLX5RT and TLX5RB lymphomas *in vitro* to BCNU, CCNU, chloroethylisocyanate and cyclohexylisocyanate at an estimated 3 log cell kill is

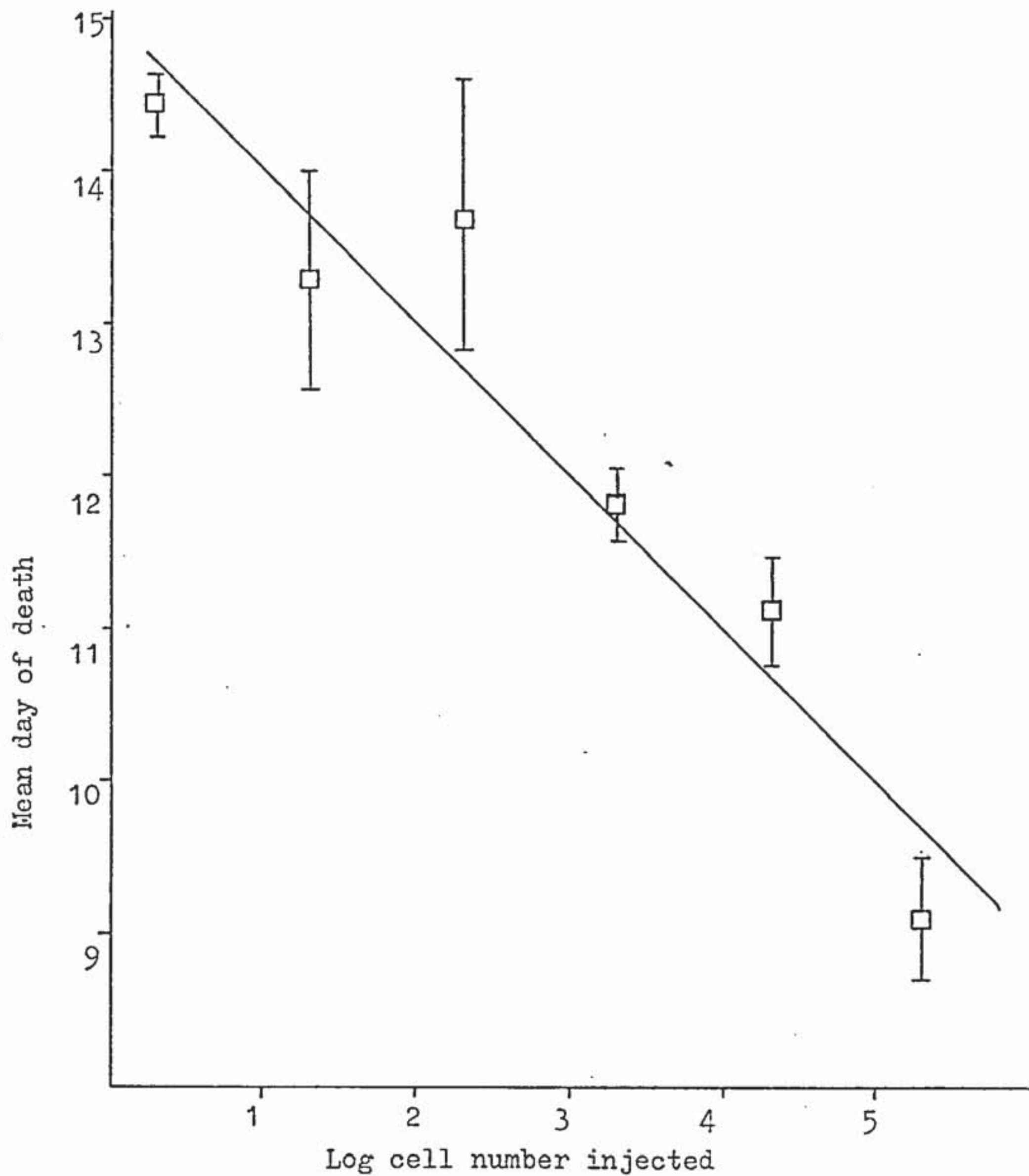


FIGURE 9

Linear relationship between the number of TLX5S lymphoma cells innoculated i.p. and the mean day of death of CBA/CA mice. Line was fitted by linear regression analysis r = -0.952. Each point is the mean and s.d. of three separate experiments.

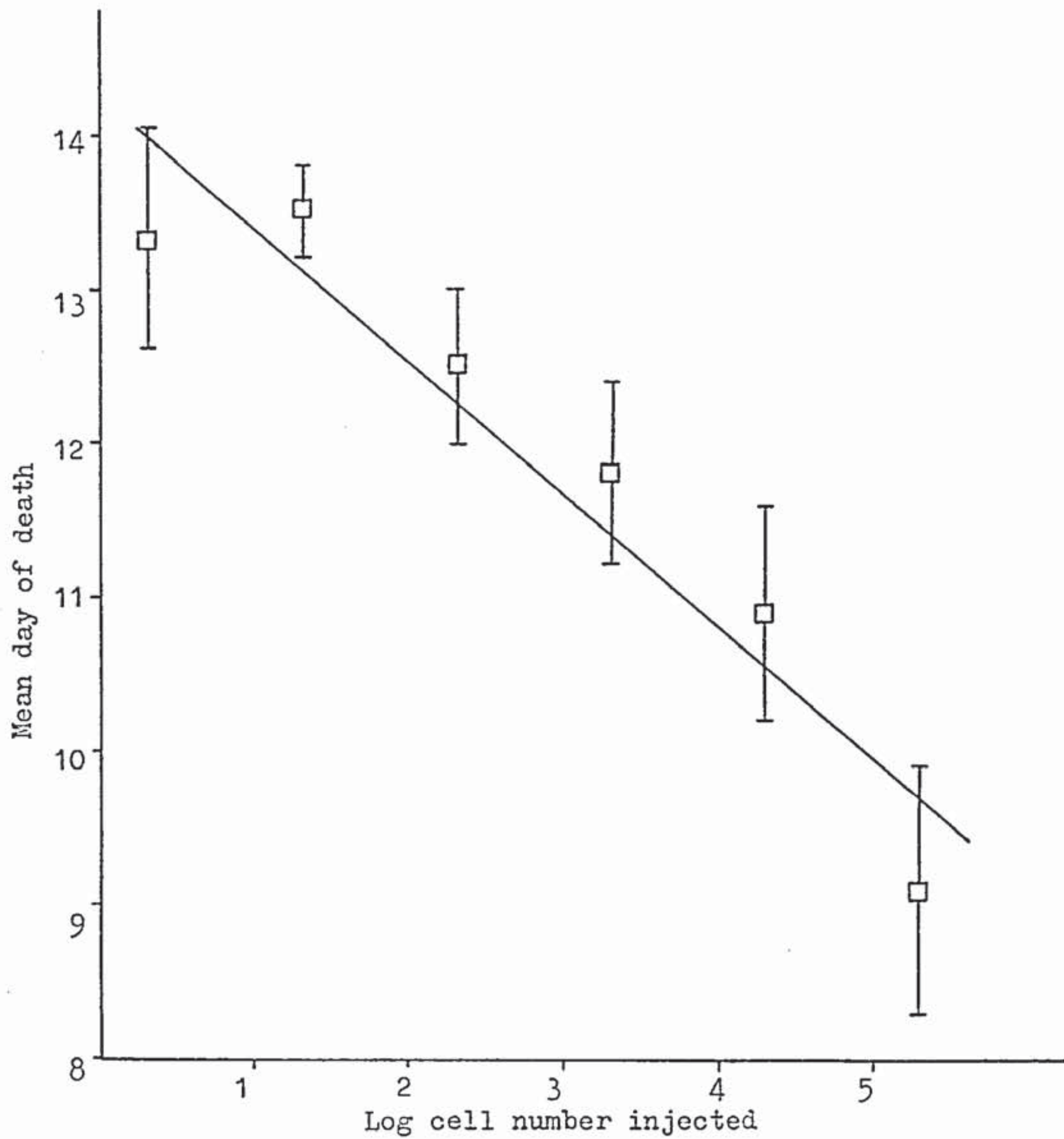


FIGURE 10 Linear relationship between the number of TLX5RT lymphoma cells innoculated i.p. and the mean day of death of CBA/CA mice. Line was fitted by linear regression analysis r = -0.952. Each point is the mean and s.d. of three separate experiments.

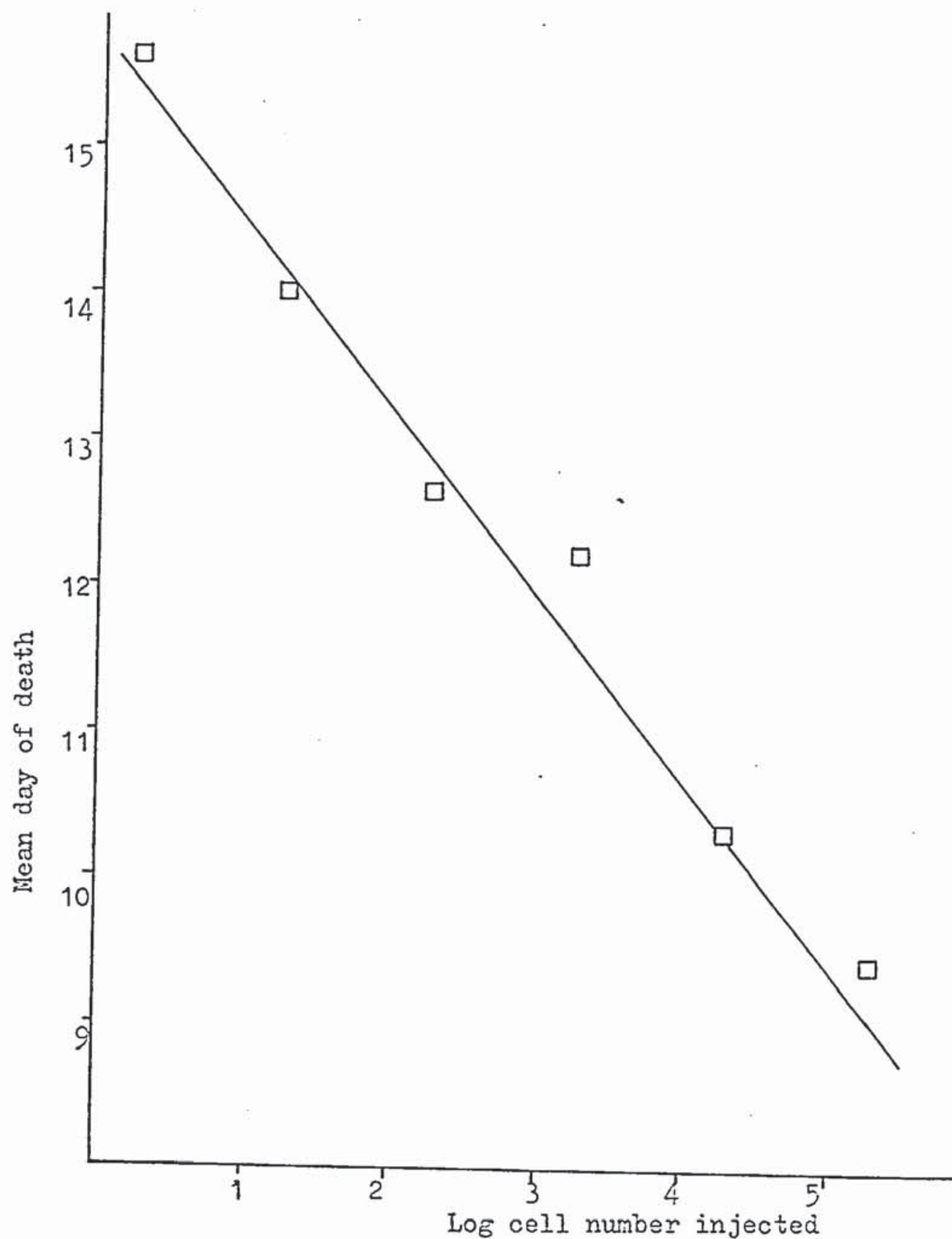


FIGURE 11 Linear relationship between the number of TLX5RB lymphoma cells injected i.p. and the mean day of death of CBA/CA mice. Line was fitted by linear regression analysis r = −0.985. Each point is the mean of at least five animals.

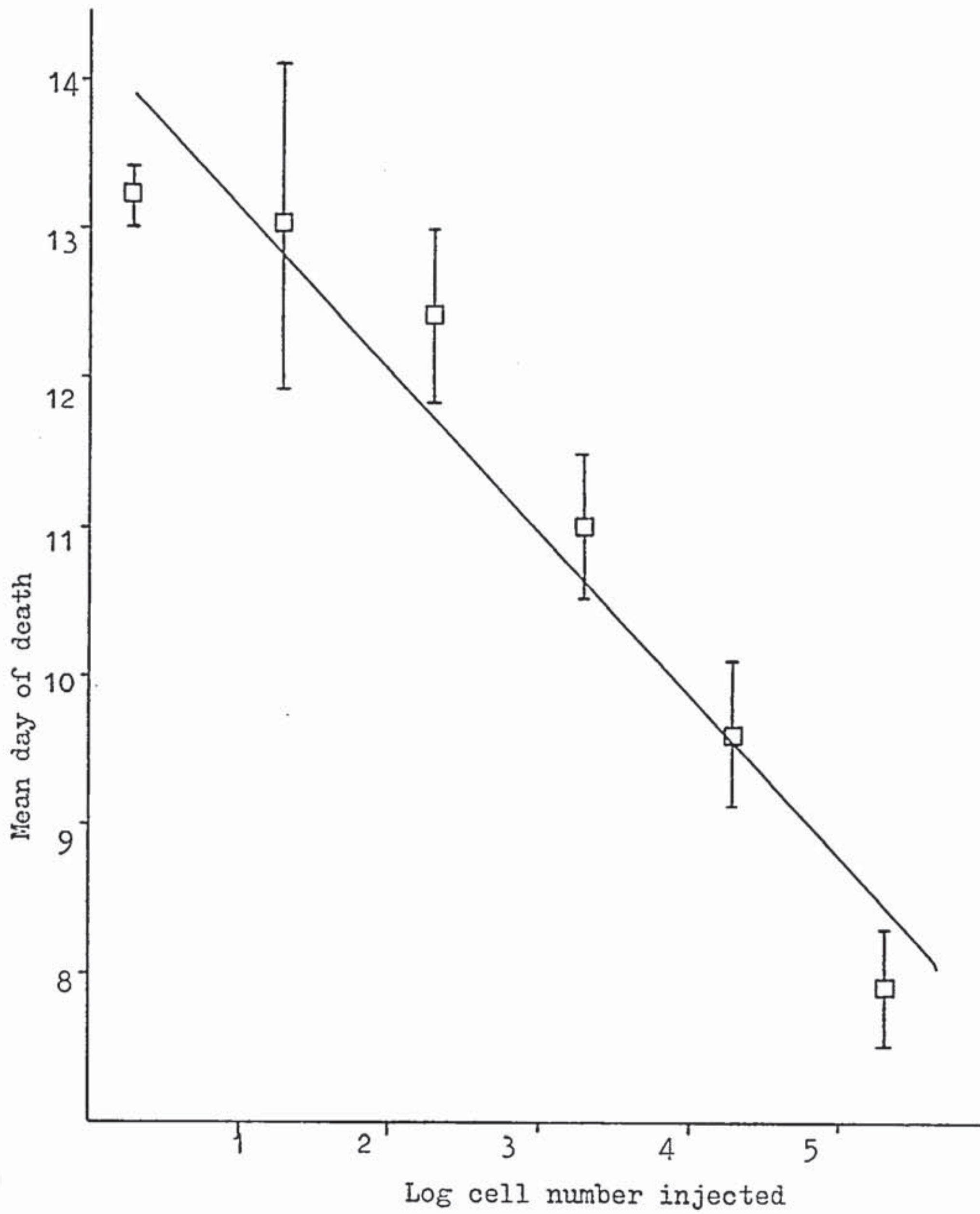


FIGURE 12

Linear relationship between the number of L1210S leukaemia cells innoculated i.p. and the mean day of death of BDF1 mice. Line was fitted by linear regression analysis r = -0.967. Each point is the mean and s.d. of three separate experiments.

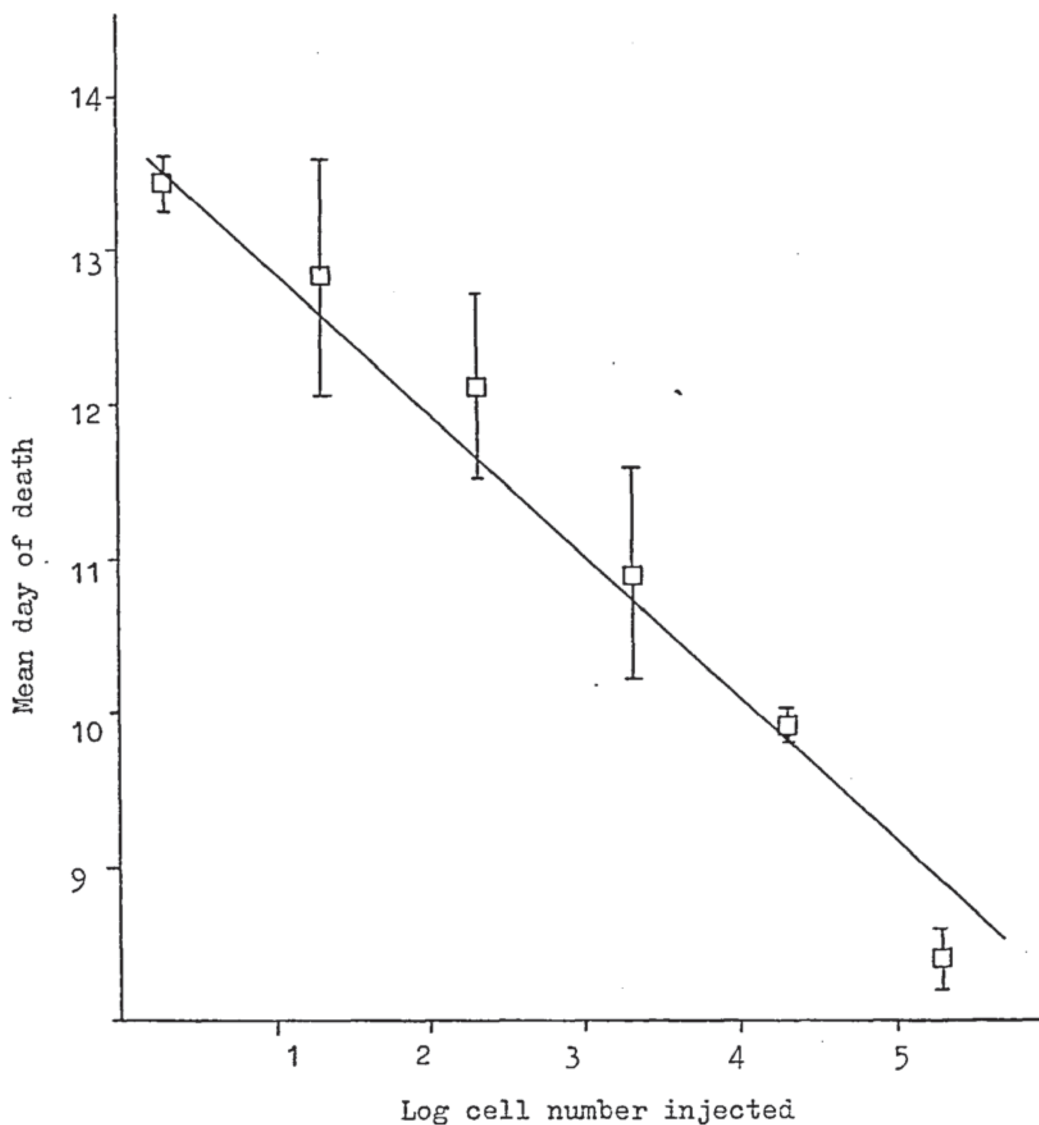


FIGURE 13

Linear relationship between the number of L1210R leukaemia cells innoculated i.p. and mean day of death of BDF1 mice. Line was fitted by linear regression analysis r = −0.985. Each point is the mean and s.d. of three separate experiments.

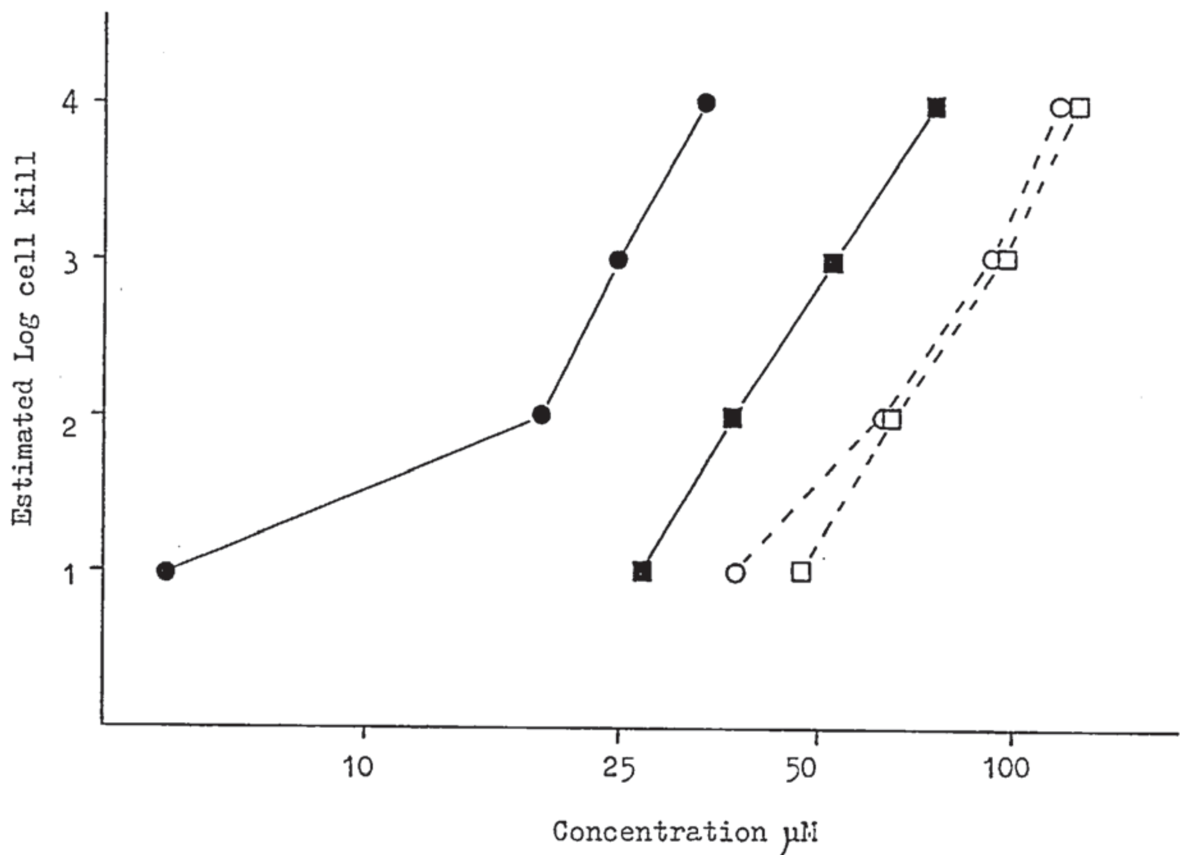


FIGURE 14

In vitro - In vivo cytotoxicity assay's of BCNU against the TLX5S, —●—, and TLX5RT, --○--, and of chloroethylisocyanate against TLX5S, —■—, and TLX5RT, --□--, lymphomas. Each point is the mean of three separate experiments.

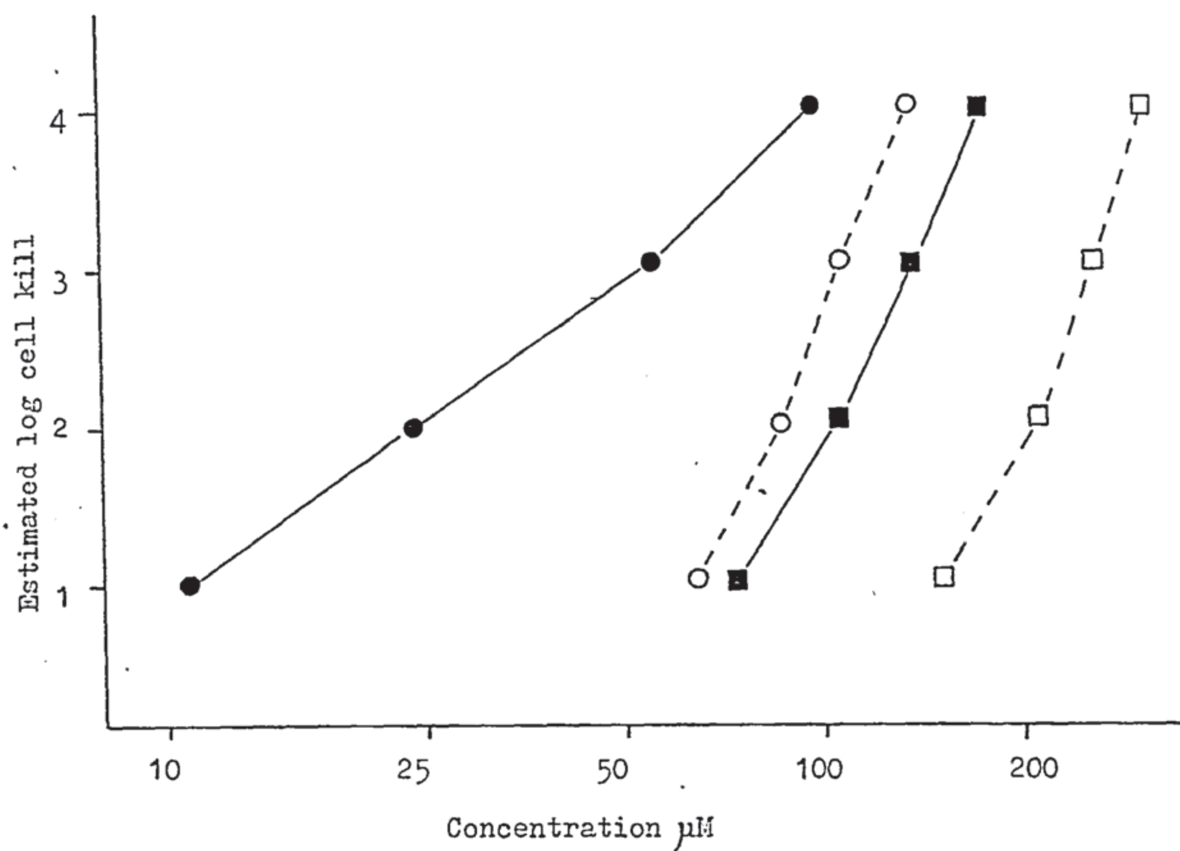


FIGURE 15 In vitro – In vivo cytotoxicity assay's of CCNU against the TLX5S, —●— , and TLX5RT, --○-- , and of cyclohexylisocyanate against the TLX5S, —■— , and TLX5RT, --□-- , lymphomas. Each point is the mean of three separate experiments

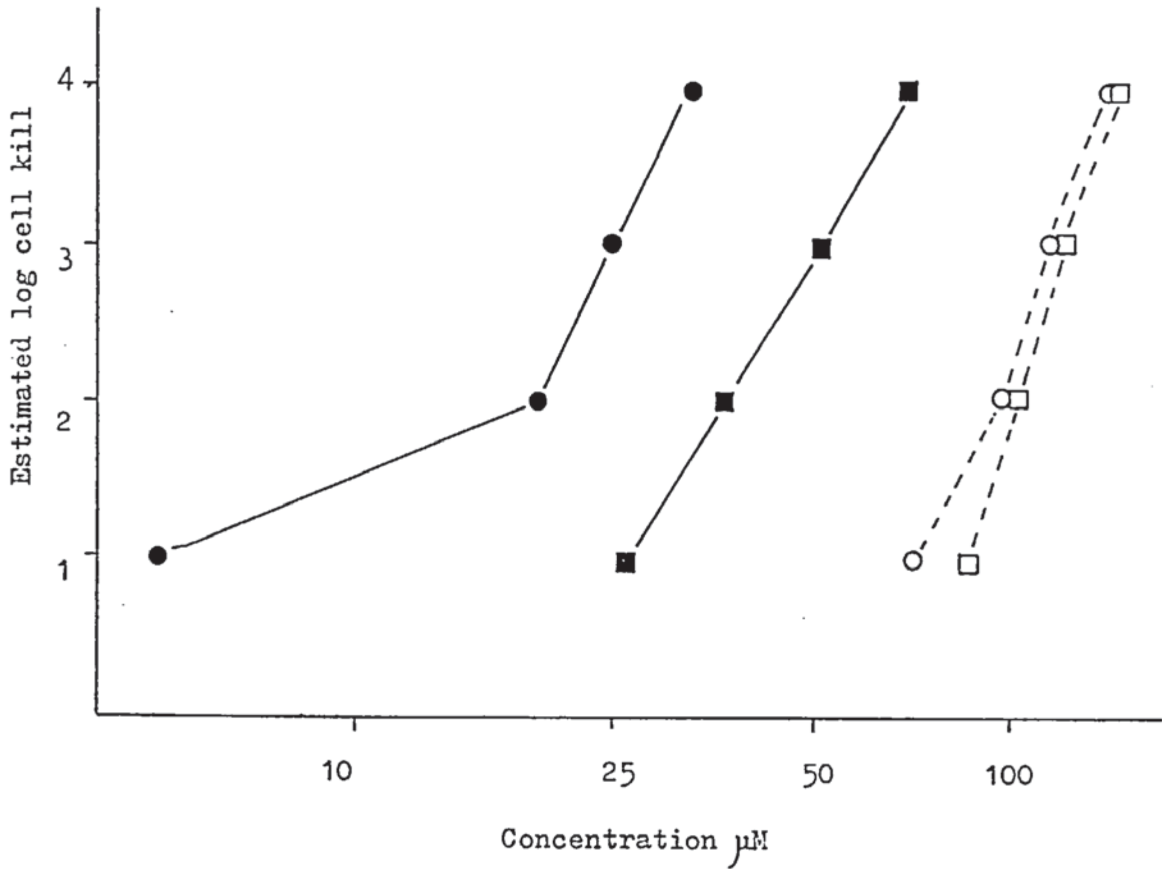


Figure 16 In vitro - In vivo cytotoxicity assay's of BCNU against the TLX5S, —●— , and TLX5RB ---○--- , and of chloroethylisocyanate against the TLX5S, —■— , and TLX5RB --□--- lymphomas. Each point is the mean of at least two separate experiments.

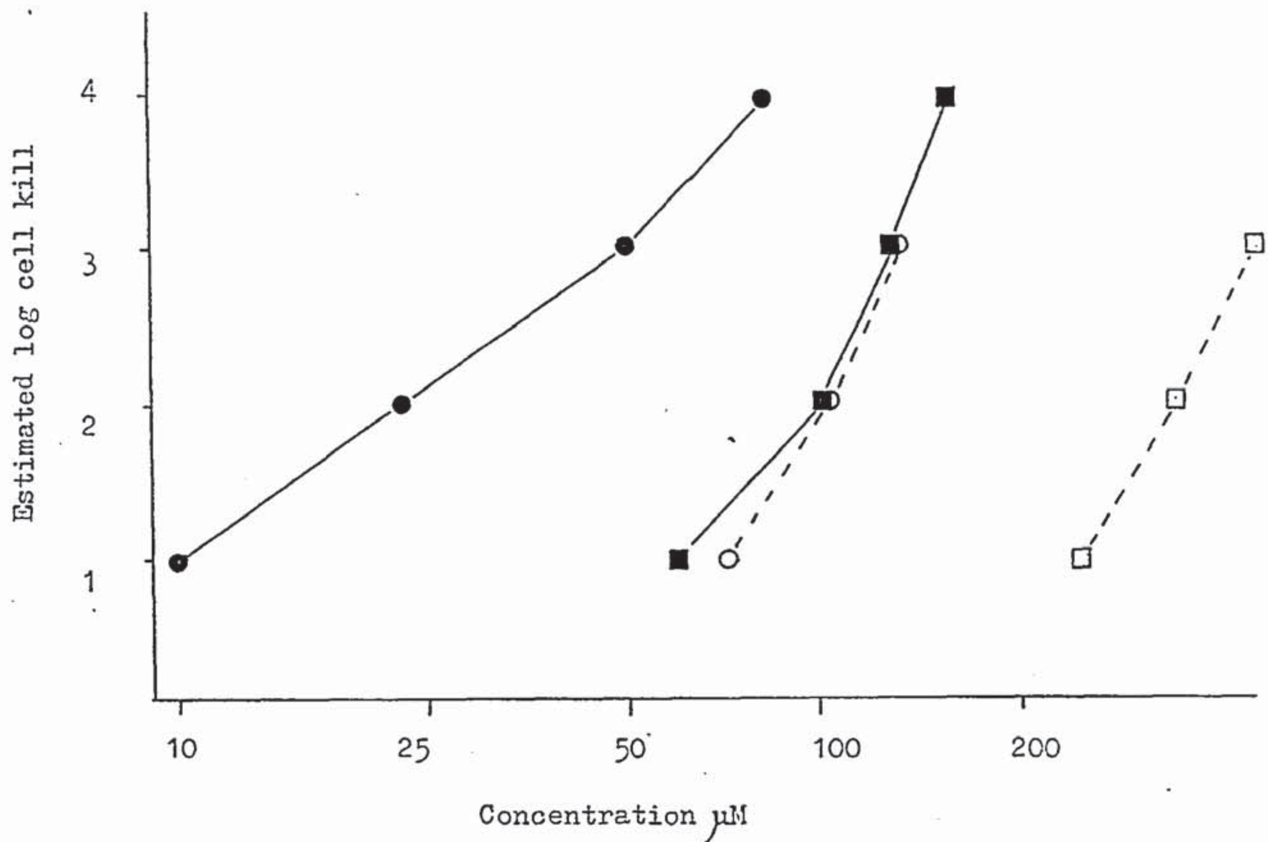


FIGURE 17 In vitro - In vivo cytotoxicity assay's of CCNU against the TLX5S, —●— , and TLX5RB, --O--, and of cyclohexylisocyanat against the TLX5S, —■— , and TLX5RB, --□--, lymphomas. Each point is the mean of at least two separate experiments

shown on Table 17.

4.2.3 *In vitro* - *In vivo* cytotoxicity assay of BCNU, CCNU chloroethylisocyanate, and cyclohexylisocyanate against the L1210S and L1210R leukaemias

The estimated log cell kill of L1210S and L1210R leukaemias by BCNU, CCNU, chloroethylisocyanate and cyclohexylisocyanate are shown in Figures 18 and 19. The L1210R leukaemia which was resistant *in vivo* to BCNU and CCNU (Tables 13 and 14) was also resistant *in vitro* (Figures 18 and 19). In contrast to the TLX5 resistant lymphomas the L1210R leukaemia was not cross resistant *in vitro* to cyclohexylisocyanate (Figure 19) although some cross resistance to chloroethylisocyanate was observed (Figure 18). The degree of resistance of the L1210R leukaemia *in vitro* to BCNU, CCNU, chloroethylisocyanate and cyclohexylisocyanate at an estimated 3 log cell kill is shown in Table 17.

4.2.4 *In vitro* - *In vivo* cytotoxicity assays of BFNU, FCNU and chlorozotocin against the TLX5S, TLX5RT lymphomas and the L1210S and L1210R leukaemias

The estimated log cell kill of TLX5S and TLX5RT lymphomas and of the L1210S and L1210R leukaemias by BFNU and FCNU are shown in Figure 20 and 21. The TLX5RT lymphoma which was resistant to BFNU and FCNU *in vivo* (Table 4 and 5) was also resistant *in vitro* (Figures 20 and 21). The degree of resistance of the TLX5RT lymphoma and

Table 17 The ratio of the concentration of the nitrosoureas and isocyanates required to produce an estimated 3 log cell kill of the resistant and sensitive cell lines

COMPOUND	TLX5RT / TLX5S	TLX5RB / TLX5S	L1210R / L1210S
BCNU	3.8	5.3	3.6
CCNU	2.0	2.0	5.5
Chloroethylisocyanate	1.9	2.3	1.5
Cyclohexylisocyanate	1.9	2.0	0.9
BFNU	2.2	-	2.2
FCNU	2.0	-	2.6
Chlorozotocin	2.5	-	-

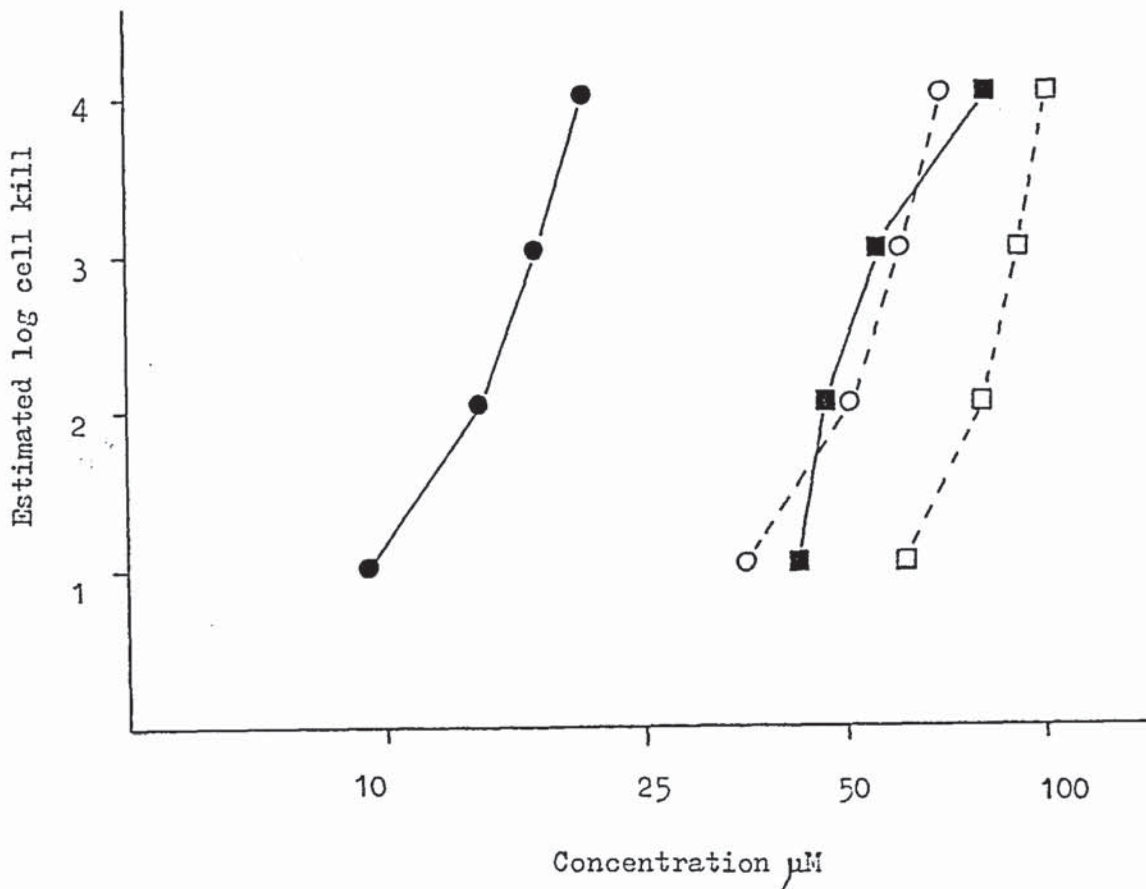
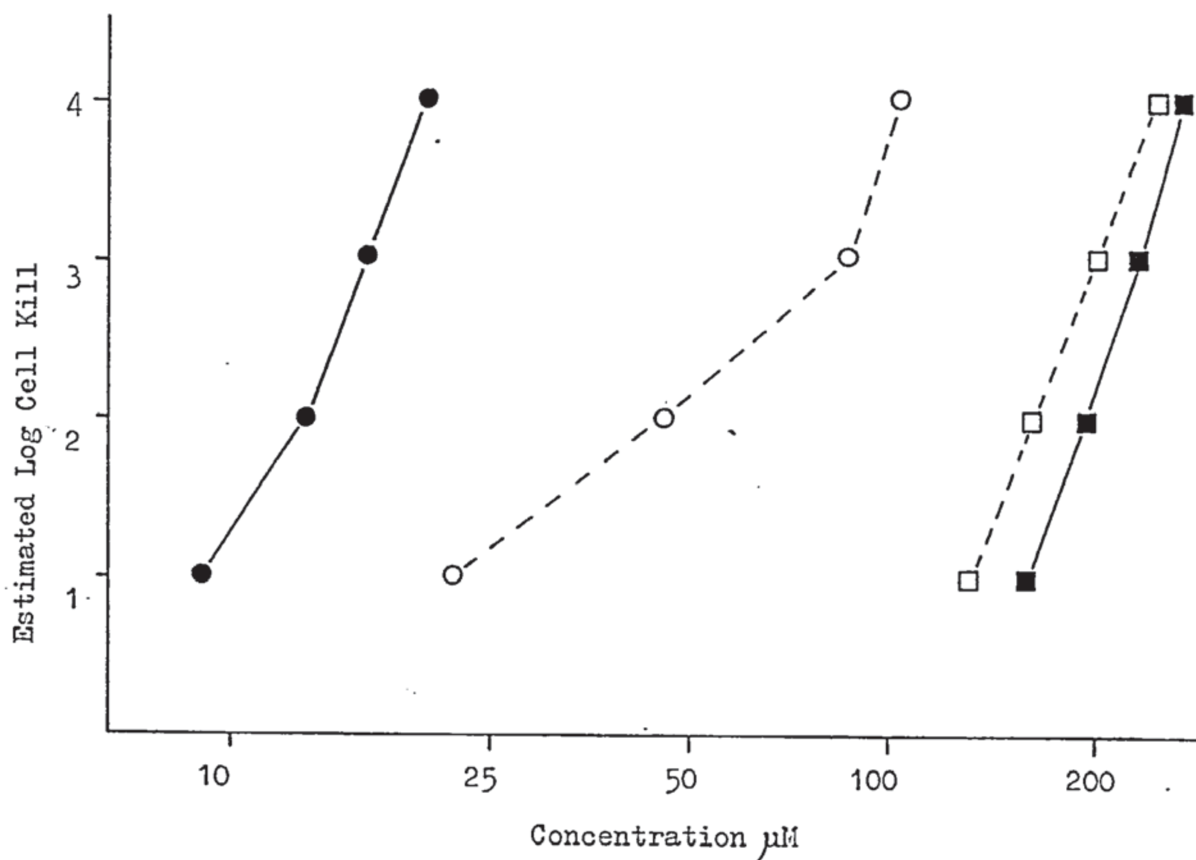


FIGURE 18

In vitro - In vivo cytotoxicity assay's of BCNU against the L1210S, —●— , and L1210R, - -O- - , and of chloroethylisocyanate against the L1210S, —■— , and L1210R, - -□- - , leukaemias. Each point is the mean of at least two separate experiments.



<u>FIGURE 19</u> <u>In vitro</u> - <u>In vivo</u> cytoxicity assay's of CCNU against the L1210S, —●— , and L1210R, --○--, and of cyclohexylisocyanate against the L1210S, —■— , and L1210R, --□--, leukaemias. Each point is the mean of at least two separate experiments.

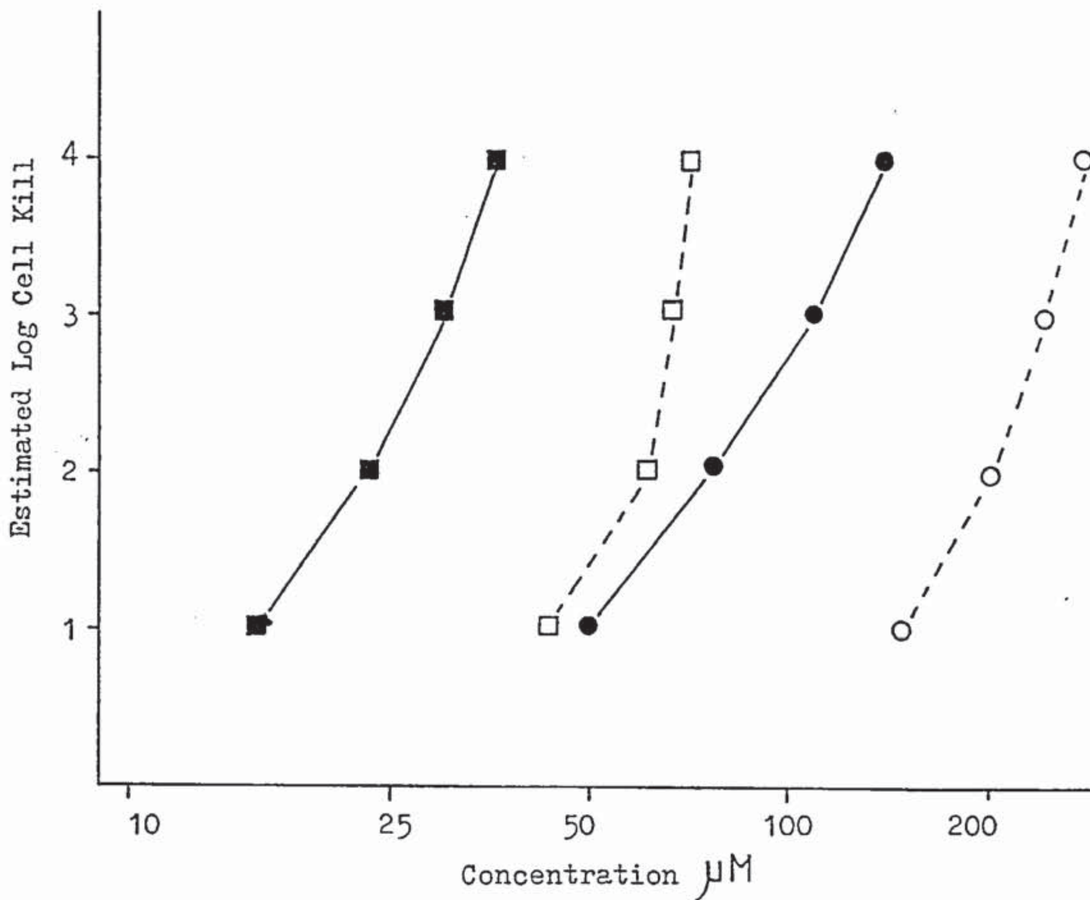


FIGURE 20 In vitro - In vivo cytotoxicity assay's of BFNU against the TLX5S, —●— , and TLX5RT, --○-- , lymphomas and against the L1210S, —■— , and L1210R, --□-- , leukaemias. Each point is the mean of at least two separate experiments.

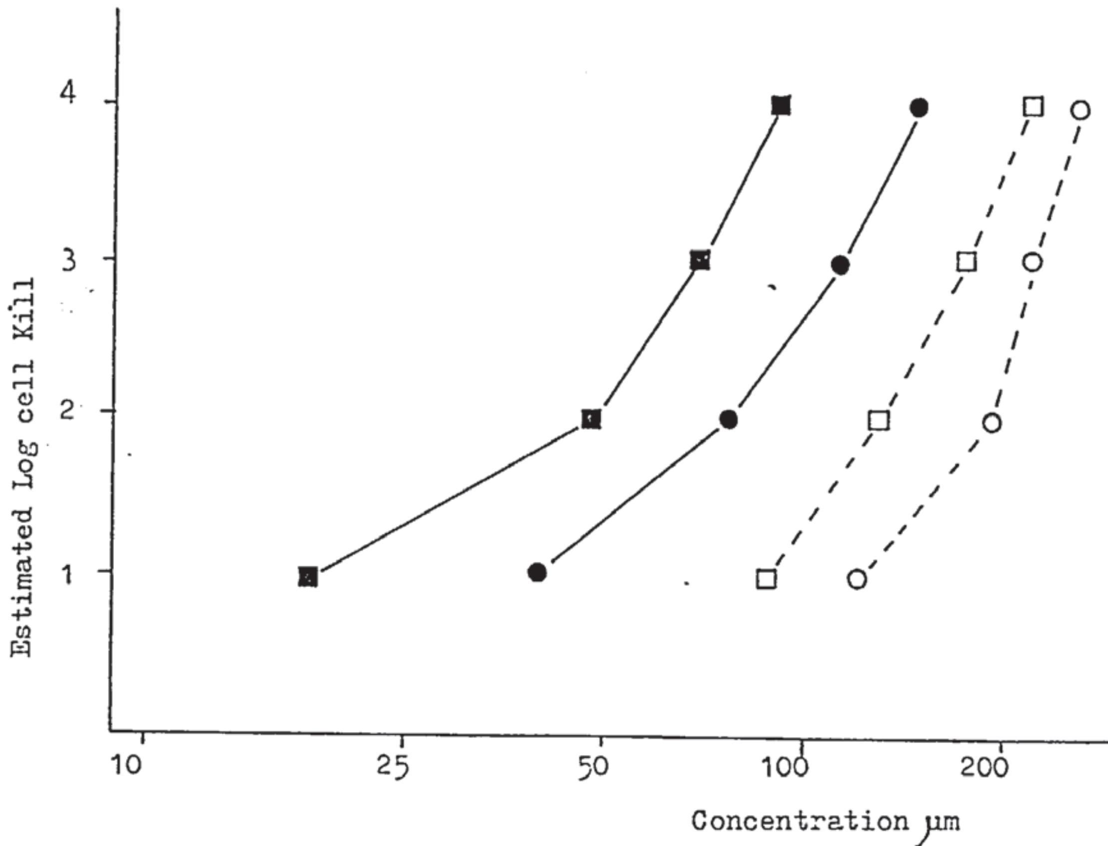


FIGURE 21 In vitro - In vivo cytotoxicity assay's of FCNU against the TLX5S, —●— , and TLX5RT, - -O- - , lymphomas and against the L1210S, —■— , and L1210R, - -□- -, leukaemias. Each point is the mean of at least two separate experiments.

L1210R leukaemia to BFNU and FCNU at an estimated 3 log cell kill is shown on Table 17.

The estimated log cell kill of TLX5S and TLX5RT lymphomas by chlorozotocin is shown in Figure 22. Chlorozotocin was consistently more toxic to the TLX5S lymphoma in vitro than the TLX5RT lymphoma. The degree of resistance of the TLX5RT lymphoma to chlorozotocin at an estimated 3 log cell kill is shown on Table 17. The degree of resistance of the TLX5RT lymphoma observed with chlorozotocin was similar to that observed with BCNU and CCNU.

4.2.5 In vitro - In vivo cytotoxicity assays of nitrogen mustard and formaldehyde against the TLX5S and TLX5RT lymphomas and L1210S and L1210R leukaemias

The estimated log cell kill of TLX5S and TLX5RT lymphomas and of the L1210S and L1210R leukaemias by nitrogen mustard are shown in Figure 23. Nitrogen mustard was equitoxic to both the L1210S and L1210R leukaemias in vitro. The TLX5RT lymphoma was more sensitive in vitro to nitrogen mustard than the TLX5S lymphoma (Figure 23). This result is in agreement with that of Connors and Hare (1974) who showed that the TLX5RB lymphoma was more sensitive in vitro to chlorambucil than the TLX5S lymphoma. In addition, Connors and Hare (1975) showed that the TLX5RB lymphoma was more sensitive to cyclophosphamide in vivo than the TLX5S lymphoma.

Formaldehyde was observed to be equitoxic to both the TLX5S and TLX5RT lymphomas in vitro at all estimated log cell kills (Figure 24).

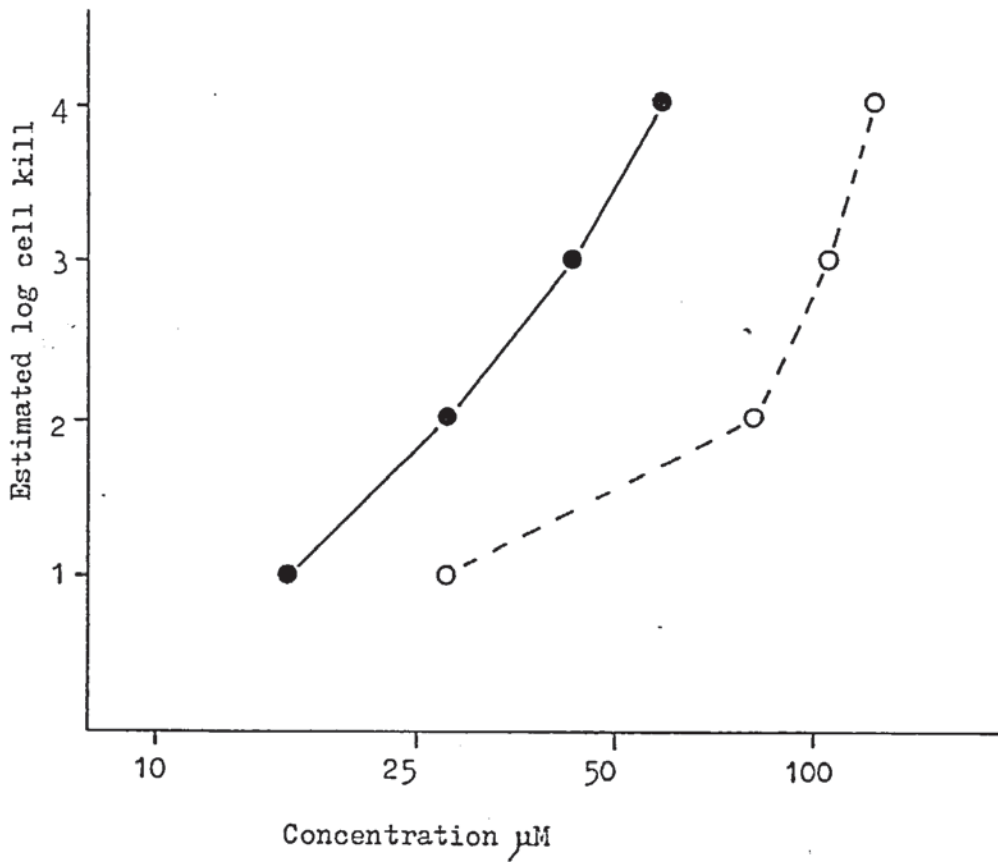


Figure 22 In vitro - In vivo cytotoxicity assay's of chlorozotocin against the TLX5S —●— , and TLX5RT --O-- , lymphomas. Each point is the mean of two separate experiments

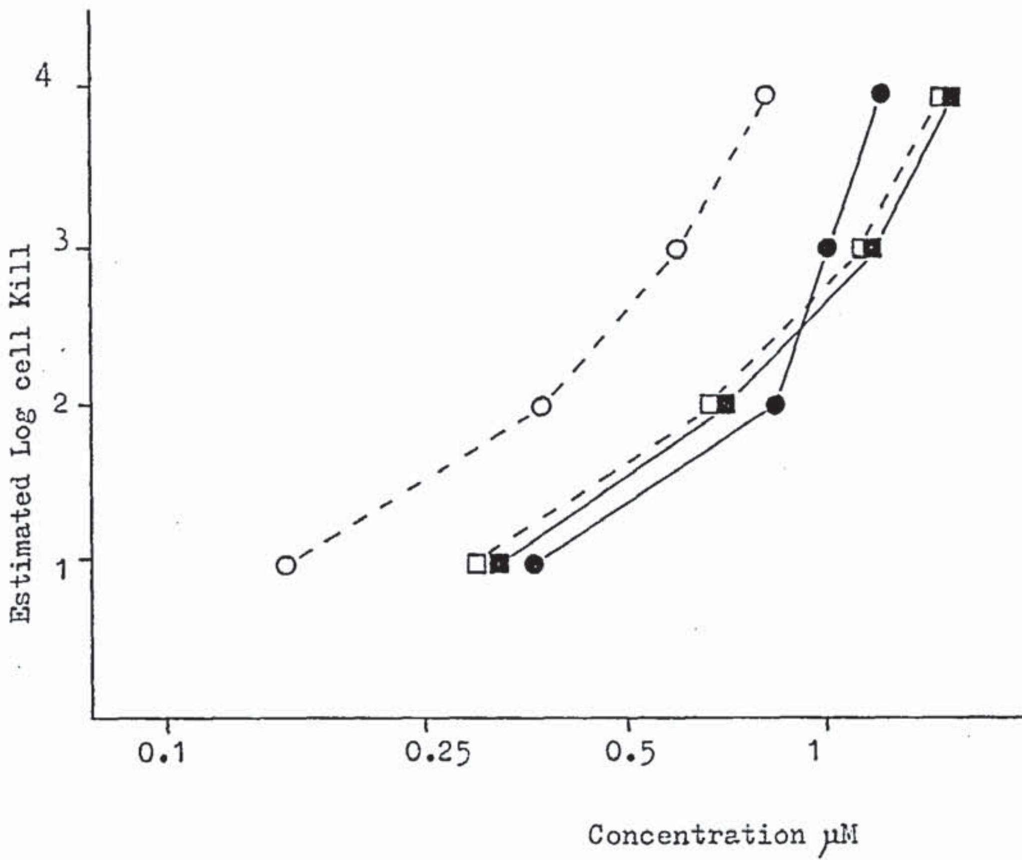


FIGURE 23 - *In vitro* - *In vivo* cytotoxicity assay's of nitrogen mustard against the TLX5S, —●—, TLX5RT, - - o - -, lymphomas and the L1210S, —■—, and L1210R, - - □ - -, leukaemias. Each point is the mean of five estimates.

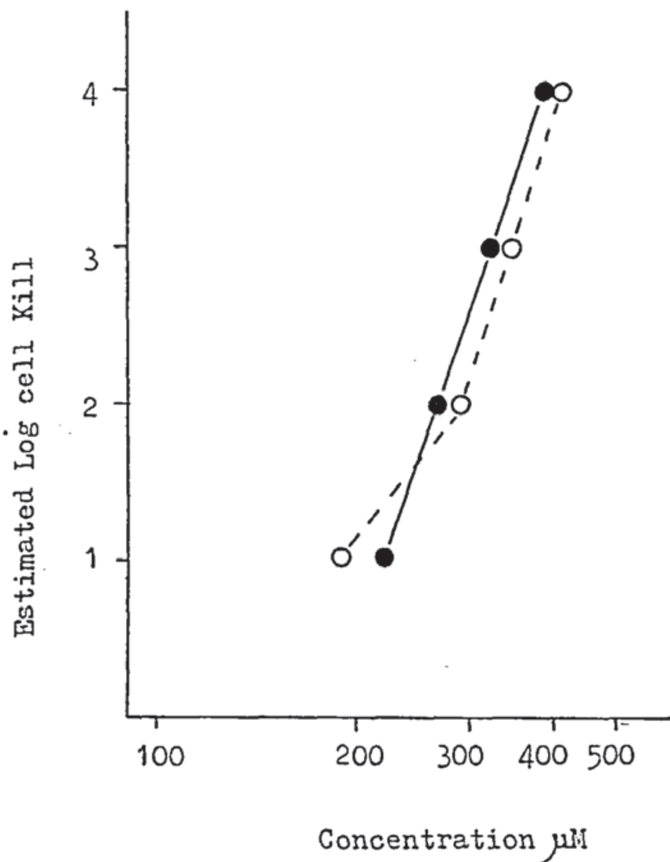


FIGURE 24 In vitro - In vivo cytotoxicity assay's of formaldehyde against the TLX5S, —●— , and TLX5RT,- -O- - lymphomas. Each point is the mean of five estimates.

Hence no cross resistance pattern between the nitrosoureas and other electrophilic species such as nitrogen mustard and formaldehyde was observed. In addition, Gescher et al (1981) have shown that a monomethyltriazene, which is considered to be an active metabolite of the corresponding dimethyltriazene and is capable of acting as a methylating agent, has equal toxicity to the TLX5S and TLX5RT lymphomas _in vitro_.

4.2.6 _In vitro_ - _In vivo_ cytotoxocity assays of cyclohexylamine, DCyU, and DCyNU against the TLX5S and TLX5RT lymphomas

Cyclohexylisocyanate, a degradation product of CCNU, itself further decomposes to both cyclohexylamine and 1,3-dicyclohexylurea (DCyU)(Section 1.2 Scheme V). Cyclohexylamine was found to be non toxic to the TLX5S and TLX5RT lymphomas even at concentrations up to 0.8mM (Table 18). DCyU was also found to be non toxic to the TLX5S and TLX5RT lymphomas at concentrations reaching 1.8mM (Table 18). These concentrations are far in excess of those that would be expected upon the decomposition of toxic concentrations of eith CCNU or cyclohexylisocyanate.

Table 18 The *in vitro* - *in vivo* cytotoxicity assay's of cyclohexylamine, DCyU, and DCyNU against the TLX5S and TLX5RT lymphomas

Compound	Concentration μM	TLX5S		TLX5RT	
		M.D.D.	% I.S.T.	M.D.D.	% I.S.T.
Cyclohexylamine	Control	9.0	-	8.4	-
	100	9.0	0	8.6	2
	200	9.6	7	8.4	0
	400	8.4	-7	8.4	0
	800	9.2	2	8.0	-5
DCyU	Control	10.0	-	9.0	-
	446	10.0	0	9.0	0
	892	9.6	-4	9.0	0
	1784	9.2	-8	10.2	14
DCyNU	Control	9.2	-	8.2	-
	19.8	9.0	-2	8.2	0
	39.5	9.0	-2	8.2	0
	79	9.6	4	8.2	0
	158	13.8	50	9.0	10
	316	60	200	9.2	12

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

DCyNU was observed to be consistently more cytotoxic to the TLX5S lymphoma *in vitro* than the TLX5RT lymphoma (Table 18) presumably as a direct consequence of the generation of cyclohexylisocyanate.

4.2.7 *In vitro* - *In vivo* cytotoxicity assays of CENA and CF_3CNU against the TLX5S and TLX5RT lymphomas

CENA, as previously suggested (Section 1.5.1) produces a chloroethyldiazohydroxide species upon its decomposition (Hecht and Kozarid, 1973). This compound was observed to have greater cytotoxicity to the TLX5S lymphoma *in vitro* than the TLX5RT lymphoma (Table 19).

1-(3,3,3-trifluoropropyl)-3-cyclohexyl-1-nitrosourea (CF_3CNU) would be expected to decompose to produce cyclohexylisocyanate but not a haloalkyldiazohydroxide that could cross link DNA. An investigation of its *in vitro* cytotoxicity was thought to help elucidate the species responsible for the *in vitro* cytotoxicity of analogous haloalkylnitrosoureas such as CCNU. CF_3CNU was found to be toxic to the TLX5S lymphoma *in vitro* but only at concentrations of 300 μM or greater. The slow rate of decomposition of this compound (see Section 4.5) may help explain its lack of toxicity *in vitro* (Table 20).

4.2.8 Attempt to select a line of the TLX5S lymphoma with resistance to cyclohexylisocyanate

In an attempt to obtain a TLX5 lymphoma cell line with resistance

Table 19 The *in vitro* - *in vivo* cytotoxicity assays of CENA against the TLX5S and TLX5RT lymphomas

Concentration μM	TLX5S				TLX5RT			
	M.D.D.		% I.S.T.		M.D.D.		% I.S.T.	
	Experiment Number							
	1	2	1	2	1	2	1	2
Control	9.8	9.8	-	-	9.6	9.0	-	-
0.5	-	13.0	-	33	-	9.0	-	0
1	-	15.0	-	53	-	9.4	-	4
2	16.7	30	70	200	11.8	10.0	23	11
4	60	30	500	200	14.2	11.4	48	27
8	60	30	500	200	60	14.0	500	56

M.D.D. - Mean day of death % I.S.T. - Percentage increase in survival time

Table 20 The *in vitro* - *in vivo* cytotoxicity assays of CF_3CNU against the TLX5S lymphoma

Concentration μM	TLX5S			
	M.D.D.		% I.S.T.	
	1	2	1	2
Control	10.8	9.8	-	-
18.7	10.8	-	0	-
37.4	11.0	-	2	-
74.8	12.0	10.6	11	8
149.6	11.8	11.0	10	12
299	14.4	12.2	34	24
598	-	16.0	-	63

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

to cyclohexylisocyanate, TLX5S lymphoma cells were incubated with a sublethal concentration of cyclohexylisocyanate for 2 hours at 37°C (see Section 3.1.4).

In the first attempt a cell line with resistance <u>in vitro</u> to cyclohexylisocyanate was obtained after four treatment schedules (Table 21). This cell line was designated TLX5Cy1 and a comparison with the TLX5S lymphoma showed that it was less sensitive to CCNU <u>in vivo</u> (Table 22). The two experiments presented on Table 22 were performed at an interval of 21 days upon which the TLX5Cy1 lymphoma cell line had been routinely passaged with no exposure to cyclohexylisocyanate. It thus appeared that the resistance developed to cyclohexylisocyanate was probably unstable.

In a second attempt to develop a cell line with resistance to cyclohexylisocyanate the cell line obtained after eight treatment schedules was observed to be resistant to cyclohexylisocyanate <u>in vitro</u> (Table 23). However, this cell line, designated TLX5Cy2, was found to have increased sensitivity to CCNU <u>in vivo</u> when compared with the TLX5S lymphoma (Table 24).

The third attempt to develop a cell line with resistance to cyclohexylisocyanate led to cells without resistance to cyclohexylisocyanate <u>in vitro</u> (Table 25). Upon the seventh treatment schedule this cell line appeared to have lost its tumourogenicity as no cells were recovered from mice innoculated with either treated or untreated cells (Table 25).

Table 21 First attempt to select a line of TLX5 lymphoma cells resistance to cyclohexylisocyanate

Treatment Number	Concentration mcg/ml	TLX5Cy$_1$ M.D.D.	TLX5Cy$_1$ % I.S.T.
1	Control	10.0	-
	10	11.0	10
	20	10.0	0
2	Control	10.5	-
	20	11.0	4
	40	12.0	14
3	Control	9.6	-
	40	10.0	4
	80	60	500
4	Control	10.0	-
	40	10.6	6

M.D.D. - Mean day of death % I.S.T. - Percentage increase in survival time.
Each value is the mean of at least three mice.

Table 22 The antitumour activity of CCNU against the TLX5S and TLX5Cy$_1$ lymphomas

Dose mg/kg	Days of Treatment	TLX5S M.D.D.		TLX5S % I.S.T.		TLX5Cyl M.D.D.		TLX5Cyl % I.S.T.	
	Experiment Number	1	2	1	2	1	2	1	2
Control	Day 3 only	9.6	10.0	-	1	11.6	10.0	-	-
5	"	10.8	10.4	13	4	12.2	12.6	5	26
10	"	13.3	12.4	38	24	13.3	11.2	13	12
20	"	16.0	16.0	67	60	12.2	12.8	6	28
40	"	15.2	15.2	58	52	12.6	13.2	9	32
80	"	-	9.0	-	-10	-	9.0	-	-10

M.D.D. - Mean day of death, % I.S.T. - Percentage increase in survival time

Table 23 Second attempt to select a line of TLX5 lymphoma resistant to cyclohexylisocyanate

Treatment Number	Concentration mcg/ml	TLX5Cy$_2$ M.D.D.	TLX5Cy$_2$ % I.S.T.
1	Control	9.6	-
	20	10.0	4
2	Control	10.2	-
	20	12.0	18
	30	12.5	23
3	Control	9.8	-
	30	12.7	30
	40	14.5	48
4	Control	9.2	-
	40	12.7	38
5	Control	8.0	-
	40	12.0	50
6	Control	9.6	-
	40	12.0	25
	60	12.5	30
7	Control	9.0	-
	40	10.3	14
	60	14.5	61
8	Control	8.4	-
	40	9.0	7
	60	11.0	31
	80	14.0	70

Each value is the mean of at least three mice

Table 24 The antitumour activity of CCNU against the TLX5S and TLX5Cy2 lymphomas

Dose mg/kg	Days of Treatment	TLX5S		TLX5Cy2	
		M.D.D.	% I.S.T.	M.D.D.	% I.S.T.
Control	Day 3 only	10.0	-	7.8	-
5	"	10.4	4	10.0	28
10	"	12.4	24	10.8	39
20	"	16.0	60	14.8	90
40	"	15.2	52	23.0*	118*
80	"	9.0	-10	5.0	-36

* 2 survivors > Day 60, not included in results

M.D.D. - mean day of death

%I.S.T. - percentage increase in survival time

Table 25 Third attempt to select a line of the TLX5 lymphoma resistant to cyclohexylisocyanate

Treatment Number	Concentration µg/ml	$TLX5Cy_3$	
		M.D.D.	% I.S.T.
1	Control	10.4	-
	30	13.7	32
	40	> 60	> 500
2	Control	10.0	-
	30	13.0	30
	40	16.0	60
3	Control	10.3	-
	30	14.0	36
	40	16.0	55
	50	18.0	75
4	Control	9.8	-
	30	13.7	40
	40	15.0	53
5	Control	10.3	-
	40	14.0	36
	50	19.0	84
6	Control	11.0	-
	40	16.0	45
	60	> 60	> 500
7	Control	> 60	-
	40	> 60	-
	60	> 60	-

M.D.D. - Mean day of death

% I.S.T. - Percentage increase in survival time

4.2.9 Attempts to establish a TLX5 colony forming assay *in vitro* for the estimation of drug toxicity

In order to compare the *in vivo* antitumour activity and the *in vitro* - *in vivo* cytotoxicity assay's with an *in vitro* cytotoxicity assay various attempts were made to establish an *in vitro* colony forming assay with the TLX5 lymphoma.

Initially the two methods used were based on those of Hamburger and Salmon (1977) and Tatsumi et al (1979) (see Section 3.5.3.1). After 10 - 14 days no colony formation was detected and no individual viable cells appeared to remain after 14 days with both methods.

The third method used was that of Chu and Fischer (1968) (see Section 3.5.3.1). In this case colony growth was detected although the plating efficiencies were 7% (Table 26) for both the TLX5S and TLX5RT lymphoma's. However, this method was considered to be a poor comparison with both the *in vivo* antitumour tests and *in vitro* - *in vivo* cytotoxicity assays unless the plating efficiencies could be increased. Thus no *in vitro* drug toxicity data was obtained by using this method.

Table 26 The plating efficiencies of the clonogenic assays of the TLX5S and TLX5RT lymphomas

Tumour Type	Initial Cell Density	Number of Colonies Counted	Plating Efficiency
TLX5S	10000	> 100	-
	1000	62,59,58,43	5.5%
	100	7, 9, 2,10	7.0%
TLX5RT	10000	> 100	-
	1000	74,70,61,65	6.8%
	100	6, 8, 8, 3	6.3%

4.3 Measurement of the non-protein thiol content of the TLX5S and TLX5RT lymphomas and the L1210S and L1210R leukaemia

The finding that the TLX5 resistant lymphomas were cross resistant *in vitro* to the isocyanates (Section 4.2), particularly to the non alkylating cyclohexylisocyanate, suggested that this cross resistance might simply be due to an increased concentration of cellular nucleophiles. This has previously been shown to be the case on the induction of resistance to alkylating agents when the level of non-protein thiols became elevated (Connors, 1966). The non-protein thiol content, expressed in mmols of non-protein thiols per gm protein, of the TLX5S, TLX5RT lymphomas and the L1210S and L1210R leukaemias are shown in Table 27. No significant difference between the TLX5S and TLX5RT lymphomas nor between the L1210S and L1210R leukaemia was observed.

Table 27 The non-protein thiol content of lines of the TLX5S and TLX5RT lymphomas and the L1210S and L1210R leukaemias.

Tumour Line	Non-Protein Thiols (mmols/gm protein)
TLX5S	1.43 ± 0.80
TLX5RT	1.37 ± 0.63
L1210S	1.30 ± 0.46
L1210R	1.38 ± 0.29

Each value is the mean and s.e.m. of six determinations.

4.4 Investigation into the cellular uptake of $[^{14}C]$ - cyclohexyl CCNU into the TLX5S and TLX5RT lymphomas and the L1210S and L1210R leukaemias

4.4.1 Control experiments to measure the amount of drug trapped in the extracellular space between tumour cells upon centrifugation

Polyethyleneglycol (PEG) is a macromolecule which does not enter mammalian cells. Therefore its labelled isotope is used in studies with radiolabelled compounds to determine the amount of compound trapped between the cells after separation from the media. Negligible carryover of PEG was found to occur (Table 28).

In order to check that no supernatant passed through the oil layer during centrifugation $[^{14}C]$-cyclohexyl CCNU labelled media was used in control experiments with no cells present. No carryover of label from the media into the formic acid layer was seen (Table 29).

4.4.2 The cellular uptake of $[^{14}C]$-cyclohexyl CCNU into the TLX5S and TLX5RT lymphomas and the L1210S and L1210R leukaemias

Another common mechanism which is relevant to the induction of resistant cell lines is that of impaired uptake of the cytotoxic agent into the cell (Hill and Montgomery, 1980). The uptake of $[^{14}C]$-cyclohexyl CCNU into the TLX5S and TLX5RT lymphomas and the L1210S and L1210R leukaemias was measured (Figures 25 and 26). No significant difference in the uptake of $[^{14}C]$-cyclohexyl CCNU into either of the resistant cell lines with respect to their sensitive counterparts was observed. The results with the TLX5S and TLX5RT lymphomas agree with those of

Connors and Hare (1974) who showed that the binding of both the alkylating and carbamoylating moities of CCNU to cellular macromolecules was the same in the TLX5S and TLX5RB lymphomas.

Table 28 The percentage carryover of PEG into the formic acid layer

Incubation Time (mins)	TLX5S Lymphoma				TLX5RT Lymphoma			
	Disintegration per minute		%	Mean	Disintegration per minute		%	Mean
	Cell Pellet	Supernatant	Carryover	± S.D.	Cell Pellet	Supernatant	Carryover	± S.D.
0	337	90634	0.372	0.260	214	71510	0.299	0.260
	183	88060	0.201	±0.098	194	73272	0.264	±0.048
	169	82397	0.205		162	78923	0.205	
120	798	85512	0.933	0.533	337	87536	0.384	0.420
	307	95264	0.322	±0.346	382	85300	0.447	±0.033
	338	97764	0.345		341	79216	0.430	

<u>Table 29</u> The percentage carryover of labelled CCNU from the supernatant into the formic acid layer

Incubation Time (mins)	TLX5S Lymphoma				TLX5RT Lymphoma			
	Disintegration per minute		%	Mean	Disintegration per minute		%	Mean
	Cell Pellet	Supernatant	Carryover	± S.D.	Cell Pellet	Supernatant	Carryover	± S.D.
0	309	117174	0.263		228	104308	0.218	
				0.300				0.174
	328	116519	0.281		161	113209	0.142	
				±0.049				±0.040
	385	108221	0.355		187	115965	0.161	
120	89	118555	0.075		45	116856	0.038	
				0.118				0.119
	286	129333	0.221		50	115374	0.043	
				±0.090				±0.135
	75	129542	0.058		307	111016	0.276	

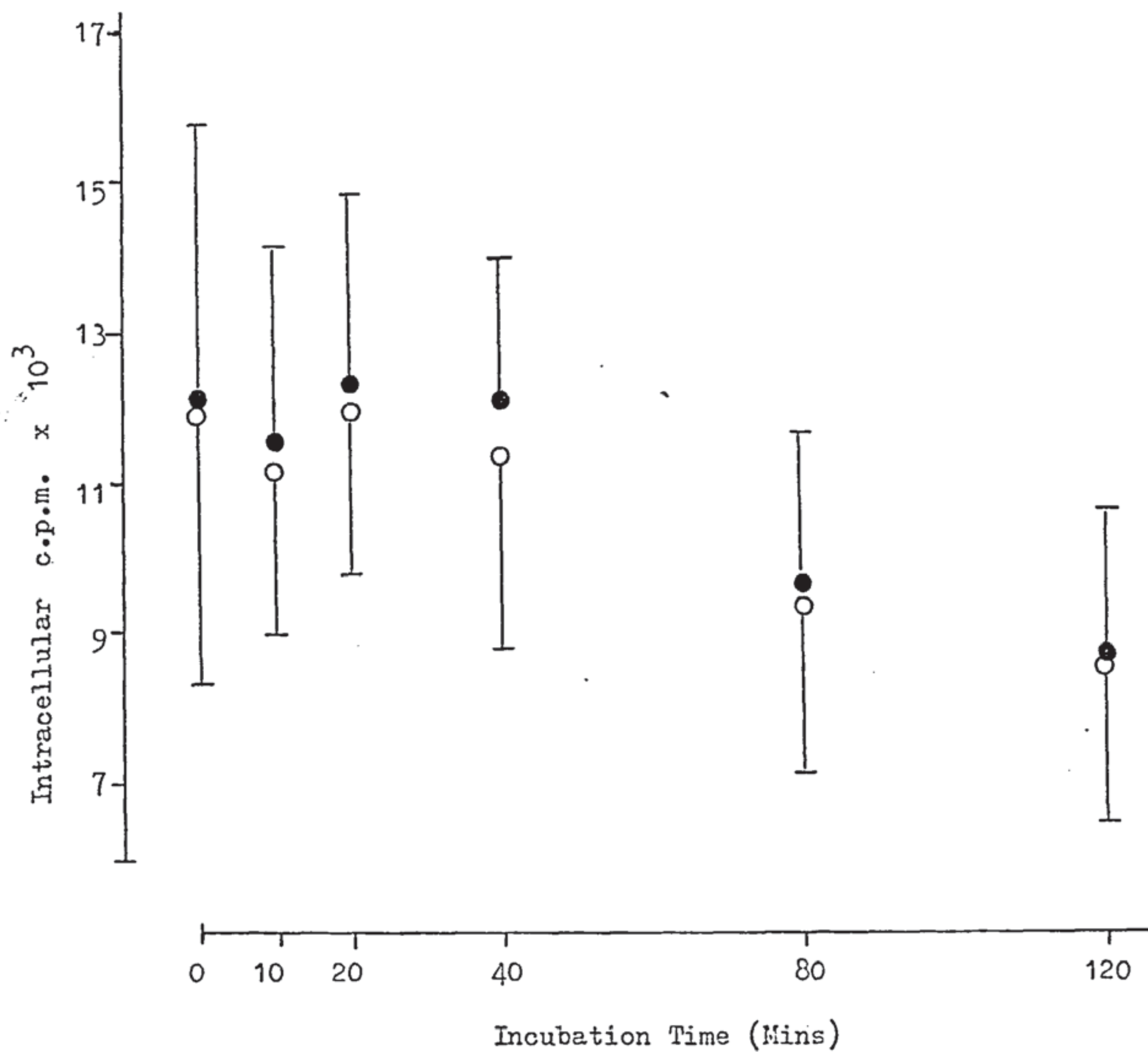


FIGURE 25 The uptake of $\left[{}^{14}C\right]$ - CCNU into TLX5S, ●, and TLX5RT, ○, lymphomas. Each point is the mean and s.d. of three separate experiments.

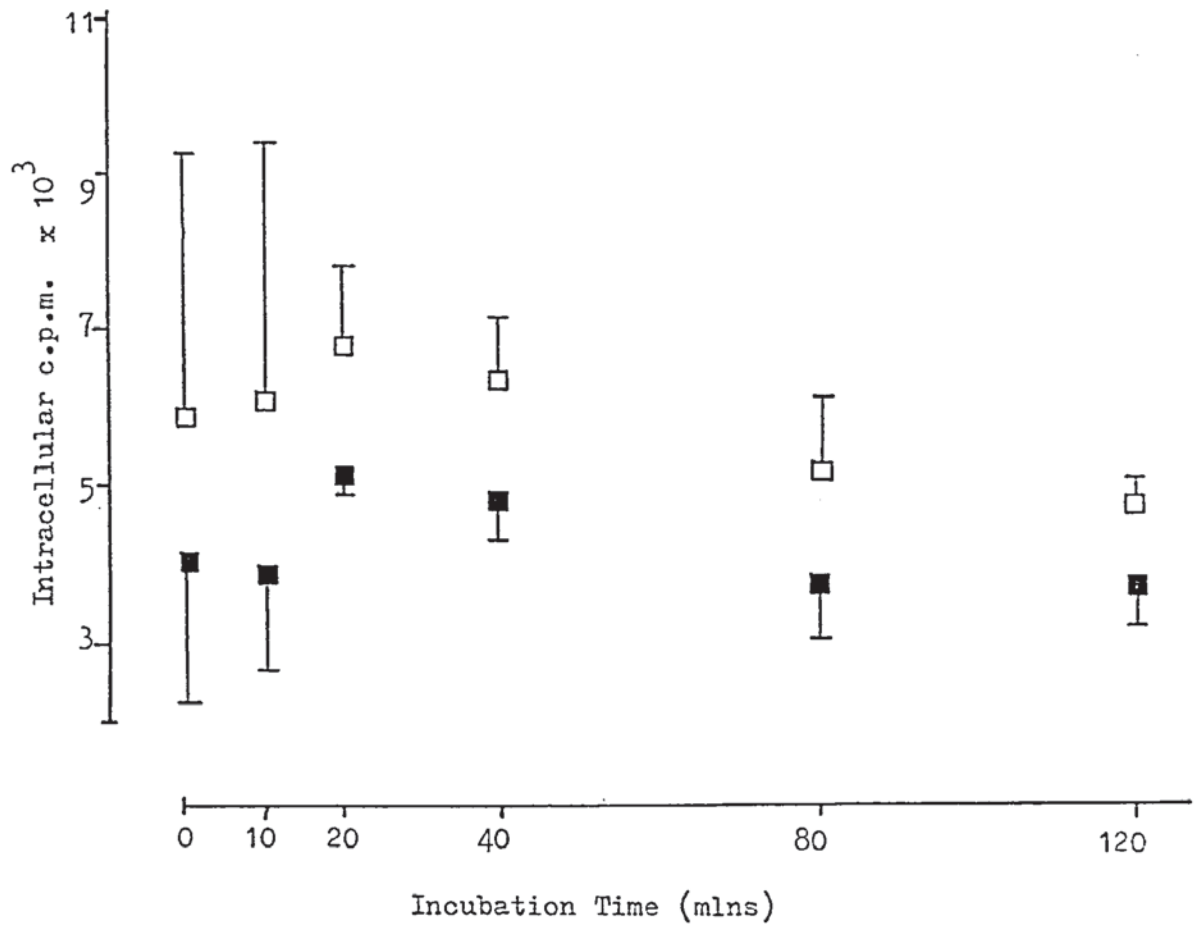


FIGURE 26 The uptake of $[^{14}C]$ - CCNU into L1210S, ■ , and L1210R, □ , leukaemias. Each point is the mean and s.d. of three separate experiments.

4.5 The determination of the chemical half lives of the haloalkylnitrosoureas in colourless R.P.M.I. media

The kinetics of decomposition in colourless R.P.M.I. 1640 media of BCNU, BFNU, CCNU, FCNU, chlorozotocin, and CF_3CNU are shown in Figures 27 - 29. The half lives and rate constants are summarised on Table 30. The results obtained in this study are very similar to those of Wheeler et al (1974) who used an analytical method different from the one used here. They measured the U.V. absorbance of the nitroso function in 5% ethanol/H_2O buffered to pH 7.4. Wheeler obtained values of 43, 52.5, and 68 minutes for BCNU, CCNU, and FCNU respectively whereas in this study the half lives of these three nitrosoureas were 44, 63 and 76 minutes respectively.

It was considered useful to determine the half lives of the nitrosoureas in a mixture of R.P.M.I. (6 parts) and horse serum (4 parts) as these were the conditions used to measure the cytotoxicity *in vitro* (Section 3.4) However this proved unsuccessful due to the inconsistent recoveries of extraction of the nitrosoureas from the R.P.M.I. and horse serum mixture into ether.

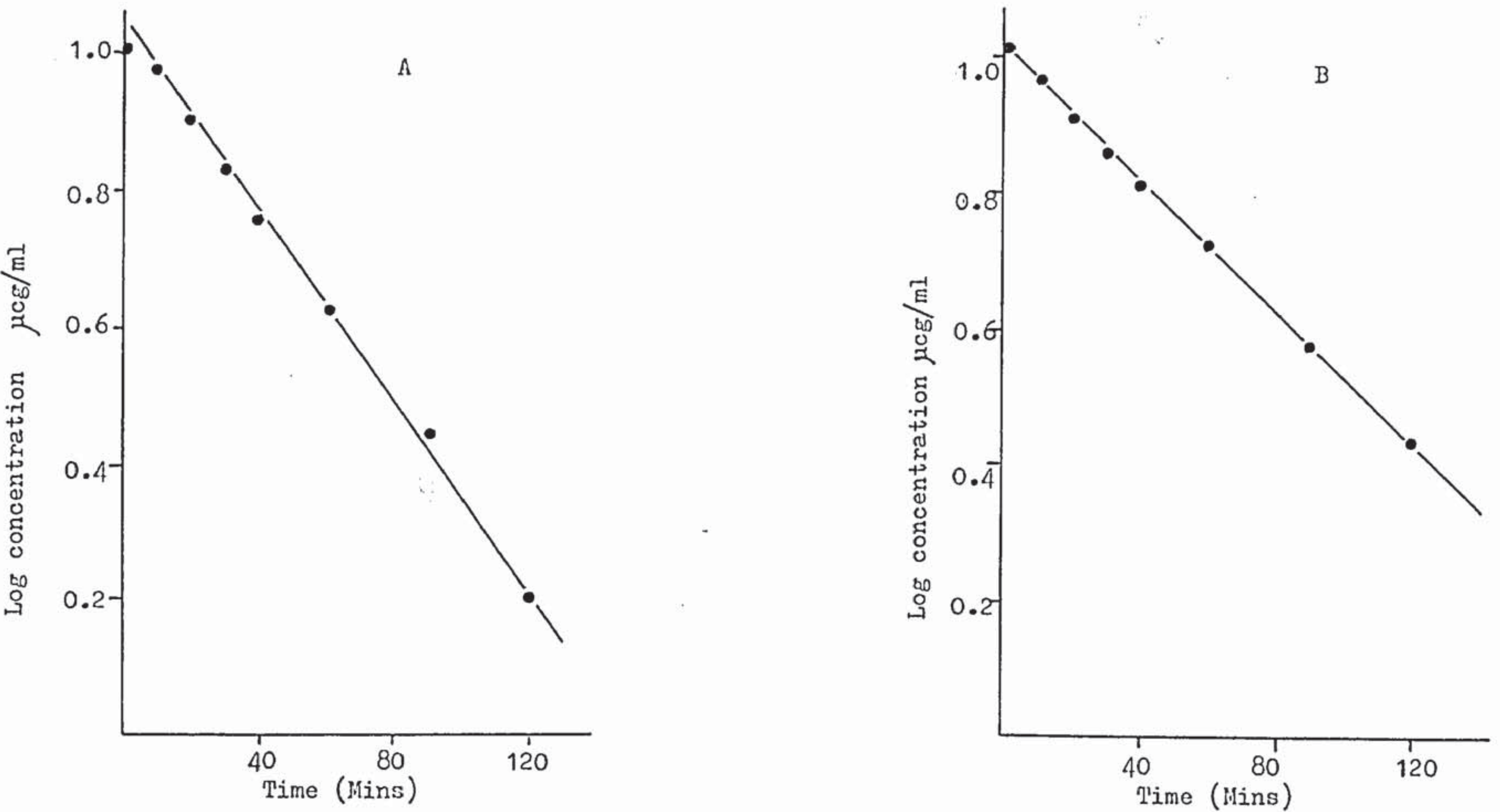


FIGURE 27 – The kinetics of decomposition of BCNU(A) and CCNU(B).
The Lines were fitted by linear regression analysis A, $r = -0.998$ and B, $r = -0.999$. Each point is the mean of three separate experiments.

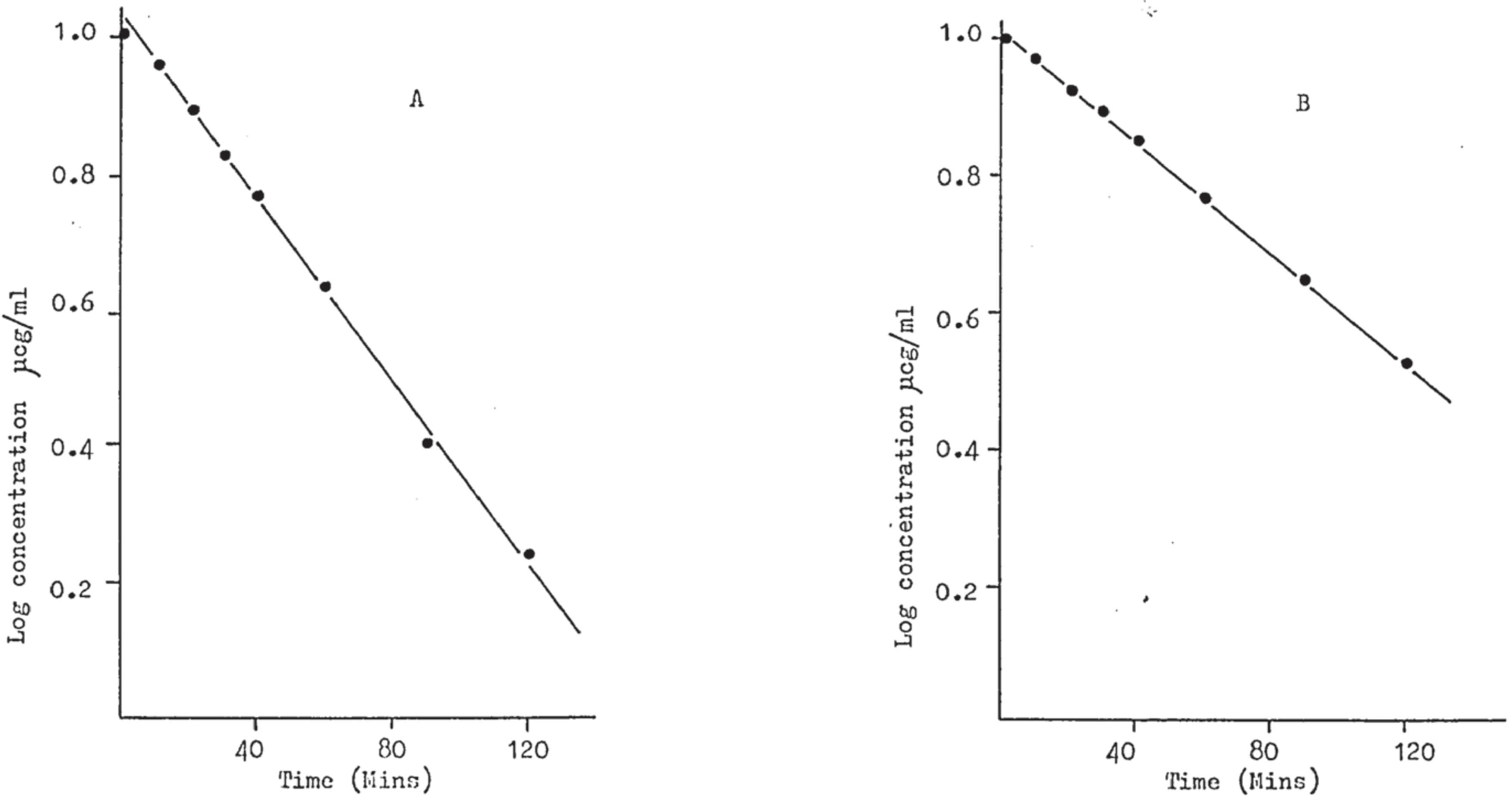


FIGURE 28 The kinetics of decomposition of BFNU(A) and FCNU(B).
Lines were fitted by linear regression analysis A, r = −0.999 and B, r = −0.999.
Each point is the mean of three separate experiments.

<u>Figure 29</u> The kinetics of decomposition of chlorozotocin (A) and CF_3NU(B). Lines were fitted by linear regression analysis A, r = −0.997 and B, r = −0.999

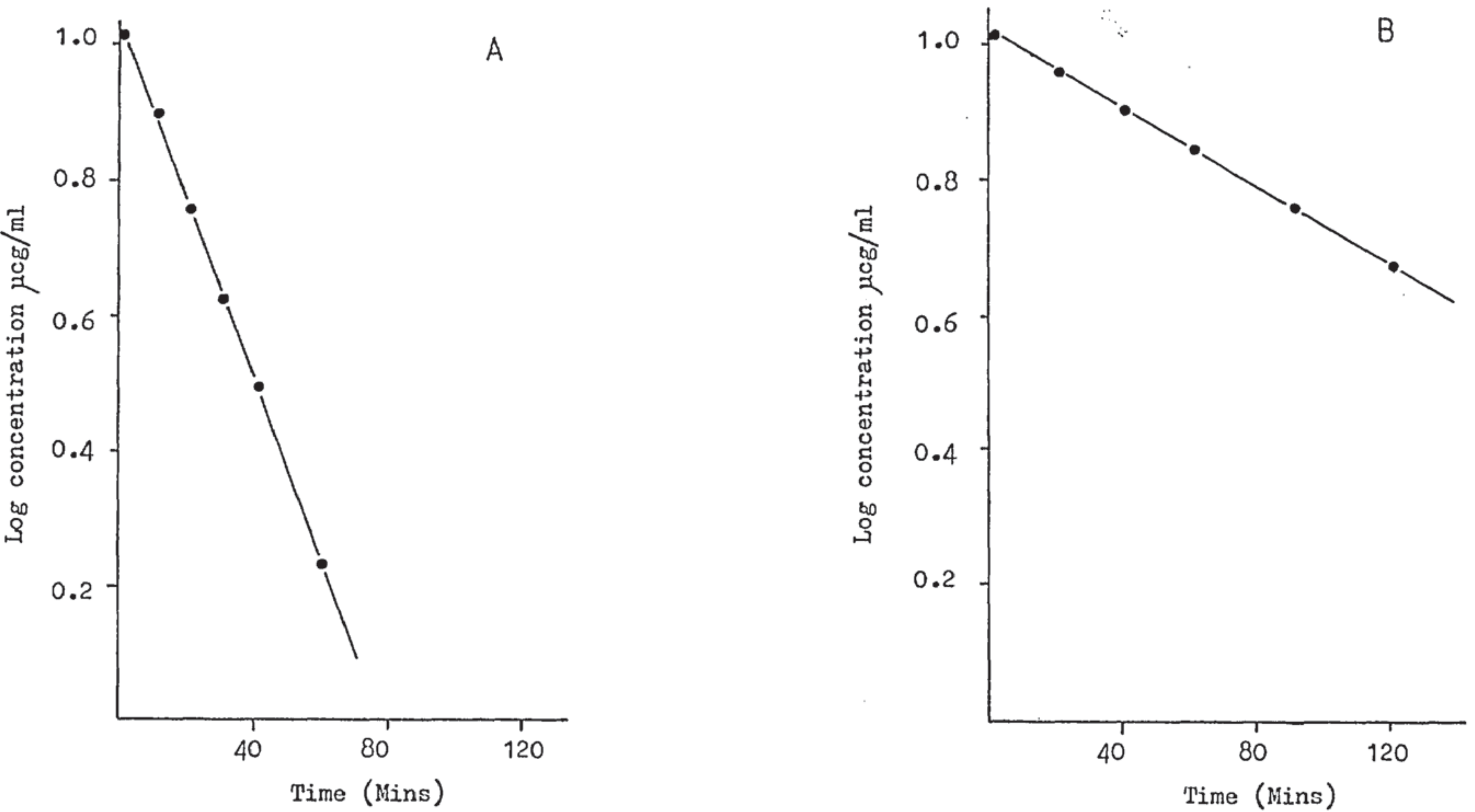


Table 30 The chemical half lives and rate constants of the haloalkylnitrosoureas

Compound	pH	Half Life $t_{\frac{1}{2}}$ min	Rate Constant k min^{-1}
BCNU	7.2	43.9 ± 3.2	0.0158 ± 0.0011
CCNU	7.2	62.8 ± 5.9	0.0111 ± 0.0010
BFNU	7.2	46.5 ± 8.1	0.0151 ± 0.0024
Chlorozotocin	7.2	24.4 ± 3.4	0.0288 ± 0.0044
FCNU	7.2	75.7 ± 3.1	0.0091 ± 0.0003
CF_3CNU	7.2	108 ± 17.0	0.0067 ± 0.0010

Each value is the mean of three separate determinations

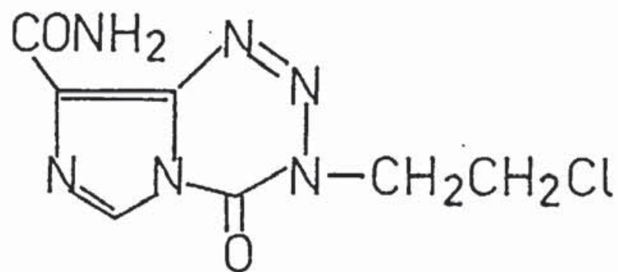
4.6 Investigation into the mechanism of action of 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d][1,2,3,5]tetrazin-4(3H)-one (Azolastone)

4.6.1 *In vivo* antitumour activity of azolastone and related compounds against the TLX5S and TLX5RT lymphomas

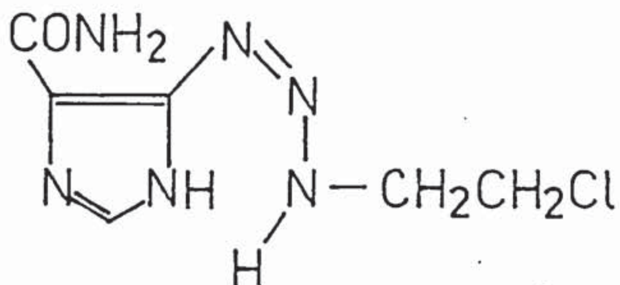
The finding that the isocyanate moiety appeared to make a significant contribution towards the cytotoxicity of the haloalkyl-nitroureas to the murine TLX5S lymphoma (Section 4.2.2) led to the testing of compounds which were synthesised as molecules which theoretically may be capable of releasing an isocyanate moiety. Azolastone (Figure 30) an imidazotetrazinone derivative was synthesised in our laboratories and was considered to be such a compound (see Discussion). Azolastone was found to have similar antitumour activity *in vivo* to the TLX5S lymphomas (Table 31) when compared with the haloalkylnitrosoureas BCNU and CCNU (Tables 2 and 3). The TLX5RT lymphoma which had been made resistant *in vivo* to a dimethyltriazene was found to be resistant to azolastone (Table 31).

8-carbamoyl-3-methylimidazo[5,1-d][1,2,3,5]-tetrazin-4(3H)-one (methazolastone)(Figure 30) is the methyl analogue of azolastone. This compound was also found to have antitumour activity against the TLX5S lymphoma *in vivo* (Table 32).

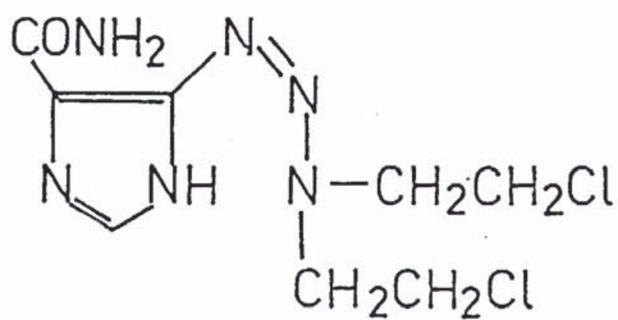
Diazo-I.C.(Figure 30) a potential decomposition product of azolastone (see Discussion Scheme VII) was found to have no antitumour activity against the TLX5S lymphoma *in vivo* (Table 33).



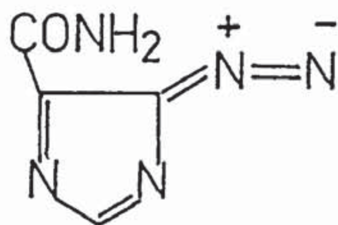
AZOLASTONE



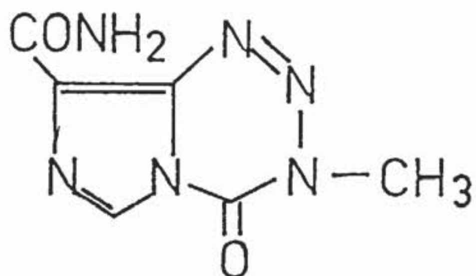
MCTIC



BCTIC



DIAZO -I.C.



METHAZOLASTONE

FIGURE 30 The structures of azolastone, MCTIC, BCTIC, DIAZO -I.C., and methazolastone

Table 31 The antitumour activity of azolastone against the TLX5S and TLX5RT lymphomas and the L1210S and L1210R leukaemias

Dose mg/kg	Days of Treatment	TLX5S			TLX5RT			L1210S			L1210R		
		M.D.D.	%I.S.T.	ΔW	M.D.D.	%I.S.T.	ΔW	M.D.D.	%I.S.T.	ΔW	M.D.D.	%I.S.T.	ΔW
Control	Day 3–Day 7	10.4	–	+2.0	10.2	–	0.6	9.6	–	4.8	9.6	–	4.7
1	"	11.6	12	+1.9	–	–	–	–	–	–	–	–	–
2	"	12.2	17	+1.5	10.4	2	0.4	10.6	10	2.0	10.2	6	4.8
4	"	14.8	42	+1.4	10.2	0	–0.5	12.6	31	1.2	10.4	8	4.0
8	"	14.8	42	–0.7	10.4	2	–0.4	18.8	96	–0.1	11.0	15	2.2
16	"	17.4	67	–0.6	11.4	12	–2.2	60	200	–1.0	12.6	31	–0.4
32	"	–	–	–	11.8	17	–3.4	14.0	46	–2.9	14.6	52	–3.5

M.D.D. – Mean day of death

%I.S.T. – Percentage increase in survival time

ΔW – Weight change between Day 0 and Day treatment ends

Table 32 The antitumour activity of methazolastone the TLX5S lymphoma and the L1210S leukaemia

Dose mg/kg	Days of Treatment	TLX5S			L1210S		
		M.D.D.	%I.S.T.	ΔW	M.D.D.	%I.S.T.	ΔW
Control	Day 3 - Day 7	12.0	-	1.0	10.3	-	3.8
5	"	14.4	20	1.1	-	-	-
10	"	17.0	42	1.5	10.8	5	2.3
20	"	18.4	53	1.4	11.8	15	1.9
40	"	17.4	45	0.6	13.2	28	0.2
60	"	-	-	-	13.7	33	-0.8
80	"	-	-	-	14.4	40	-0.5

M.D.D. - Mean day of death

%I.S.T. - Percentage increase in survival time

ΔW - weight change between Day 0 and Day treatment ends

Table 33 The antitumour activity of diazo - I.C. MCTIC and BCTIC against the TLX5S lymphoma

Compound	Dose mg/kg	Days of Treatment	TLX5S		
			M.D.D.	%I.S.T.	ΔW
Diazo - I.C.	Control	Day 3-Day 7	10.0	-	1.8
	1	"	9.4	-6	2.0
	2	"	9.8	-2	1.8
	4	"	10.4	4	1.2
	8	"	10.0	0	0.4
	16	"	9.4	-6	-1.2
MCTIC	Control	Day 3 only	12.9	-	-
	10	"	14.6	13	-
	20	"	14.2	10	-
	40	"	15.4	19	-
	80	"	15.4	19	-
	160	"	7.0	-46	-
BCTIC	Control	Day 3 only	12.8	-	-
	10	"	13.4	5	-
	20	"	13.0	2	-
	40	"	13.4	5	-
	80	"	15.0	17	-
	160	"	18.4	44	-

M.D.D. - Mean day of death

% I.S.T. - Percentage increase in survival time

ΔW - weight change between Day 0 and Day treatment ends

4.6.2 *In vivo* antitumour activity of azolastone and related compounds against the L1210S and L1210R leukaemias

Azolastone was observed to produce long term extensions in the survival times of mice innoculated with L1210S leukaemia (Table 31). The L1210R leukaemia, which was made resistant to BCNU *in vivo*, was found to be cross resistant to azolastone *in vivo* (Table 31).

Methazolastone was found to have antitumour activity against the L1210S leukaemia (Table 32) although when compared with azolastone its antitumour activity was definitely less.

4.6.3 *In vitro* - *In vivo* cytotoxicity assay's of azolastone and related compounds

4.6.3.1 The effect of metabolic activation upon the *in vitro*-*in vivo* cytotoxicity of azolastone

As azolastone is a novel compound it was thought relevant to investigate the effect of the presence of a metabolising system on its *in vitro* cytotoxicity. The addition of a liver 9000 G homogenate resulted in a slight reduction in the *in vitro* cytotoxicity of azolastone (Table 34). No enhancement of the *in vitro* cytotoxicity of azolastone was observed in the presence of a metabolising system.

4.6.3.2 *In vitro* - *In vivo* cytotoxicity assay of azolastone and diazo-I.C. against the TLX5S and TLX5RT lymphomas

The estimated log cell kill of TLX5S and TLX5RT lymphomas by azolastone and diazo-I.C. are shown in Figure 31. The TLX5RT

Table 34 The effect of metabolism on the in vitro - in vivo cytotoxicity of azolastone to the TLX5S lymphoma

Concentration mcg/ml	TLX5S	
	M.D.D.	% I.S.T.
Control	10.0	-
Control^{+o}	10.2	2
5	16.7	67
10	>60	>500
5°	15.5	55
10°	15.4	54
5^{+o}	15.2	52
10^{+o}	16.2	62

o Incubations contained 1mM NADPH and 5mM $MgCl_2$

\+ Incubation contained a 9000G supernatant equivalent to 100mg wet liver weig

Each value is the mean of five mice

M.D.D. Mean day of death,

% I.S.T. - Percentage increase in survival time

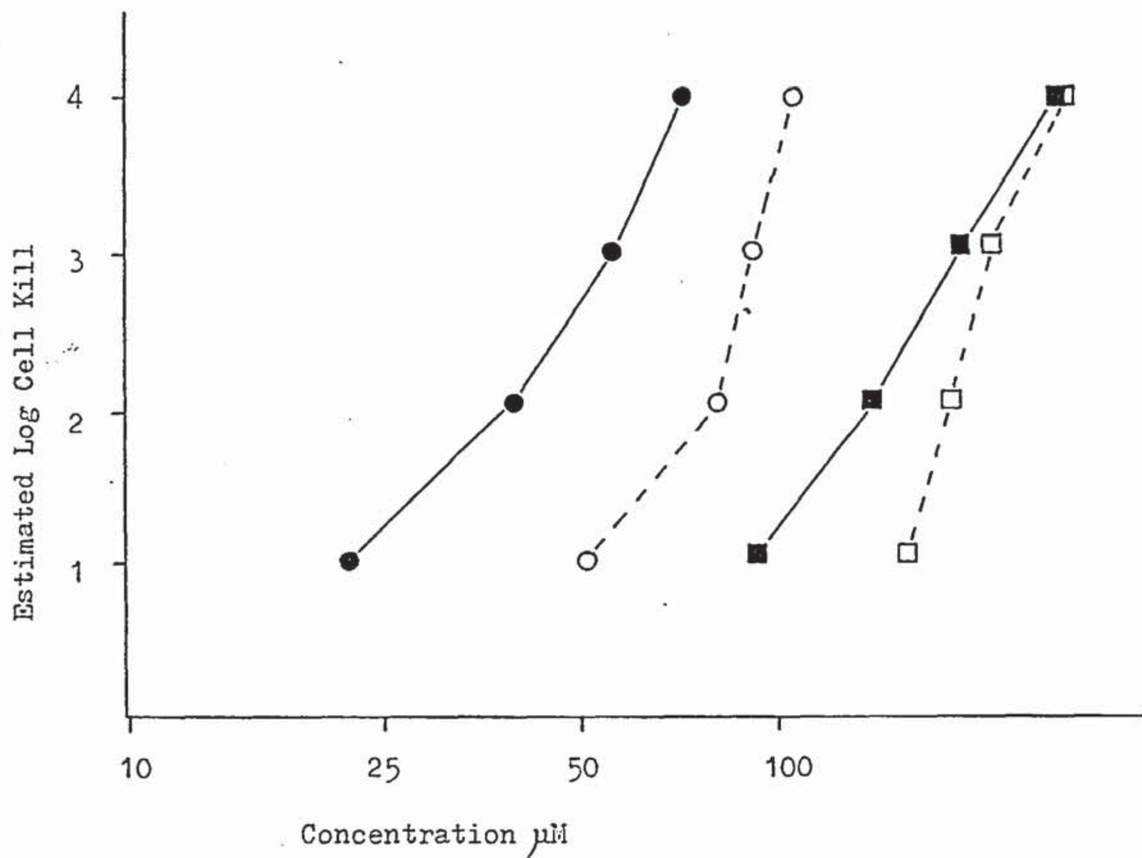


FIGURE 31 In vitro - In vivo cytotoxicity assay's of azolastone against the TLX5S, ——●——, and TLX5RT, ---○---, and of diazo - I.C. against the TLX5S, ——■——, and TLX5RT, --□--, lymphomas. Each point is the mean of five estimates.

lymphoma which was resistant *in vivo* to azolastone (Table 31) was also resistant *in vitro* (Figure 31). The concentration of azolastone required to produce a 3 log cell kill of TLX5RT lymphoma cells is 94 μM whereas an equivalent cell kill of TLX5S lymphoma cell requires 56 μM. Hence the TLX5RT lymphoma is 1.7 fold resistant to azolastone *in vitro*. Diazo-I.C. at the same log cell kill was observed to be equitoxic towards the TLX5S and TLX5RT lymphomas.

4.6.4 The *in vitro* cytotoxicity of azolastone, CNU, MCTIC and chloroethlyisocyanate against L1210 leukaemia as measured by a colony forming assay

The *in vitro* cytotoxicities of azolastone CNU, MCTIC, and chloroethylisocyanate following a 2 hour exposure of L1210 leukaemia cells to various concentrations of these agents are shown in Figure 32 and 33. The survival curves of CNU and MCTIC are very similar in profile and both compounds are more potent than either chloroethyl-isocyanate or azolastone. The concentrations of each agent required to produce a 2 log cell kill against the L1210 leukaemia *in vitro* are as follows, MCTIC 15 μM, CNU 20 μM, chloroethylisocyanate 32.5 μM, and azolastone 55 μM.

4.6.5 The investigation of DNA damage caused by azolastone, CNU, MCTIC and chloroethylisocyanate as measured by alkaline elution (see Section 3.9)

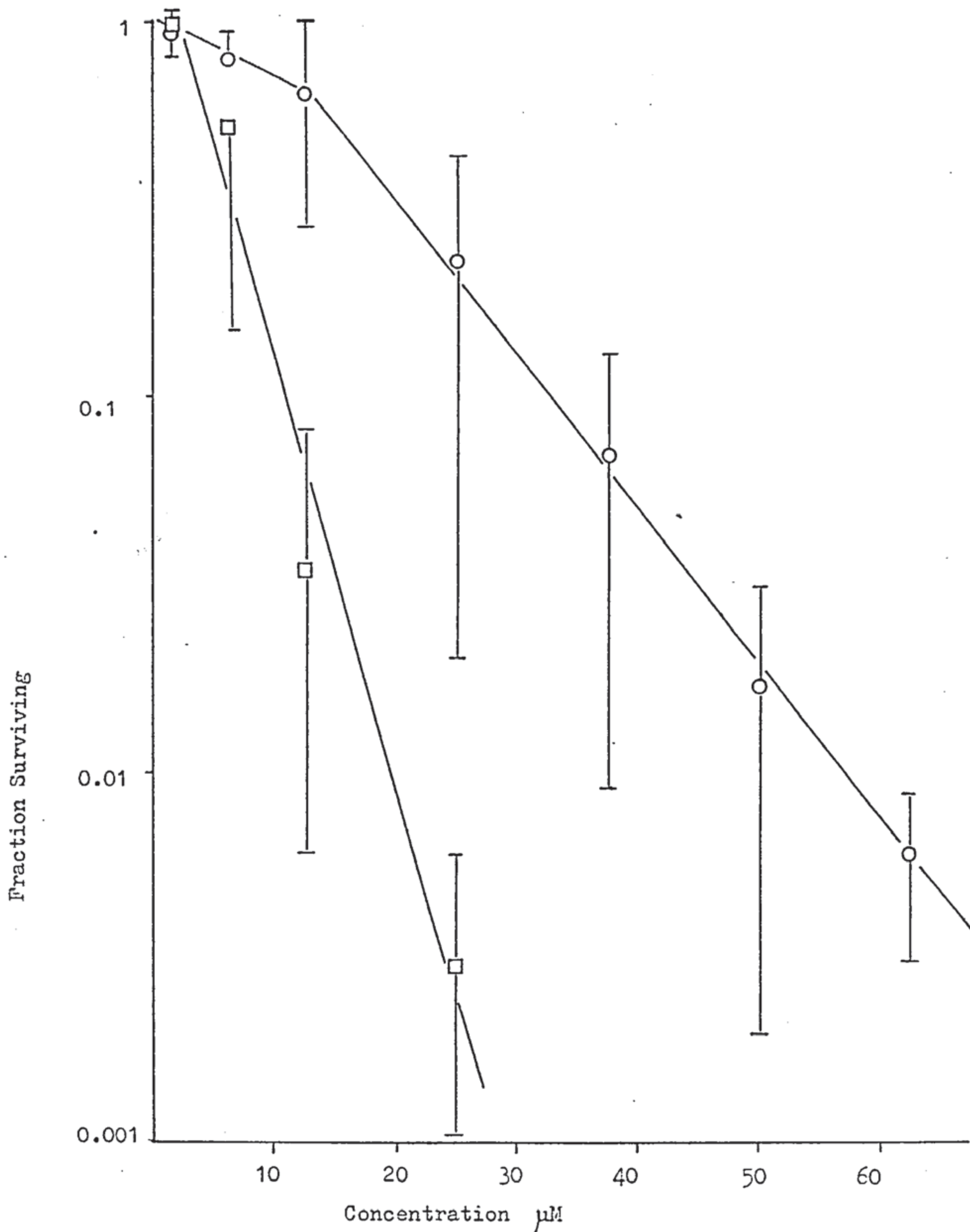
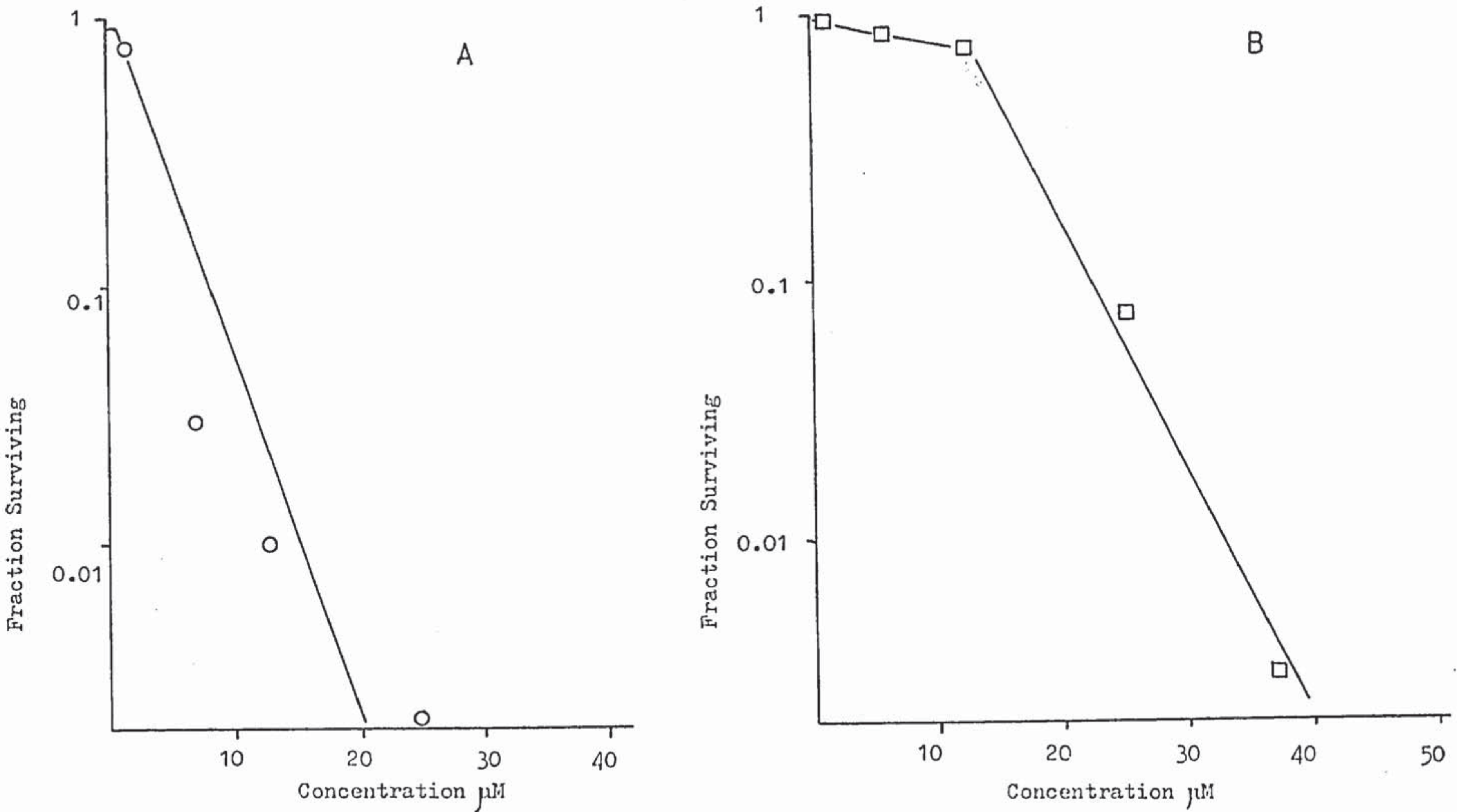


Figure 32 *In vitro* cytotoxicity of L1210 cells exposed to either azolastone, —O— , or CNU, —□— ,. Each point is the mean and s.d. of three separate experiments.



<u>FIGURE 33</u> <u>In vitro</u> cytotoxicity of L1210 cells to MCTIC (A) and chloroethylisocyanate (B). Each point is the mean of t iplicate determinations.

4.6.5.1 The estimation of DNA single strand breaks caused by azolastone, CNU, MCTIC and chloroethylisocyanate

The data is presented to show either single strand breaks (dotted lines) or DNA-DNA interstrand cross links (closed lines). L1210 cells were exposed to each agent for 2 hours and were either assayed immediately or after drug exposure were resuspended in drug free medium for set periods of time and then assayed (Section 3.9).

Single strand breaks could be observed by comparing the elution profiles of the control unirradiated cells with those of the drug treated unirradiated cells. Such comparisons revealed that CNU, MCTIC and azolastone all caused single strand breaks when measured immediately after drug treatment (Figures 34 - 36). Six hours after drug treatment the elution profiles of the drug treated unirradiated cells were similar to those of the control unirradiated cells and this indicates that these strand breaks have been repaired (Figures 34 -36).

The isocyanates have previously been shown to have a limited capability to react with DNA (Cheng et al, 1972) yet chloroethylisocyanate at concentrations which produce less than a 2 log cell kill was remarkable in that it resulted in large quantities of single strand breaks (Figure 37). Furthermore no repair as was the case with azolastone, CNU,MCTIC was observed. Indeed a slight increase in the number of strand breaks was apparent 6 hours after drug treatment (Figure 37).

4.6.5.2 The estimation of DNA-DNA interstrand cross links caused by azolastone, CNU, MCTIC and chloroethylisocyanate

Figure 34 Alkaline elution assays for DNA interstrand cross linking and single strand breaks in L1210 cells exposed to CNU both O(A) and 6 hours (B) after drug treatment. Control, ---●-- , and 50 μM, ---o--- , treated cells received no irradiation, whereas, control, —o— 25μM, —x— , and 50μM —+— , received 3Gy of X-rays

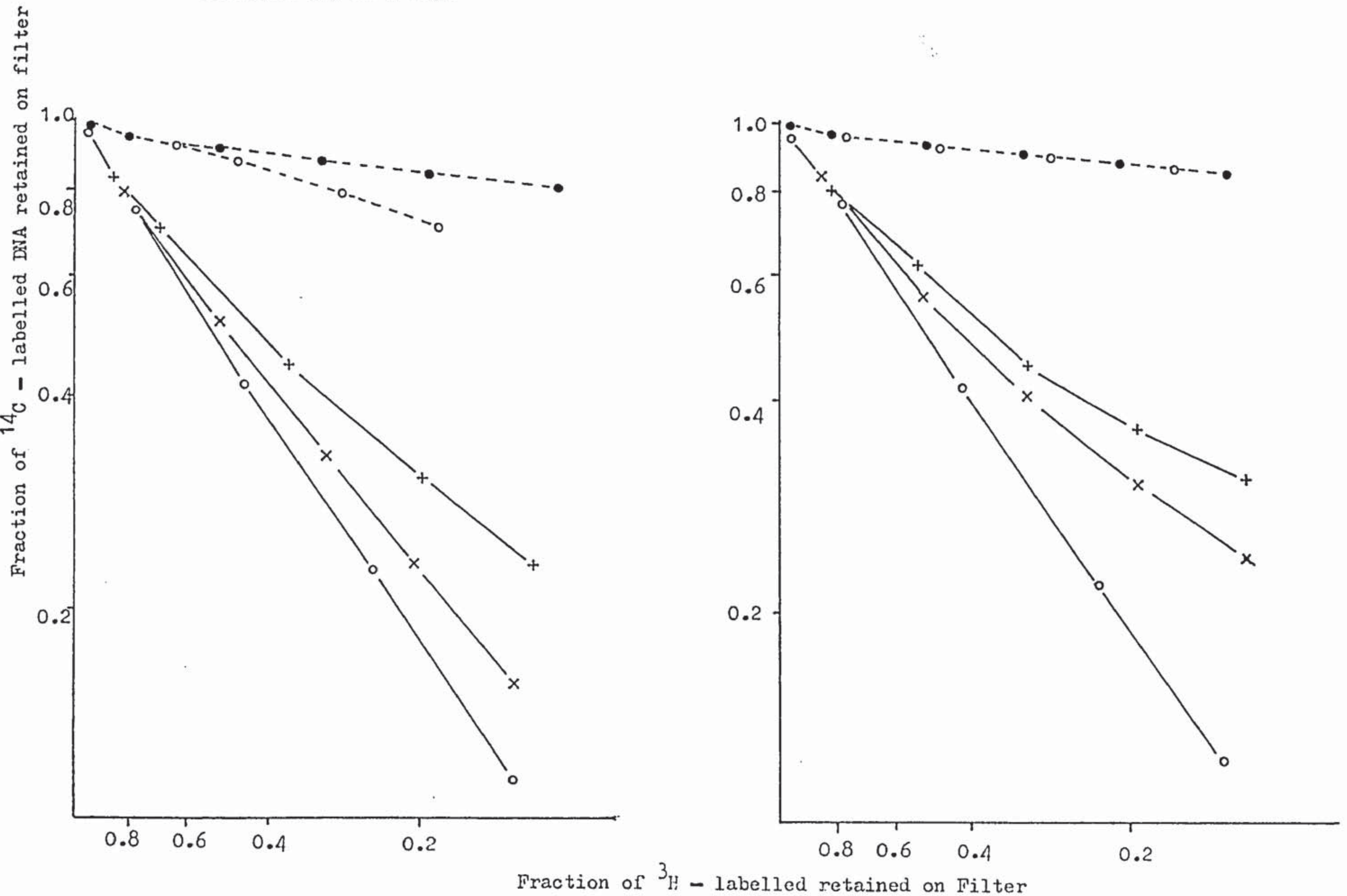


Figure 35 Alkaline elution assays for DNA interstrand cross linking and single strand breaks in L1210 cells exposed to azolastone both 0 (A) and 6 hours (B) after drug treatment. Control, - - ● - - - , and 50μM, - - o - - , treated cells received no irradiation, whereas, control, —o— , 25μM, —×— and 50μM, — + — received 3Gy of X-rays

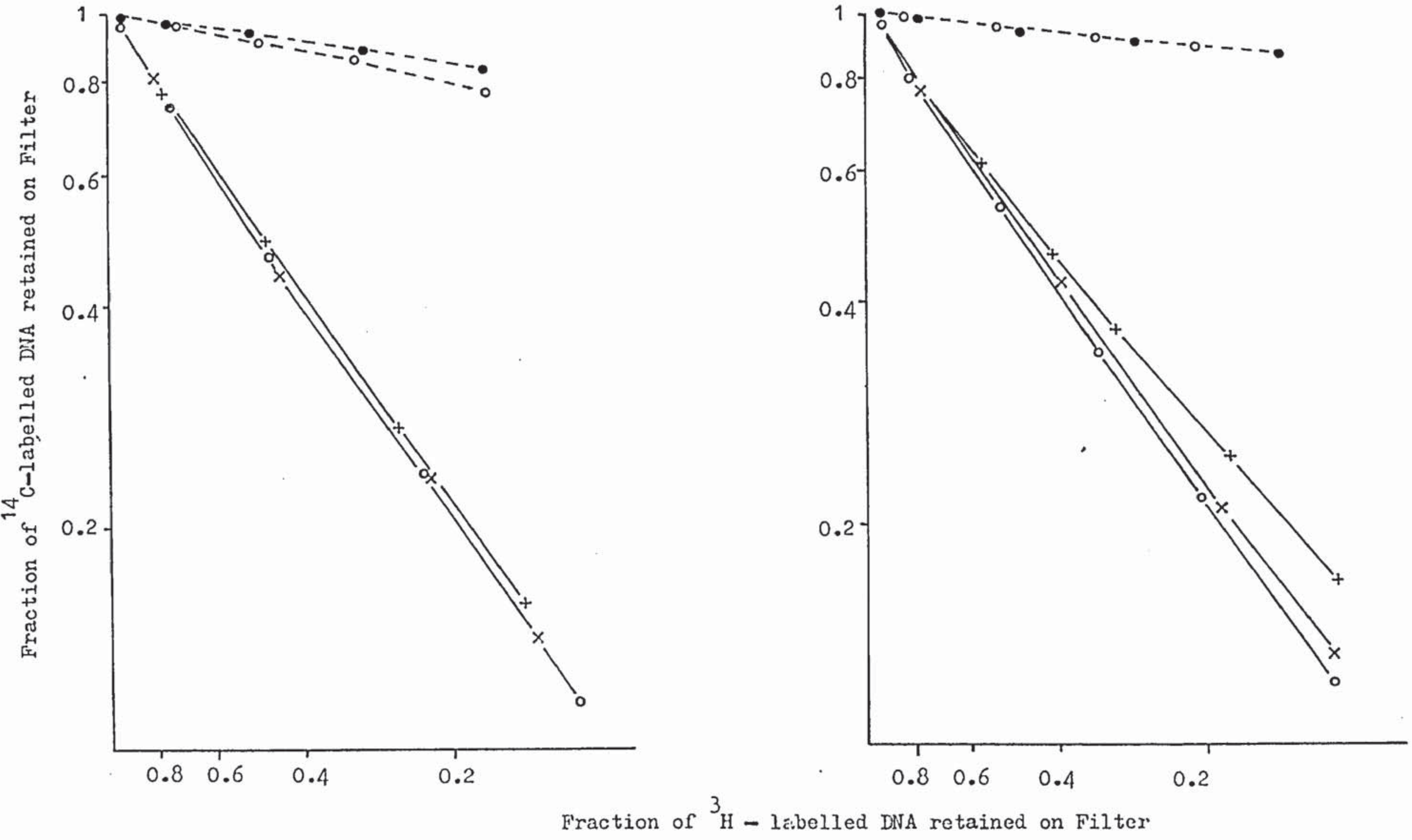
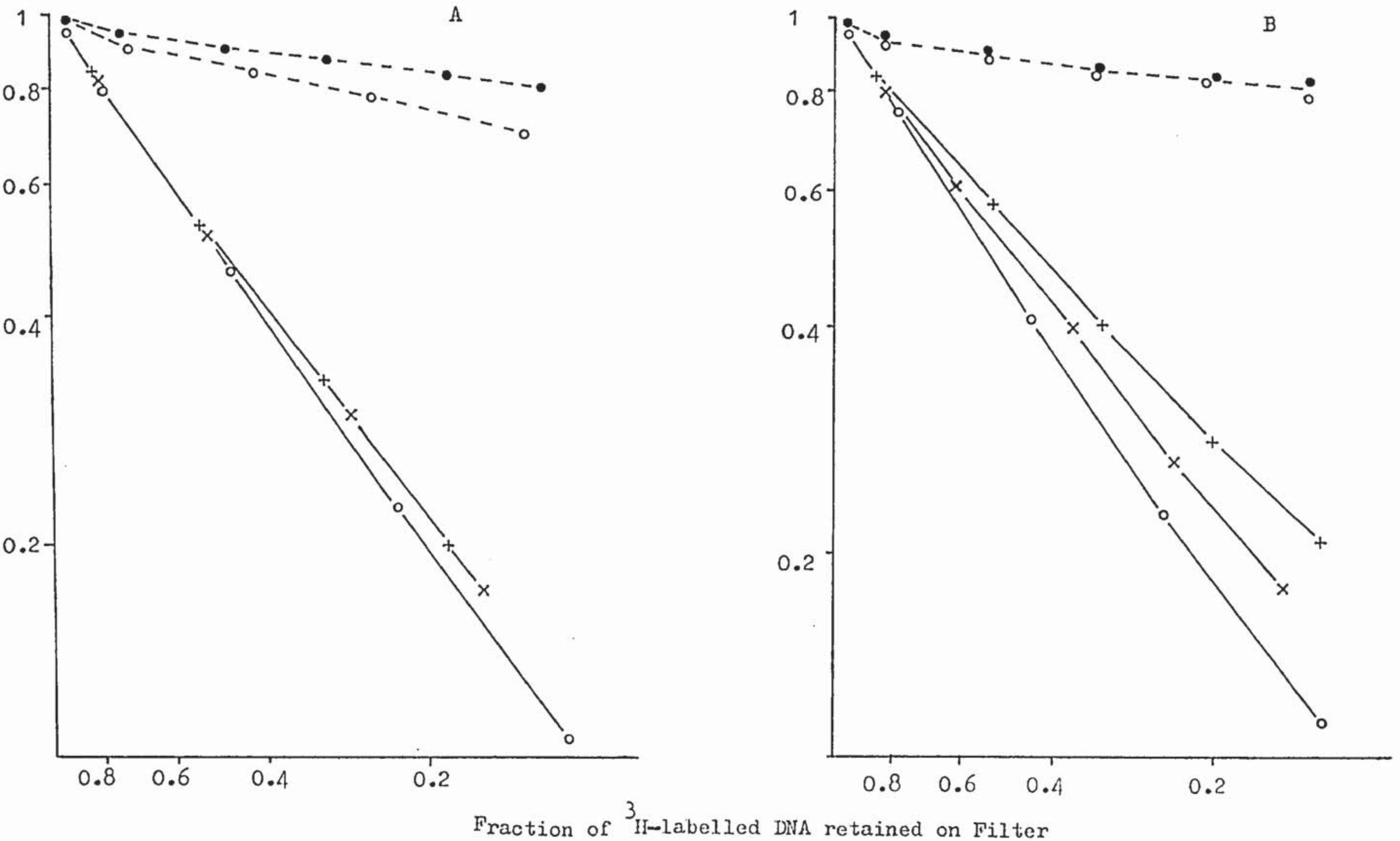
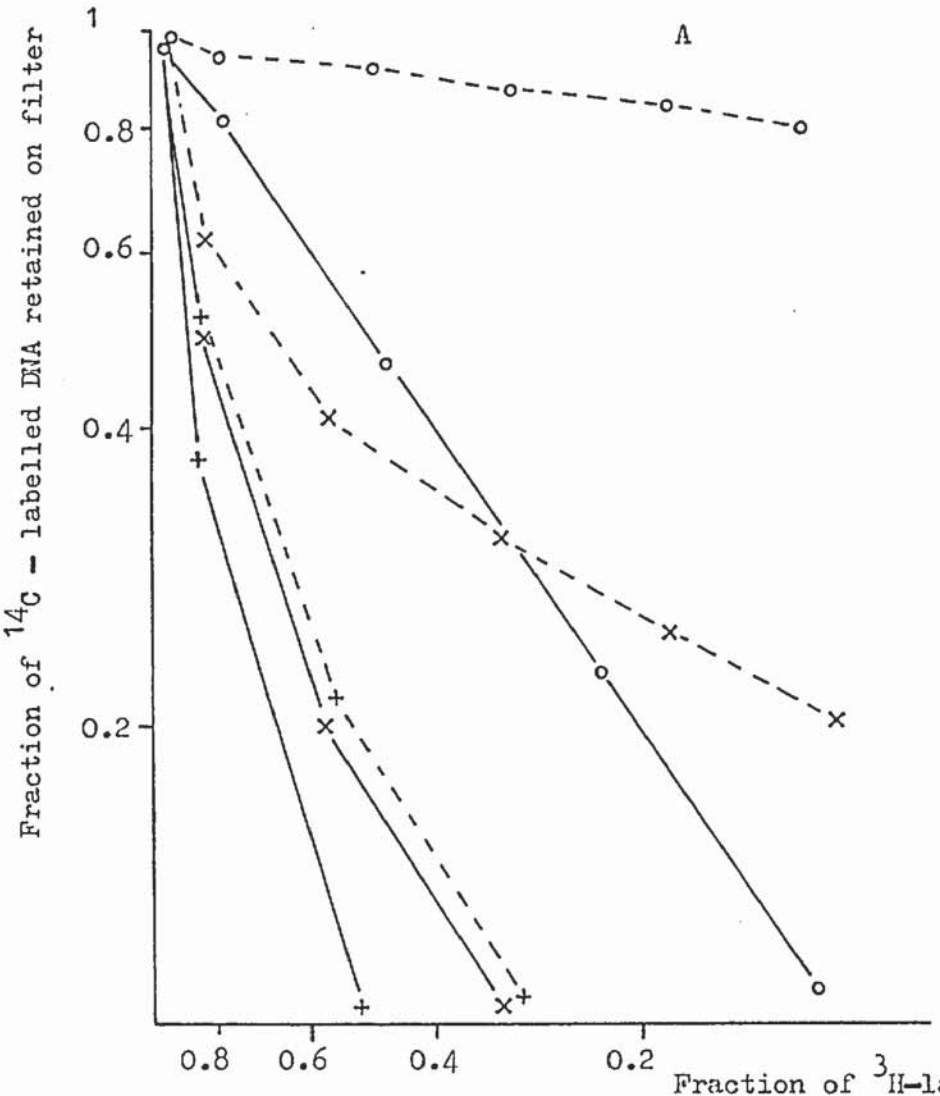


Figure 36 Alkaline elution assays for DNA interstrand cross linking and single strand breaks in L1210 cells exposed to MCTIC both 0(A) and 6 hours (B) after drug treatment. Control, ---●---, and 25μM, ---o---, treated cells received no irradiation, whereas, control, —o—, 12.5μM, —x—, and 25μM, —+—, received 3Gy of X rays.





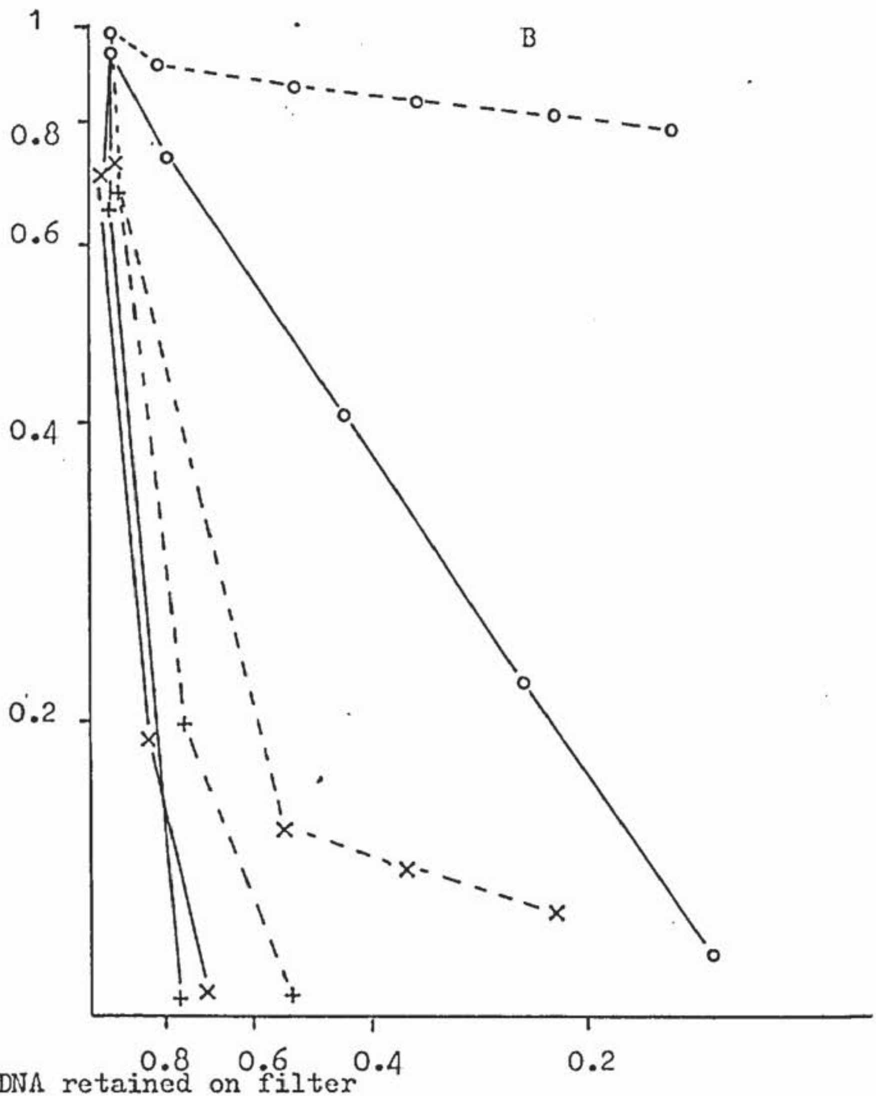


FIGURE 37 Alkaline elution assay for DNA interstrand cross linking and single strand breaks in L1210 cells exposed to chloroethylisocyanate both 0(A) and 6 hours (B) after drug treatment. Control.--o-- , 25μM,--x-- , and 50μM, --+-- .treated cells received no irradiation, whereas, , control,—o— , 25μM, —x—, and 50μM,—+—, received 3Gy of X-rays.

DNA-DNA interstrand cross linking can be observed by comparing the elution profiles of the control irradiated cells with the drug treated irradiated cells. From the elution profiles each compound can be calculated to have a DNA interstrand cross link index (Section 3.9.1).

The relationship between DNA interstrand cross link index and concentration is shown for both CNU and azolastone in Figure 38. At equimolar concentrations CNU produces a six fold greater DNA interstrand cross link than azolastone. The DNA interstrand cross link index 6 hours after drug treatment of 50 µM CNU is 0.196 and that of 50 µM azolastone is 0.032. As the relationship between DNA interstrand cross link index of CNU and azolastone at equitoxic concentration can be determined by extrapolation. At a 2 log cell kill (Section 4.6.4) CNU (20µM) and azolastone (55µM) were extrapolated to provide a DNA interstrand cross link index 6 hours after drug treatment of 0.064 and 0.030 respectively. Hence even at equitoxic concentrations CNU has at least two fold greater DNA interstrand cross link index than azolastone.

MCTIC at a concentration equivalent to a 2 log cell kill provides a DNA interstrand cross link index 6 hours after drug treatment of 0.038. The DNA interstrand cross link index of azolastone and MCTIC at equitoxic concentrations are thus very similar.

The kinetics of DNA interstrand cross link formation and removal are shown for CNU and azolastone in Figure 39. 25µMCNU consistently resulted in a greater DNA interstrand cross link index than either 25µM or 50µM azolastone at all time points studied. Indeed the interstrand cross linking caused by CNU appeared to peak 9 hours after a drug free incubation. Whereas due to the low levels of DNA

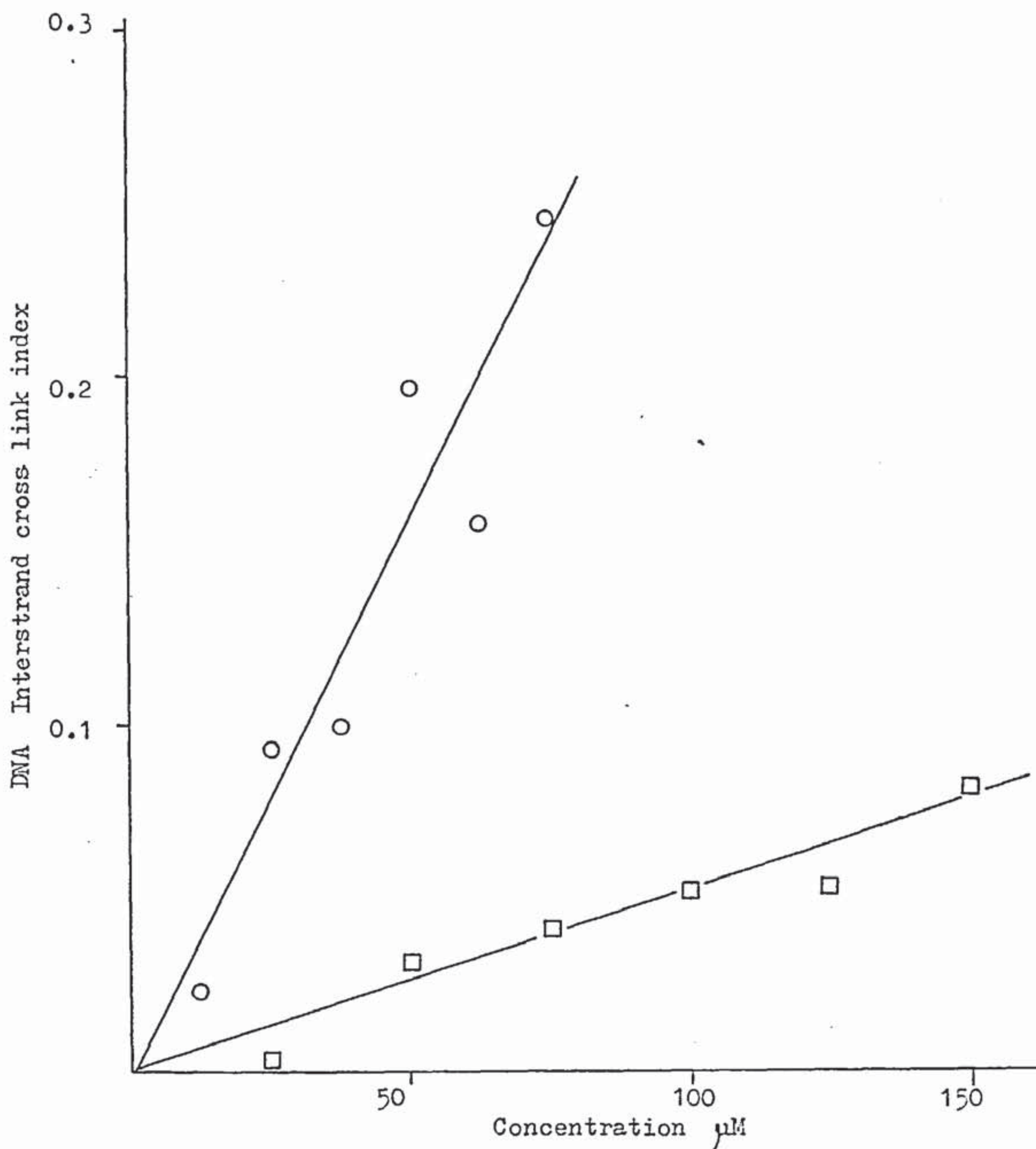


FIGURE 38 The relationship between DNA interstrand cross link index and concentration of CNU —O—, and azolastone —□—. Each point in the mean of at least two separate experiments.

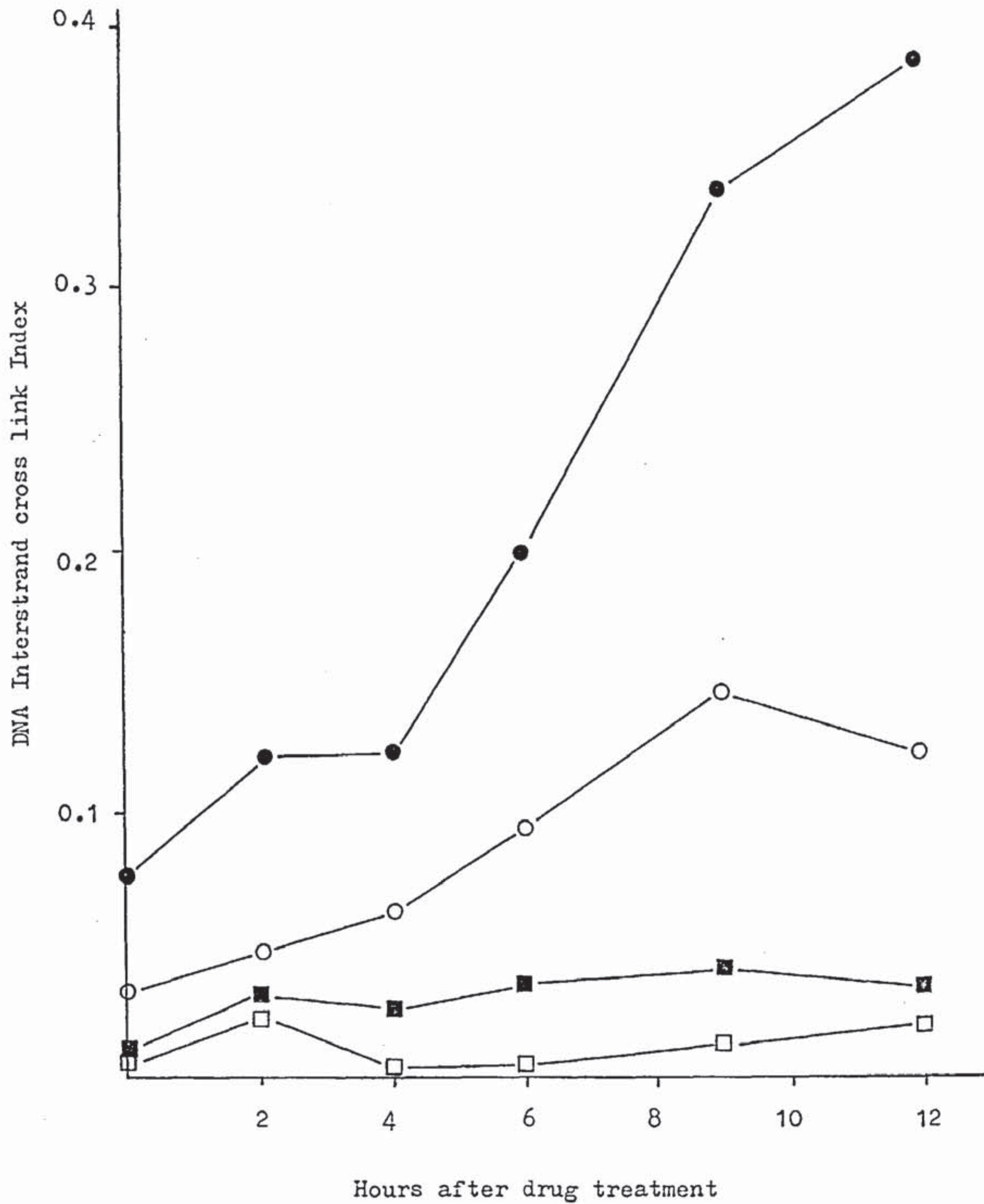


Figure 39 The relationship between DNA interstrand cross link index and time after drug treatment for CNU, 25μM —○— , and 50μM —●— , and azolastone, 25μm —□— and 50μM —■— . Each point is the mean of at least two separate experiments.

interstrand cross linking caused by azolastone no apparent peak was observed (Figure 39).

The elution profiles of the chloroethylisocyanate treated and irradiated cells (Figure 37) would seem to indicate that the isocyanate caused no DNA-DNA interstrand cross linking. The large quantities of single strand breaks caused by chloroethylisocyanate increased the rate of elution (Figure 37) whereas it would be expected that DNA-DNA interstrand cross linking would decrease the rate of elution (Section 3.9.1). Hence the large quantities of strand breaks observed with chloroethylisocyanate would mask the presence and prevent the detection of DNA-DNA interstrand cross links caused by chloroethylisocyanate.

4.6.5.3 The estimation of DNA-protein cross links caused by azolastone and CNU

The DNA-protein cross link index can be calculated as previously described (Section 3.9.2). The DNA-protein cross links observed with CNU and azolastone at both 0 and 6 hours after drug treatment are shown in Figures 40 - 43. 25µM CNU was calculated to have a DNA-protein cross link index of 0.056 and 0.067 at 0 and 6 hours after drug treatment. 50µM azolastone was calculated to have a DNA-protein cross link index of 0.016 and 0.052 at 0 and 6 hours after drug treatment. At equimolar concentrations CNU was found to have a 3 fold greater DNA-protein cross link index than azolastone 6 hours after drug treatment. No data was obtained for these compounds at equitoxic concentrations.

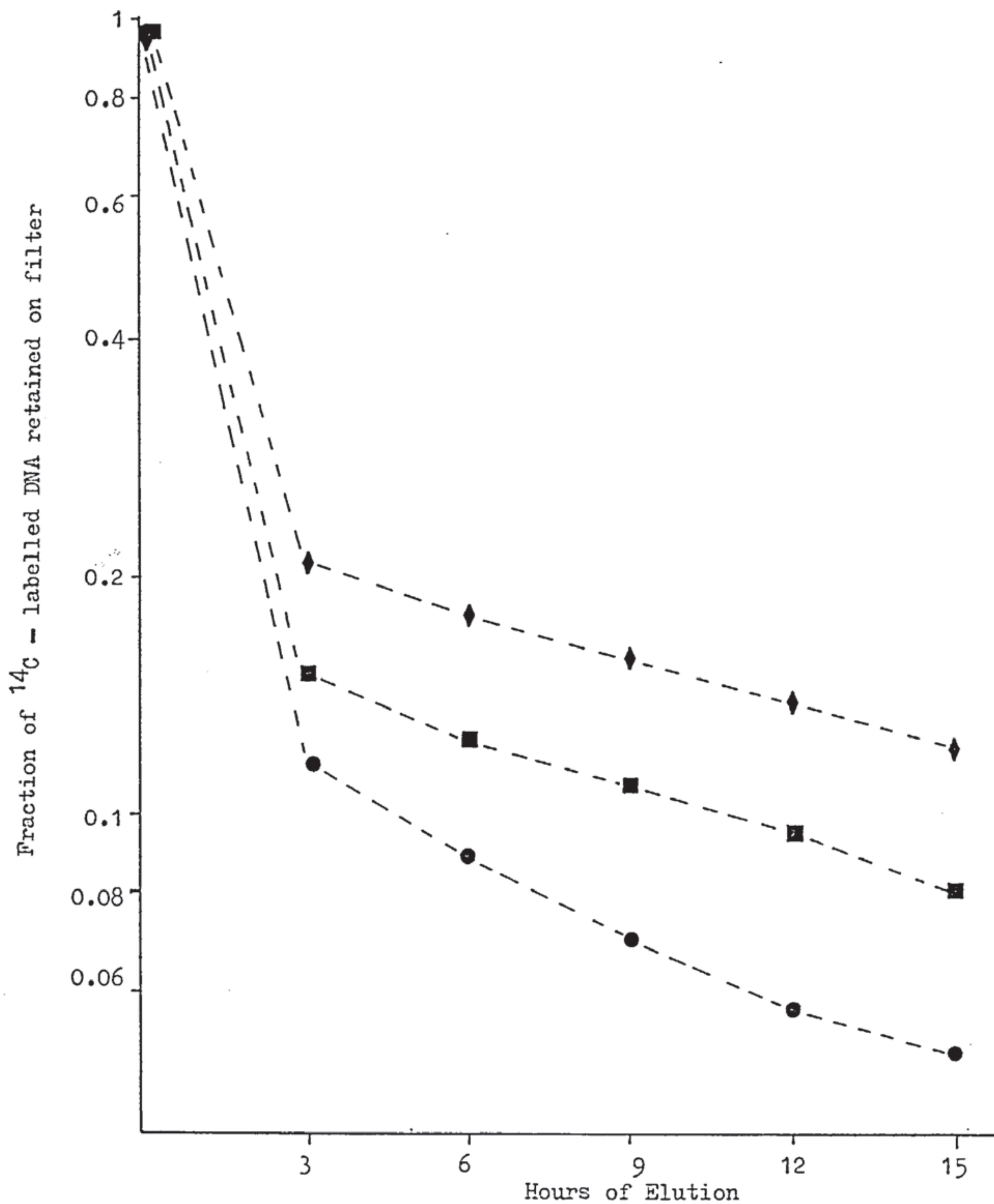


FIGURE 40

Alkaline elution assay for DNA - protein cross linking in L1210 cells exposed to CNU 0Hours after drug treatment. Control, —●— , 25μM, —■— , and 50μM, —♦— treated cells all received 30Gy of X-rays.

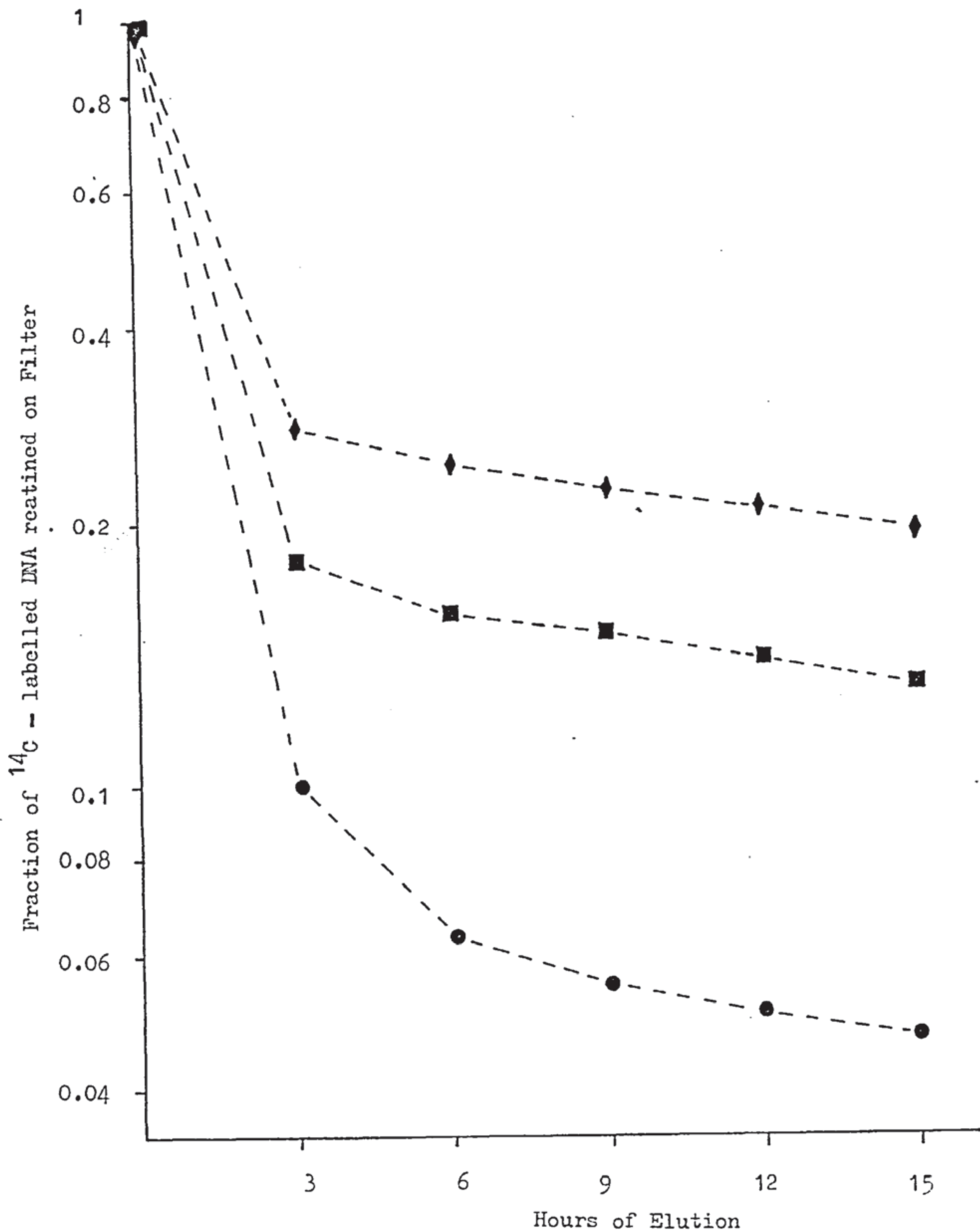


FIGURE 41 Alkaline elution assay for DNA-protein cross linking in L1210 cells exposed to CNU, 6 hours after drug treatment. Control, --●-- , 25µM,--■-- , and 50µM, --♦--, treated cells all received 30Gy of X-rays.

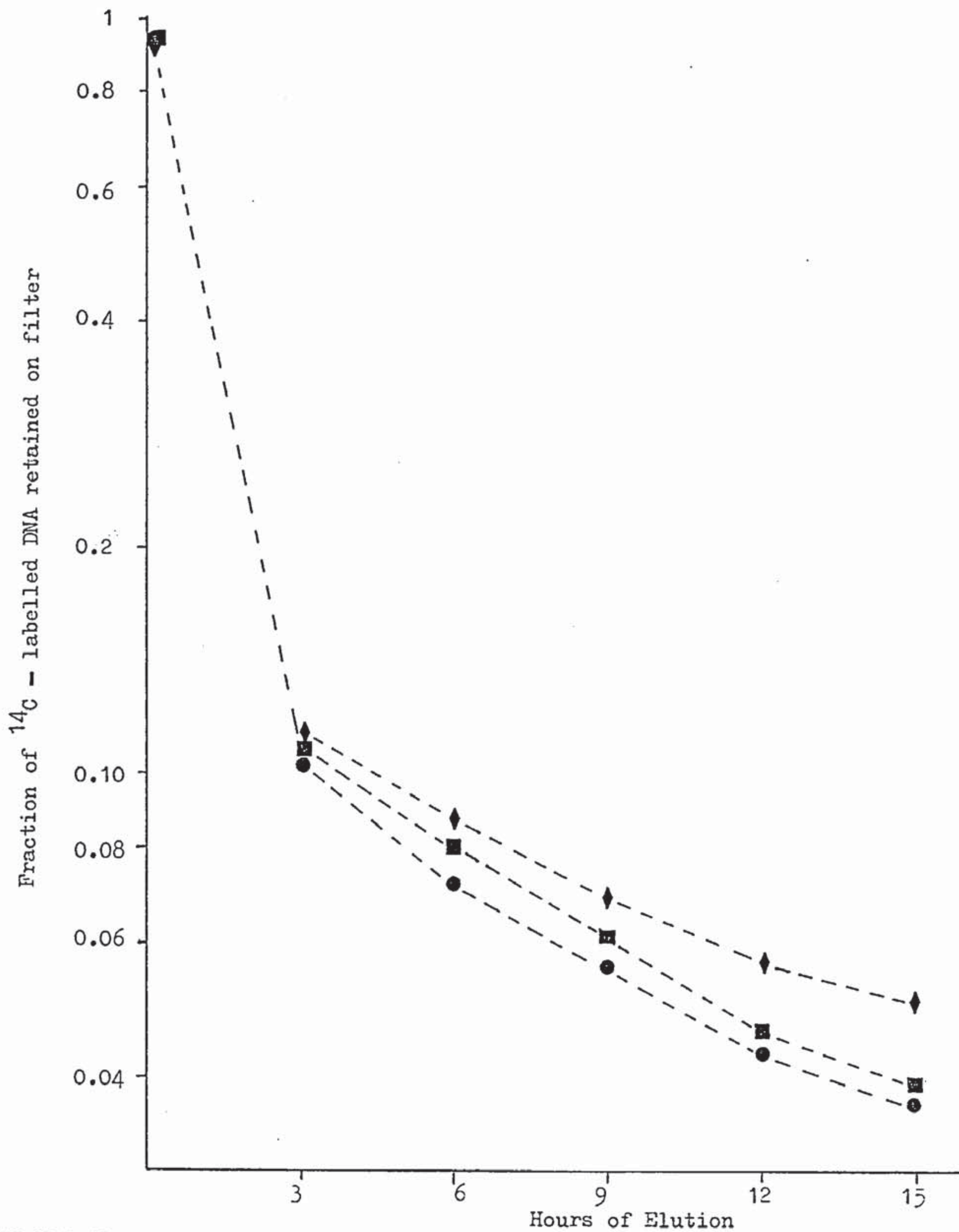


FIGURE 42

Alkaline elution assay for DNA - protein cross linking in L1210 cells exposed to azolastone, 0hours after drug treatment. Control,--●--, 25μM,--■--, and 50μM,--♦--, treated cells received 30Gy of X-rays.

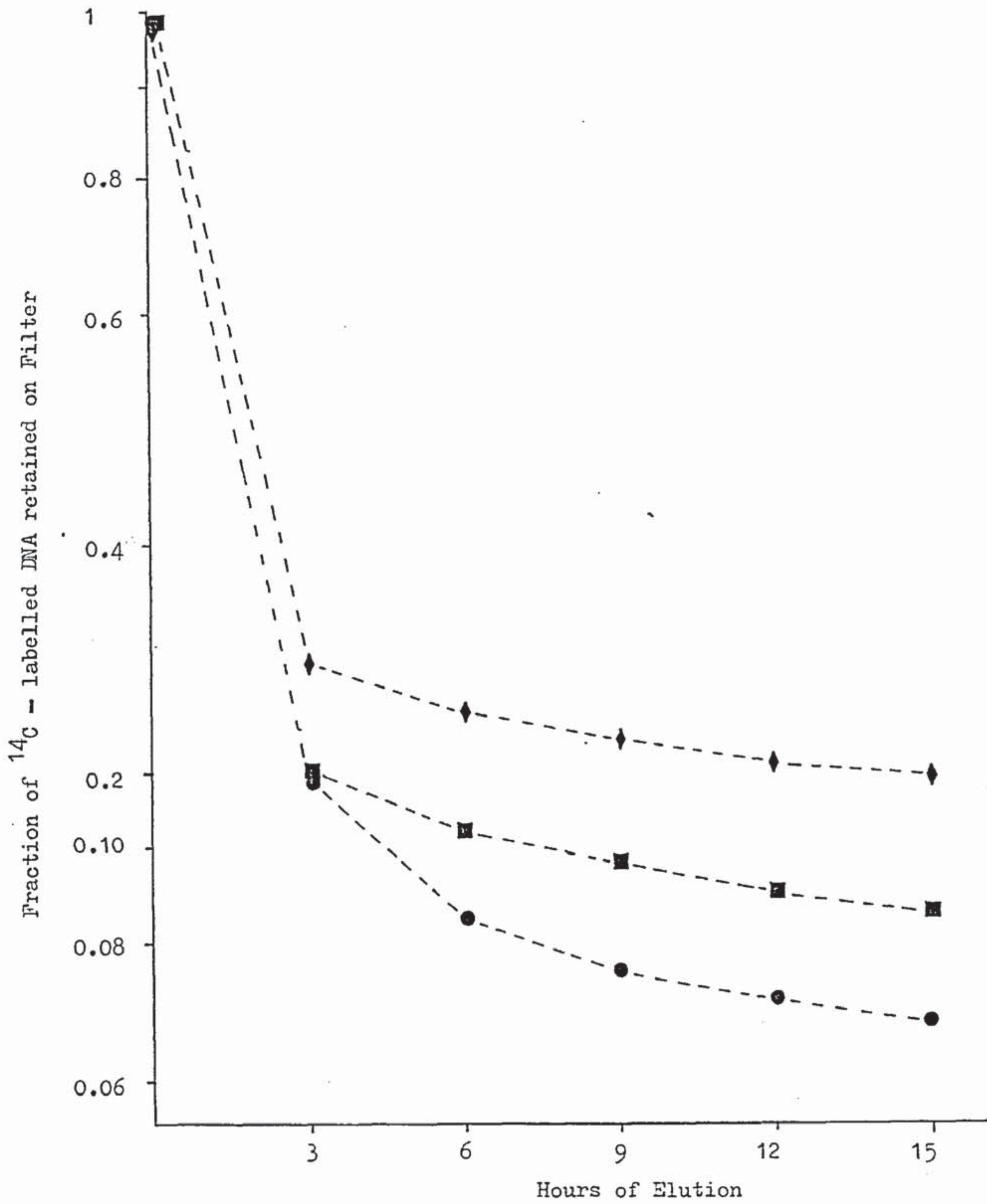


FIGURE 43 Alkaline elution assay for DNA - protein cross linking in L1210 cells exposed to azolastone, 6 hours after drug treatment. Control,--●-- , 25μM,--■-- , and 50μM, --♦--, treated cells received 30Gy of X-rays

4.6.5.4 The estimation of total DNA cross linking caused by azolastone and CNU

The total DNA cross links which includes both DNA-protein cross links and DNA-DNA interstrand cross links, and which were calculated in the same manner as the DNA interstrand cross link index (Section 3.9.1) are shown in Table 35. 25μM CNU was found to have a total DNA cross link index of 0.227 and 0.393 at 0 and 6 hours after drug treatment. 50μM azolastone had a total DNA cross link index of 0.033 and 0.219 at the same time points. At equimolar concentrations CNU was found to have a 3 fold total DNA cross link index than azolastone 6 hours after drug treatment.

Table 35 The total DNA cross link index of CNU and azolastone at 0 and 6 hours after drug treatment

Compound	Concentration µM	Hours after Treatment	Total Cross link Index
CNU	25	0	0.227
	50	0	0.425
	25	6	0.393
	50	6	0.730
Azolastone	25	0	0.011
	50	0	0.033
	25	6	0.122
	50	6	0.219

SECTION 5

DISCUSSION

The haloalkylnitrosoureas, such as BCNU and CCNU decompose to produce two major reactive intermediates, a haloalkyldiazohydroxide and an isocyanate species (Section 1.2), each of which is potentially capable of contributing towards the mechanism of antitumour action of the parent compound. The mechanism of the cytotoxic action of the haloalkylnitrosoureas is generally considered to be related to their ability to alkylate and then cross link DNA (Kohn et al, 1981). However, there is evidence which indicates that this may not fully explain their cytotoxicity (Section 1.5.5) and the ability of the haloalkylnitrosoureas to perform carbamoylation reactions may be of additional importance to their mechanism of action (Section 1.5.7 - 1.5.9).

The basis of the present investigation was to compare the *in vitro* cytotoxicity of the two major reactive intermediates produced upon haloalkylnitrosourea decomposition to cell lines which are either sensitive or resistant to the progenitor compound *in vivo*. The rational behind this approach is that, as previously stated (Section 1.6) if a cytotoxic species is observed which preferentially kills cells of sensitive lines *in vitro* in comparison to those which are resistant then this species maybe likely to be responsible for some or all of the selective cytoxicity or antitumour effect *in vivo*. In addition, when a species has equivalent *in vitro* cytotoxicity to the sensitive and resistant lines it was argued that this species is responsible for non-selective cytotoxicity of the parent molecule (Gescher et al, 1981).

As previously mentioned the mechanism of action of haloalkylnitrosoureas is generally considered to be due to their ability to

perform alkylation reactions. It was thus considered relevant to investigate the mechanism of action of the haloalkylnitrosoureas against the L1210 leukaemia, which is naturally sensitive to alkylating agents (Schabel, 1976) and the TLX5 lymphoma which is naturally insensitive to alkylating agents (Connors and Hare, 1975). Both these cell lines are sensitive to haloalkylnitrosoureas *in vivo* (Schabel, 1976; Connors and Hare, 1975). In addition an L1210 leukaemia and a TLX5 lymphoma with induced resistance to BCNU *in vivo* were utilised in this study (Schabel et al, 1978; Connors and Hare, 1975).

Of additional interest was the finding that a TLX5 lymphoma with induced resistance to a dimethyltriazene *in vivo* was cross resistant to the nitrosoureas *in vivo* (Audette et al, 1973). It was thus considered that an *in vitro* analysis of the cytotoxic effects of the isolated cytotoxic fragments generated from a nitrosourea, particularly the isocyanates, to this triazene resistant tumour would be interesting because unlike the other resistant cell lines studied this cell line had not been exposed to either the alkylating or carbamoylating fragments of the nitrosoureas *in vivo* as resistance was induced. Furthermore it was considered that the formation of a TLX5 lymphoma cell line resistant to cyclohexylisocyanate may provide an indication of the role of this isocyanate in the mechanism of action of CCNU.

The advantage of the *in vitro* - *in vivo* cytotoxicity assay system used in this study is that tests were performed on cells which have not been selected for long term growth *in vitro* but

were instead the same as those used in the *in vivo* antitumour tests. Hence the comparisons between the *in vivo* antitumour tests and the *in vitro* - *in vivo* cytotoxicity assays would seem to be more applicable than those which utilise cell lines which have been adopted for growth in culture media.

Using logarithmic dilutions of cells from each cell line, estimates were made of an apparent logarithmic cell kill achieved *in vitro*. This method assumes that increases in survival times of animals which have received treated cells are due to a reduction of viable cells rather than a slowing of the cell cycle time or a change in the immune response of the host to those treated cells. This assumption is also applicable to the standard *in vivo* antitumour tests which measure survival time as their end point. Clonogenic assays performed *in vitro* (rather than *in vivo* which is what the *in vitro* - *in vivo* cytotoxicity assay essentially does) suffer from the problem that cellular adaptation and selection may be necessary for growth *in vitro*. In the case of L1210 leukaemia, for example, mercaptoethanol treatment of ascites cells was required in order to obtain growth in vitro (Ishii et al, 1981). Various attempts in the present study to establish a consistent clonogenic assay for the TLX5 lymphomas have so far proved unsuccessful, although it would appear that the method of Chu and Fischer (1968) may provide such an assay albeit with very low plating efficiencies (Table 26).

The *in vitro* cloning of certain other tumour cell lines has also presented problems to other workers. The culture requirements of mouse plasmacytoma cells *in vitro* have been shown to be stringent

(Park et al, 1971a). Colony formation occurred when cultures were replenished daily with media stored at $-20^{o}C$ but not $4^{o}C$. In addition colony formation was greatest in the presence of L-ascorbic acid (Park et al, 1971a). A mouse kidney tubule feeder layer was found to be essential for the growth of mouse myeloma cells (Park et al, 1971b). Furthermore, human serum promoted and fetal calf serum inhibited colony formation of mouse myeloma cells _in vitro_ (Park et al, 1971b). It has been reported that autoclaved agar contains an inhibitor of granulocyte-macrophage colony growth _in vitro_ (Dixon et al, 1981).

Thus the interpretation of data obtained from such _in vitro_ systems may not relate to the _in vivo_ situation as the growth conditions _in vitro_ appear unrelated to those _in vivo_. Moreover, significant differences in the pattern of drug sensitivity obtained _in vitro_ can result from variations in clonogenic assay procedures (Hepburn et al, 1982). Hence in this study the _in vitro_ - _in vivo_ cytotoxicity assay was considered a more suitable assay to use in comparison with the _in vivo_ antitumour tests especially as the assumptions which have to be made are the same for both systems.

The results presented in this study (Tables 2 - 8) confirm the findings of Connors and Hare (1974, 1975) that the TLX5S lymphoma is naturally insensitive _in vivo_ to alkylating agents of the 2-chloroethylamine type, such as cyclophosphamide and chlorambucil (Table 7 and 8), but is highly sensitive _in vivo_ to the haloalkylnitrosoureas (Table 2 - 5). In addition the

L1210S leukaemia has been shown to be highly sensitive *in vivo* to alkylating agents such as cyclophosphamide (Table 16) and to haloalkylnitrosoureas (Table 13-14). Furthermore the patterns of resistance, as previously reported by Connors and Hare (1974, 1975) and Schabel et al (1978), of the resistant cell lines used in this study were identical (Tables 2-5, 13 and 14).

This study has also confirmed the findings of Audette et al (1973) that the TLX5RT lymphoma, a cell line with induced resistance to a dimethyltriazene *in vivo*, was cross resistant to the haloalkylnitrosoureas *in vivo* (Tables 2 - 5). This finding is particularly interesting as a L1210 leukaemia cell line with induced resistance to a dimethyltriazene *in vivo* was shown to retain sensitivity to haloalkylnitrosoureas (Kline et al, 1971). As neither of these cell lines had previously been exposed to a nitrosourea *in vivo* the results may suggest that there is a common link between the mechanism of action of the haloalkylnitrosoureas and the dimethyltriazenes *in vivo* against the TLX5 lymphoma cell line, but not the L1210 leukaemia cell line.

The *in vitro* - *in vivo* cytotoxicity assay was used to estimate the degree of resistance of the TLX5RT and TLX5RB lymphomas *in vitro*. The fact that the TLX5RT lymphoma was cross resistant to both the haloalkylnitrosoureas and the isocyanates (Figures 14 and 15) suggest that the isocyanates may play a role in the *in vitro* cytotoxicity and possibly therefore *in vivo* antitumour activity of the haloalkylnitrosoureas. Indeed, in the case of cyclohexylisocyanate

the degree of resistance of the TLX5RT lymphoma when an estimated 3 log cell kill was obtained _in vitro_ was similar to that observed with its progenitor nitrosourea CCNU (Table 17). The finding that the TLX5RB lymphoma was also cross resistant to both the nitrosoureas and isocyanates (Figures 16 and 17) would appear to strengthen the hypothesis that the isocyanates are involved in the _in vitro_ cytotoxicity and _in vivo_ antitumour activity of the nitrosoureas.

Generally the concentrations of the isocyanates required to give cytotoxicity equivalent to that of the nitrosoureas against the TLX5 lymphomas was 2 to 3 times that of the progenitor nitrosourea (Figures 14 - 17). It has been argued that isocyanates play a minor role in the cytotoxicity of the nitrosoureas since little toxicity was observed when the isocyanates were added to L1210 cells over a time period calculated to give a concentration x time profile similar to that of the release from their progenitors (Hilton et al, 1978). However, the nitrosoureas are latentiated forms of isocyanates and are transported intact into tumour cells (Begleiter et al, 1977), thus the addition of isocyanates extracellularly at similar concentrations may not be equivalent to the addition of an intact nitrosourea which is capable of constantly releasing the isocyanate intracellularly.

The lack of cross resistance between the nitrosoureas and other electrophilic compounds such as nitrogen mustard and formaldehyde (Figure 23 and 24) suggests that the cross resistance

to the isocyanates (Figure 14 – 17)., is specific for this chemical species. These results would also suggest that the *in vitro* – *in vivo* cytotoxicity assay is capable of identifying those species which are selectively cytotoxic *in vitro* and may thus have antitumour activity *in vivo*. It is particularly interesting that nitrogen mustard, a compound which is known to alkylate and cross link DNA (Ewig and Kohn, 1977), should have a greater *in vitro* cytotoxicity to the TLX5RT lymphoma than to the TLX5S lymphoma (Figure 23). The TLX5RB lymphoma has also been shown to have a greater sensitivity *in vitro* to chlorambucil than the TLX5S lymphoma (Connors and Hare, 1974). These results are both examples of collateral sensitivity, a phenomenon which has been observed with many resistant tumour cell lines (Schmid and Hutchinson, 1972).

Formaldehyde, a compound which can react with DNA (Chaw et al, 1980) and cause DNA-protein cross links (Fox and Bedford, 1981), was equally toxic to the TLX5S and TLX5RT lymphomas *in vitro* (Figure 24). In addition, a monomethyltriazene, which is capable of acting as a methylating agent, has equal toxicity to the TLX5S and TLX5RT lymphomas *in vitro* (Gescher et al, 1981). These results together with that for nitrogen mustard (Figure 23) would suggest that alkylation of cellular targets is a non-selective cytotoxic event for the TLX5 lymphoma.

The results dicussed so far suggest that a species other than the haloalkyldiazohydroxide alkylating species, probably the

isocyanates, produced upon nitrosourea decomposition may contribute substantially to the *in vitro* cytotoxicity and *in vivo* antitumour activity of the nitrosoureas to the TLX5 lymphoma. This hypothesis along with other results presented in this thesis (Section 4.2.5), appear to contradict the generally accepted hypothesis that the cytotoxicity of the nitrosoureas is essentially a direct result of their ability to alkylate and cross link DNA (Kohn et al, 1981).

The failure to find cross resistance between the haloalkylnitrosoureas and isocyanates *in vitro* using the L1210 leukaemia cell lines (Figure 18 and 19) suggest that, as is generally accepted, alkylation may be the major cause of nitrosourea cytotoxicity to this tumour. Thus the results obtained with the *in vitro* - *in vivo* cytotoxicity assay are dependent not only on the chemical species but also on the cell line used. Hence the specificity of this assay would appear to suggest that it is a valid assay to use in studies similar to this one. The L1210 leukaemia resistant to BCNU showed some degree of cross resistance to chloroethylisocyanate (Figure 18) and it may be that the ability of this molecule to cross link cellular macromolecules is of relevance. Thus the cross resistance to the isocyanates observed in this study (Figures 14 - 17) as well as being a specific feature of the isocyanate species is also largely specific to the TLX5 lymphoma (cf Figures 18 and 19).

The findings that nitrogen mustard was equally cytotoxic

to the L1210S and L1210R leukaemias in vitro (Figure 23) and that cyclophosphamide showed similar in vivo antitumour activity to those two cell lines (Table 16) are not consistent with the view that alkylation is a selective cytotoxic process involved in the mechanism of action of the nitrosoureas to the L1210 leukaemia. As the isocyanates are known to inhibit the repair of DNA lesions (Kann et al, 1980b) it may be possible that the acquired resistance of the L1210 leukaemia to BCNU is related to the cell's ability to overcome the inhibition of DNA repair enzymes by the isocyanates. However, if the nitrosoureas do act as alkylating agents so as to be cytotoxic to the L1210 leukaemia then the lack of cross resistance between the nitrosoureas and cyclophosphamide in vivo (Section 4.1.2.) and nitrogen mustard in vitro (Section 4.2.3. and 4.2.5) support the concept that cross resistance among alkylating agents is not universal (Schmid et al, 1980) although it must be borne in mind that these alkylating agents may alkylate by different mechanisms.

The results presented in this study indicate that the validity of hypotheses concerning the mechanism of cytotoxic action of the nitrosoureas is dependent upon the nature of the cell line chosen for investigation. It is hardly surprising that the mechanism of action of the nitrosoureas is considered to be due to alkylation and cross linking of DNA (Kohn et al, 1981), when the majority of studies have utilised L1210 leukaemia (Kohn et al, 1981; Hilton et al, 1979)(see Section 1.5), a cell line which is highly sensitive to alkylating agents (Schabel, 1976).

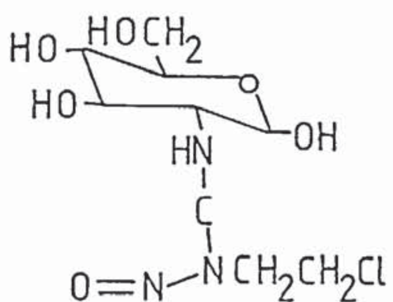
Further evidence in support of the view that alkylation and cross linking of DNA may not be important in the mechanism of action of the nitrosoureas to the TLX5 lymphoma was obtained in a study of the in vivo antitumour activity of the flouroethylnitrosoureas BFNU and FCNU (Section 4.1.1.). BFNU and FCNU are as active in vivo against the TLX5S lymphoma (Tables 4 and 5) as BCNU and CCNU (Tables 2 and 3). Furthermore the TLX5RT lymphoma is cross resistant in vitro to BFNU and FCNU (Figures 20 and 21). As the floureothylnitrosoureas have been shown to have little cross linking activity (Kohn, 1977; Lown et al, 1978; Sharkey et al, 1982) these results argue against the hypothesis that the mechanism of action of the nitrosoureas involves cross linking of DNA (Kohn et al, 1981) although alkylation of DNA cannot be ruled out (Section 1.5.5).

If the hypothesis that carbamoylation may be important in the mechanism of action of the nitrosoureas to the TLX5 lymphoma is valid then nitrosoureas with low carbamoylating activity such as chlorozotocin (Figure 2) would be expected to have no in vivo antitumour activity against the TLX5 lymphoma. It may also be expected that the TLX5RT lymphoma would not be cross resistant to chlorozotocin in vitro. Chlorozotocin was found to have antitumour activity against the TLX5S lymphoma in vivo (Table 6), although its activity was less than that of BCNU or CCNU (Tables 2 and 3). Furthermore the TLX5RT lymphoma was found to be cross resistant to chlorozotocin in vitro (Figure 22).

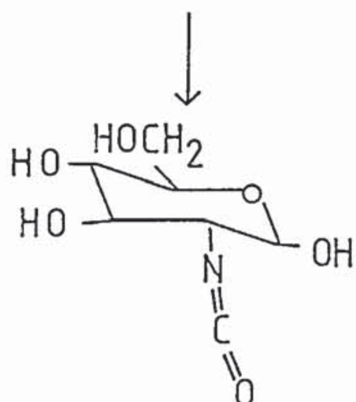
The low carbamoylating activity of chlorozotocin as measured

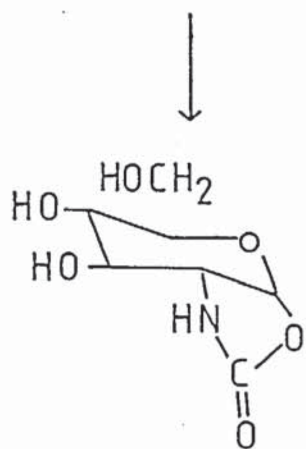
by its ability to react with radiolabelled lysine *in vitro* (Wheeler et al, 1975), is suggested to be a result of the fact that chlorozotocin is capable of carbamoylating itself intra-molecularly (Figure 44)(Montgomery, 1976). The results of chlorozotocin presented in this study (Table 6, Figure 22) raise some doubts about the importance of carbamoylation in the mechanism of action of the nitrosoureas to the TLX5 lymphoma. These results are somewhat equivocal given that doubts exist concerning the correlation between the failure of chlorozotocin to carbamoylate lysine *in vitro* and certain of its biological effects which suggest that it may be capable of carbamoylating cellular constituents (Tew and Wang, 1982). Similar doubts were raised concerning the inactivation of glutathione reductase by the so called "non-carbamoylator" ACNU, an inactivation presumably caused by the formation of an isocyanate (Babson and Reed, 1978). In addition the inactivation of chymotrypsin by cyclohexylisocyanate *in vitro* was not affected by the addition of a 70 fold excess of lysine (Babson et al, 1977) Yet the extent to which lysine is carbamoylated *in vitro* by various nitrosoureas is still used as an indication of the carbamoylating ability of these nitrosoureas that are capable of degrading via isocyanates (Wheeler et al, 1975). The modification of lysine *in vitro* would thus appear to be an unsatisfactory method to determine the carbamoylating potential of nitrosoureas.

Various groups of workers have argued that as chlorozotocin has excellent antitumour activity agains the L1210 leukaemia *in vivo* then the mechanism of action of the nitrosoureas is related to its ability to perform alkylation reactions and not carbamoylation



Chlorozotocin





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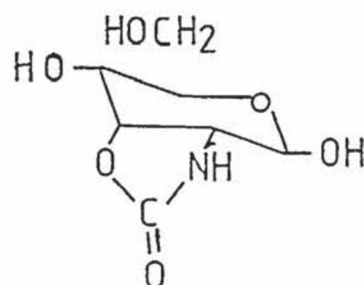

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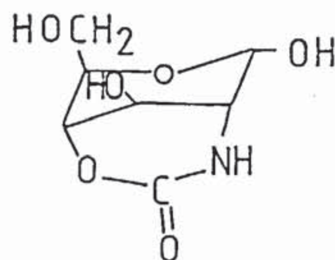


FIGURE 44 Intramolecular carbamoylation of chlorozotocin

reactions (Wheeler et al, 1974; Montgomery, 1976; Erickson et al, 1978c). However, surprisingly in this study chlorozotocin was found to have marginal antitumour activity against the L1210 leukaemia (Table 15). In previous studies chlorozotocin was injected i.p. into the site of tumour innoculation (Montgomery, 1976). In this study chlorozotocin was administered i.p. but the tumour was innoculated s.c. (Section 3.3.). Hence the differences in the extent of the <u>in vivo</u> antitumour activity of chlorozotocin may be related to the site of tumour innoculation. In addition chlorozotocin was found to have no significant antitumour activity <u>in vivo</u> against a C1498 myeloid leukaemia, a TM2 mammary carcinoma, and a B16 melanoma all of these cell lines being innoculated s.c. with chlorozotocin administered i.p. (Hayet et al, 1979). Therefore conclusions that because chlorozotocin has low carbamoylating activity, it is the alkylating activity of the nitrosoureas that is responsible for their antitumour activity are only relevant to the L1210 leukaemia with a particular protocol and care should be applied in extrapolating the results to other cell lines.

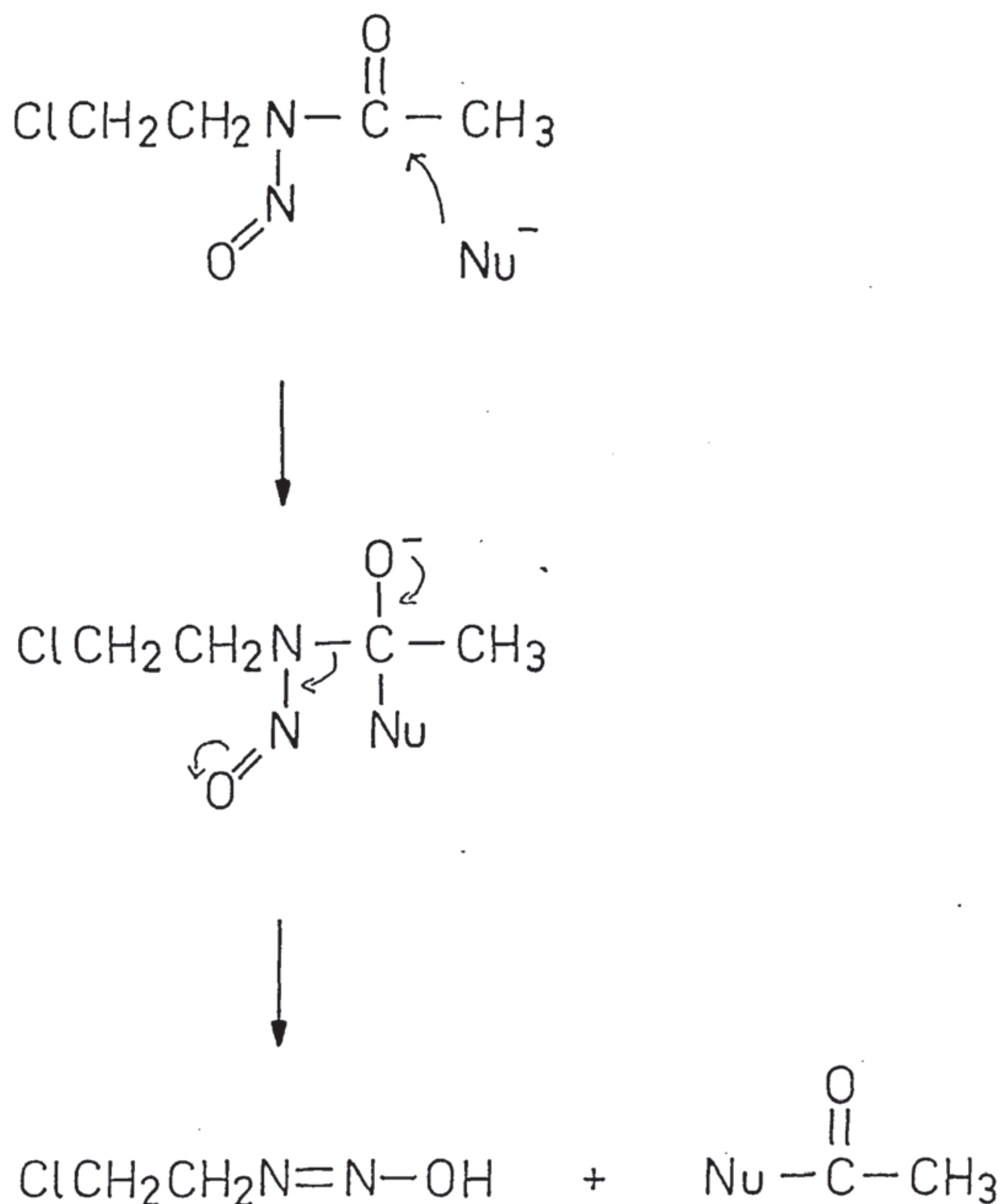
Furthermore chlorozotocin when administered i.p. was found to have no antitumour activity against the Walker 256 carcinoma which had been innoculated s.c., whereas BCNU also administered i.p. showed good <u>in vivo</u> antitumour activity (Fiebig et al, 1980). As both these nitrosoureas decompose to produce a chloroethyldiazohydroxide alkylating species and only BCNU has carbamoylating activity then these results may suggest that the ability of the nitrosoureas to perform carbamoylation reactions may be of importance in their mechanism of action to the Walker 256 carcinoma. Indeed the

importance of the carbamoylation reactions of the nitrosoureas towards the cytotoxicity of the Walker 256 Carcinoma has recently been shown by Tew and Wang (1982)(See Section 1.5.9). Thus the carbamoylating activity of the nitrosoureas may constitute a major element of their cytotoxicity to both the TLX5 lymphoma and the Walker 256 carcinoma.

However, it was still considered relevant to investigate the importance of the haloalkyldiazohydroxide alkylating species, produced upon the decomposition of BCNU and CCNU (section 1.2) in the mechanism of action of these nitrosoureas.

CENA is a compound which has been suggested to decompose to produce a chloroethyldiazohydroxide species (Hecht and Kozarid, 1973; Douglas et al, 1978) as do BCNU and CCNU (Lown et al, 1979). Yet CENA does not decompose to produce an isocyanate (Douglas et al, 1978)(Scheme VI) and has therefore no carbamoylating activity and as such was considered an ideal compound to use in the study of the importance of the chloroethyldiazohydroxide species.

CENA was found to have no _in vivo_ antitumour activity (Table 10) yet was interestingly found to have a greater _in vitro_ cytotoxicity to the TLX5S lymphoma than the TLX5RT lymphoma (Table 19). Thus it would appear the CENA is selectively cytotoxic to the TLX5S lymphoma _in vitro_. This is particularly interesting as it has been argued in this thesis that a compound which is selectively cytotoxic to the sensitive cell line _in vitro_, but not to the resistant line,



SCHEME VI - Postulated mechanism by which CENA could perform acetylation reactions

is likely to be responsible for the selective element of overall cytotoxicity _in vitro_ or antitumour activity _in vivo_. As CENA has no antitumour activity _in vivo_ yet is selectively cytotoxic _in vitro_ then these results contradict the above argument. However, CENA was found to be extremely toxic to the host animal (Table 10) and this may account for the lack of antitumour activity observed. Furthermore the biodistribution of this compound may be different from the nitrosoureas and explain the absence of antitumour activity _in vivo_.

CENA has a half life of 90 minutes at physiological pH and temperature (Hecht and Kozarid, 1973) and the rate of decomposition of CENA is almost twice that of the nitrosoureas BCNU and CCNU (Section 4.5). It may thus be postulated that it is the parent molecule which is selectively cytotoxic _in vitro_ and not the chloroethyldiazohydroxide species produced upon its decomposition. Indeed CENA may be postulated to acetylate nucleophilic sites (Scheme VI) and as such may inactivate the same sites that the isocyanates carbamoylate. Furthermore the toxicity to the host may then be accounted for by the production of the chloroethyldiazohydroxide alkylating species (Scheme VI). If acetylation is responsible for the selective cytotoxicity of CENA _in vitro_ then these results may not be inconsistent with the hypothesis that carbamoylation, like acetylation an acylation reaction, might be important in the expression of the nitrosourea antitumour activity _in vivo_.

This study has shown that the isocyanates themselves and not

their breakdown products are cytotoxic *in vitro* (Figures 14-19). The lack of toxicity of either of the decomposition products of cyclohexylisocyanate, DCyU and cyclohexylamine supports this view (Table 18). However, the isocyanates failed to show any antitumour activity *in vivo* (Table 10), mainly as a result of their toxicity to the host. When compared with CENA this result suggests that carbamoylation and acetylation are very reactive processes. However to achieve a selective toxicity *in vivo* the slow intracellular release of compounds capable of such acylation reactions may be necessary.

To further investigate the role of carbamoylation in the cytotoxicity of the nitrosureas towards the TLX5 lymphoma, compounds which would decompose to an isocyanate species yet have no alkylating activity were investigated. DCyNU produces cyclohexylisocyanate upon its decomposition and has been shown to have no alkylating activity (Wheeler, 1976). This compound was thus considered a good model compound to use in the investigation of the importance of carbamoylation for cytotoxicity to the TLX5 lymphoma.

DCyNU was found to have no significant *in vivo* antitumour activity against the TLX5S lymphoma (Table 11). This result initially suggests that the release of cyclohexylisocyanate is not responsible for the *in vivo* antitumour activity of the nitrosourea CCNU. However, DCyNU was found to be selectively cytotoxic to the TLX5S lymphoma when compared with the TLX5RT lymphoma (Table 18) presumably as a result of the generation of cyclohexylisocyanate.

The lack of *in vivo* activity of this compound may again be related to its distribution *in vivo* which may prevent it attaining a cytotoxic concentration within the tumour cell.

The inhibition of glutathione reductase *in vitro* is an assay which has recently been used to determine the carbamoylating ability of a compound (Eisenbrand, personal communication). The finding that two of the most potent carbamoylators, BCPD and CCI, as predicted by this assay (Eisenbrand, personal communication) were inactive against the TLX5S lymphoma (Table 12) was a surprising result. However these compounds are very unstable in aqueous conditions (Eisenbreand, personal communication) and this fact may help explain their lack of activity *in vivo*. In addition the ability of a compound to inhibit glutathione reductase *in vitro* may however be unrelated to the biological effects these compounds display *in vivo*.

In this study it was decided to explore the development of resistance of the TLX5S lymphoma to cyclohexylisocyanate *in vitro* and to investigate the cross resistance patterns of the derived cell lines *in vivo*. It was hoped that by doing so the importance of cyclohexylisocyanate in the mechanism of action of the nitrosourea CCNU would be clarified.

Two cell lines with resistance to cyclohexylisocyanate were obtained *in vitro* (Tables 21 and 23) and these cell lines were found to be dissimilar in their sensitivity to CCNU *in vivo*. Hence no conclusions about the role of the isocyanate in the mechanism of

action of the nitrosoureas could be made. This strange phenomena is not unique as it has been reported that various attempts to develop resistant cell lines of the Lewis Lung carcinoma to MeCCNU has resulted in several tumour lines which vary widely in their sensitivity to MeCCNU (Peacock et al, 1982). Development of resistance can arise either by selection of cells which survive treatment or by genetic or epigenitic changes induced by the treatment. For instance, resistant L1210 leukaemia cells have been shown to differ in their immunogenicity when compared with their parent L1210 cell line (Mihich and Kitano, 1971). It has also been suggested that such an altered immunogenicity of resistant cell lines may influence the relationship between tumour and host (Mihich, 1969). A third attempt to develop a cell line resistant to cyclohexylisocyanate in vitro resulted in a cell line which lost its tumourogenicity (Table 25). This may be explained by the suggestions, as outlined above, of Mihich (1969).

One of the most widely accepted mechanisms of resistance is that of impaired uptake of the cytotoxic agent into the cell (Hill and Montgomery, 1980). The cross resistance of the TLX5 lymphoma in vitro to the nitrosoureas and isocyanates observed in this study could not be explained by a difference in the cellular transport of the nitrosourea into either TLX5S or TLX5RT lymphoma cells (Figure 25). In addition no significant difference in the distribution of the nitrosourea CCNU was found in the TLX5S and TLX5RB lymphomas by Connors and Hare (1974). Furthermore the resistance of the L1210R leukaemia utilised in this study could not be explained by any difference in the cellular transport of

the nitrosoureas (Figure 26). These results would suggest that the resistance of cell lines used in this study is a result of a mechanism other than impaired drug uptake.

Elevation of non-protein thiols is also a process which has previously been linked to resistance (Connors, 1966; Suzakake et al, 1982). Non-protein thiols such as gluthathione, may inactivate both the alkylating and carbamoylating activity of the nitrosoureas. However, there was no significant difference in the non-protein thiol content when the resistant and sensitive cell lines used in this study were investigated (Table 27).

The rates of decomposition and the chemical half lives of the nitrosoureas used in this study have been investigated in R.P.M.I. media (Section 4.5). The half lives of the nitrosourea in this media (Table 30) have been found to be similar to those previously reported (Wheeler et al, 1974). Decomposition of the nitrosourea has been shown to be an important process in the generation of cytotoxic species (Section 1.2). Indeed, the <u>in vitro</u> cytotoxicity of four chloroethylnitrosoureas to a 9L brain glioma has been found to be similar when the drug concentration and exposure period are correlated with reference to the rates of decomposition (Weinkam and Deen, 1982). These authors found that exposure of 9L glioma cells to 50 uM BCNU ($t\frac{1}{2}$ = 50mins) for 1 hour would result in equivalent <u>in vitro</u> cytotoxicity as exposure of cells to 50 uM PCNU ($t\frac{1}{2}$ = 25mins) for 30 minutes. The four chloroethylnitrosoureas studied all decompose to produce a chloroethyldiazohydroxide yet

differed in the isocyanate produced. This led to the proposal that it is the chloroethyldiazohydroxide species which is responsible for the _in vitro_ cytotoxicity of these compounds to 9L glioma cells (Weinkam and Deen, 1982).

If this were the case here then one would expect chlorozotocin ($t\frac{1}{2}$ = 25 mins) to have a greater _in vitro_ cytotoxicity to the TLX5S lymphoma than CCNU ($t\frac{1}{2}$ = 60 mins) after a 2 hour drug exposure. This however was not so (cf Figure 15 and 22) and may imply that the chloroethyldiazohydroxide species is not responsible for the _in vitro_ cytotoxocity of these nitrosoureas to the TLX5S lymphoma.

The results discussed so far indicate that isocyanates play a major role in the mechanism of action of the nitrosoureas towards the TLX5 lymphoma. This hypothesis was strengthened by the findings that the TLX5RT lymphoma, a cell line never previously exposed to the nitrosoureas, was cross resistant to both the nitrosoureas and isocyanates (Section 4.2.2). A further test of this hypothesis and a logical conseqence of these findings was the screening of novel molecules which potentially release an isocyanate moiety but not a haloalkyldiazohydroxide.

A study conducted in our laboratories, in parallel with the pharmacological work described here dealt with the chemical synthesis and properties of imidazo [5,1-d] [1,2,3,5] tetrazin - 4(3H)-ones. The synthesis was achieved by the reaction of diazo-I.C. with an aryl or alkylisocyanate (Stone, 1981). Of particular interest was the

synthesis of 8-carbamoyl-3-(2-chloroethyl)imidazo [5, 1-d] [1,2,3,5] tetrazin - 4(3H) - one (azolastone)(Figure 30). This synthesis was accomplished by reaction of diazo-I.C. with chloroethylisocyanate (Scheme VII).

The finding that azolastone showed good _in vivo_ antitumour activity to the TLX5S lymphoma (Table 31) was a very exciting result. The activity of azolastone to this cell line was found to be similar to that of the nitrosoureas BCNU and CCNU (of Tables 2 and 3). The TLX5RT lymphoma was cross resistant to both the nitrosoureas BCNU and CCNU (Tables 2 and 3) and azolastone (Table 31). If the release of isocyanates from azolastone was important for its antitumour activity _in vivo_ then azolastone might not be expected to be active against the L1210S leukaemia. However, azolastone was found to have good antitumour activity against the L1210S leukaemia (Table 31). Again its activity was found to be similar to that of BCNU and CCNU (Tables 13 and 14). In addition, an L1210R leukaemia with induced resistance to BCNU _in vivo_, was also resistant to azolastone _in vivo_ (Table 31). The fact that cyclophosphamide had similar _in vivo_ antitumour activity to the L1210S and L1210R leukaemias may suggest the release of isocyanates from nitrosoureas and their possible release from azolastone may be more important in the mechanism of action of these agents to the L1210 leukaemia than previously thought. The results obtained in this study show a strong similarity to the spectrum of activity of azolastone and the nitrosoureas.

Independent screening of azolastone at Rhone - Poulene, France, has also shown that azolastone has significant level of activity against the L1210S leukamia. Administration of 10mg/kg azolastone

SCHEME VII - Synthesis and possible decomposition pathway of azolastone (Taken from Stone, 1981)

on days 1,2,3 and 4 after tumour innoculation resulted in 10 out of 10 survivors on day 35. Activity was also observed against a cyclophospha mide-resistant L1210 cell line with 10mg/kg/day of azolastone resulting in 9 out of 10 survivors on day 35.

It is of interest to note that both the cyclophosphamide - resistant L1210 leukaemia and the TLX5S lymphoma are all cell lines which are resistant to alkylating agents of the 2-chloroethylamine type but are sensitive to both nitrosoureas and azolastone. Recent screening studies have shown that azolastone has good in vivo antitumour activity against the P388 leukaemia, M5076 sarcoma, and the Lewis Lung carcinoma. Azolastone has also been found to have antitumour activity against B16 melanoma and a mouse colon 38 cell line (Atassi, personal communication). Nitrosoureas have also been shown to have activity against the majority of these cell lines (Schabel, 1976). These results also indicate the possibility of a link between the mechanism of action of the nitrosoureas and azolastone.

The therapeutic index of azolastone against the M5076 sarcoma is equivalent to that of cyclophosphamide (Hickman et al, unpublished observations). Furthermore azolastone has far superior activity against the Lewis Lung carcinoma than MeCCNU when compared under identical conditions (Hickman et al, unpublished observations). In view of the fact that MeCCNU was previously considered the most active compound tested against the Lewis Lung carcinoma (Carter et al, 1972) and the relevance of the Lewis Lung carcinoma in

predicting compounds for use in human solid tumours (Venditti, 1977) suggests that azolastone rates among the most potent compounds screened for potential use in man.

Studies to investigate the mechanism of cytotoxic action of azolastone were undertaken as part of this work (see Section 4.6).

The lack of antitumour activity of diazo-I.C. (Table 33) suggests that this potential decomposition product (see Scheme VII) may be of no importance in the antitumour activity of azolastone. Further evidence in support of this suggestion is the finding that azolastone has a greater cytotoxicity to the TLX5S lymphoma than the TLX5RT lymphoma *in vitro* (Figure 31) whereas diazo-I.C. has equal cytotoxicity to both these cell lines *in vitro* (Figure 31).

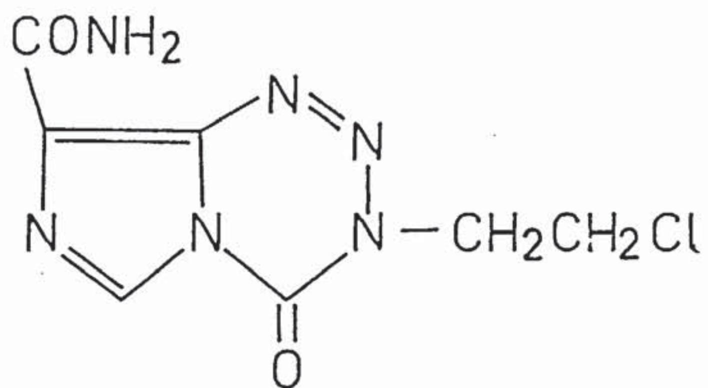
It has previously been shown in this study that chloroethylisocyanate is more cytotoxic to the TLX5S lymphoma than the TLX5RT lymphoma *in vitro* (Figure 14). Therefore it may be suggested that the similarity in the *in vitro* cytotoxicity to these cell lines observed with azolastone may be related to its presumed ability to produce chloroethylisocyanate upon decomposition. It is worth noting that this decomposition has not been verified for azolastone under physiological conditions.

As previously suggested in this thesis the cross resistance of the TLX5RT lymphoma to the dimethyltriazenes and nitrosoureas may indicate some common mechanism in the expression of their antitumour activity. In addition the fact that this cell line is also resistant

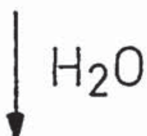
to azolastone (Table 31) may suggest that all three classes of compound have some common link in their mechanism of action. Further evidence in support of this view is that the methyl analogue of azolastone, 8-carbamoyl-3-methylimidazo[5,1-d][1,2,3,5]tetrazin-4(3H)-one (methazolastone, Figure 30)(Table 32), a momomethyltriazene (Gescher et al, 1981) and 1-methyl-1-nitrosourea (MNU) (Connors and Hare, 1975) are all active antitumour agents against the TLX5S lymphoma. It is worth noting that none of these compounds would be expected to cross link DNA. Indeed no DNA-DNA interstrand cross links were observed with MNU (Kohn, 1977).

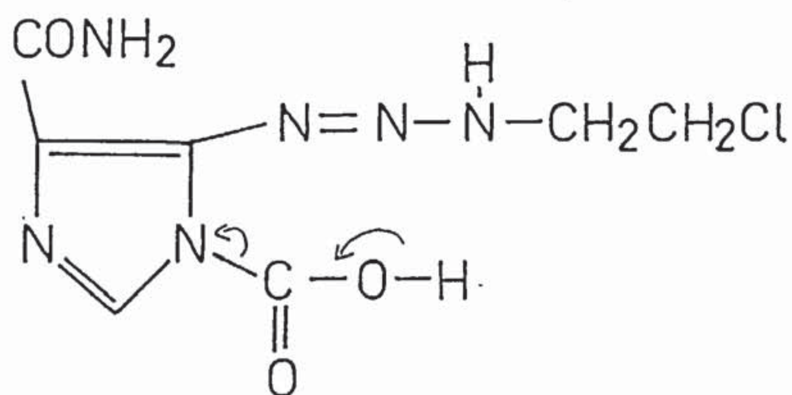
As well as decomposing to produce diazo-I.C. and probably chloroethylisocyanate (Scheme VII), azolastone in aqueous conditions could be postulated to decompose to produce 5-[3-(2-chloroethyl)triazen-1-yl]imidazo-4-carboxamide (MCTIC, Figure 30) and carbon dioxide (Scheme VIII). MCTIC was found to be inactive against the TLX5S lymphoma <u>in vivo</u> (Table 33) yet MCTIC has been reported to have good activity against the L1210S leukaemia <u>in vivo</u> (Shealy et al, 1975). These results may suggest that MCTIC is not involved in the expression of the antitumour activity of azolastone to the TLX5S lymphoma but may be involved against the L1210 leukaemia. However, the sites of tumour innoculation in this study differ from those of Shealy et al, (1975) and as such these results are not strictly comparable.

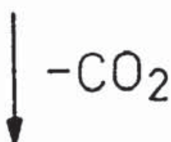
Another compound which is structurally related to both azolastone and MCTIC is 5-[3,3-bis(2-chloroethyl)triazen-1-yl]imidazo-4-carboxamide

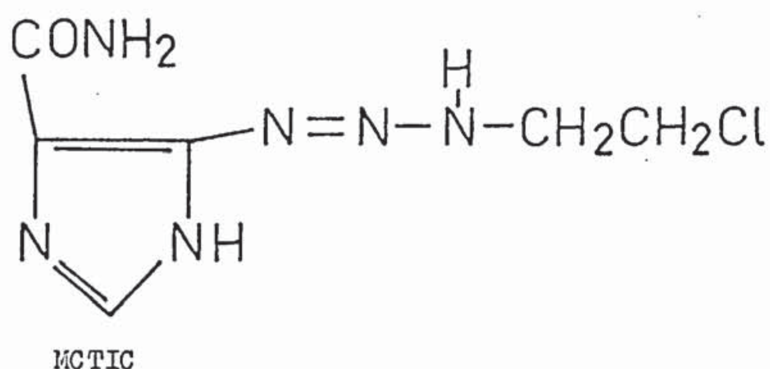


AZOLASTONE









MCTIC

SCHEME VIII - Postulated decomposition pathway of azolastone in aqueous conditions

(BCTIC, Figure 30). BCTIC was found to have good *in vivo* antitumour activity against the TLX5S lymphoma (Table 33) as well as the L1210 leukaemia (Shealy et al, 1975). There is no apparent mechanism by which azolastone could form BCTIC and thus their mechanism of antitumour activity would appear unrelated. However, BCTIC can also produce MCTIC upon metabolic dealkylation (Shealy et al, 1975) and thus there may indeed be a possible connection between the *in vivo* antitumour activity of azolastone and BCTIC. The fact that MCTIC is inactive against the TLX5S lymphoma (Table 33) would seem to argue against the possibility that this compound is the active species produced upon the decomposition of either azolastone or BCTIC.

As the majority of studies to investigate the mechanism of action of azolastone would utilise *in vitro* assays it was considered relevant to investigate the effect of the presence of a hepatic metabolising system upon the *in vitro* cytotoxicity of azolastone to the TLX5S lymphoma. The failure to influence the cytotoxicity of azolastone by the addition of a liver homogenate fraction in the *in vitro* - *in vivo* cytotoxicity assay (Table 34) suggests that azolastone is directly cytotoxic and does not require metabolic activation. The slight reduction in cytotoxicity seen upon the addition of a liver homogenate (Table 34) may be due to non-selective reaction of liver proteins with azolastone. This type of effect has previously been reported in the attempted metabolic activation of a monomethyltriazene (Gescher et al, 1981).

Adriamycin, bleomycin, mitomycin-C, cis-platinum, haloalkylnitrosoureas and the nitrogen mustard type alkylating agents are all

antitumour agents which have the potential to react with DNA (for review see Kohn, 1979). Indeed alkylating agents of the nitrogen mustard type are thought to kill cells by cross linking the paired DNA strands (Kohn etal, 1966). Using the alkaline elution technique (see Section 1.5.4) the reaction of azolastone and related compounds with DNA was investigated (Section 4.6.5).

Alkaline elution experiments appear to distinguish between four types of DNA strand lesions produced in mammalian cells (Figure 8, Section 1.5.4): DNA strand breaks, alkali-labile lesions, DNA-DNA interstrand cross links and DNA-protein cross links.

DNA strand breaks and alkali-labile sites have both been shown to increase the rate of DNA elution (Kohn, 1979). Alkali-labile sites would continuously generate breaks under the alkaline conditions of this assay ;and cause the rate of elution of DNA to increase with time. Kinetics of this type have previously been shown with MNU (Ewig and Kohn, 1977). MNU would be expected to produce alkali-labile sites both in the form of apurinic sites (Verly, 1974) and in the form of alkylated phosphates (Shooter, 1976).

In this investigation the reaction of azolastone, MCTIC (Figure 30), chloroethylisocyanate and CNU with DNA were studied. MCTIC and chloroethylisocyanate were chosen as model compounds particularly in view of their possible formation in the decomposition of azolastone (Schemes VII and VIII). CNU was chosen as a model compound as the previous findings have indicated that azolastone and the haloalkynitrosoureas may have some common link in their mechanism of action.

An increase in the rate of elution of DNA was observed with azolastone, MCTIC, CNU and chloroethylisocyanate (Figures 34-37). In the case of azolastone, MCTIC, and CNU these increases were relatively small (Figures 34-36), so that it was not possible to distinguish between alkali-labile lesions and DNA strand breaks.

Chloroethylisocyanate, however, produced very large increases in the rate of elution of DNA (Figure 37), which in profile were dissimilar to ionising radiation (Figure 37). The effect of X-rays on DNA elution has previously been shown to be first order with respect to time and that the distribution of breaks caused by X-rays suggested to be random (Kohn et al, 1976). A comparison of the elution curves of ionizing radiation and chloroethylisocyanate shown in Figure 37 suggests that the distribution of breaks caused by the isocyanate is non-random. It is particularly interesting that the concentrations of chloroethylisocyanate required to produce the large quantities of DNA damage are those which result in less than a 2 log cell kill (Figure 33). The biphasic nature of the elution curves after treatment with the isocyanate are consistent with the possibility that the DNA of these treated cells consists of a mixture of undamaged and severely damaged components. This result would be expected if the cells were heterogenous in sensitivity to the isocyanate. This type of phenomenon has previously been reported for bleomycin (Iqbal et al, 1976). It is also known that other cell populations have been observed to be made up of sensitive and resistant components (Drewinko et al, 1972).

However, one major difference between the effects seen in this

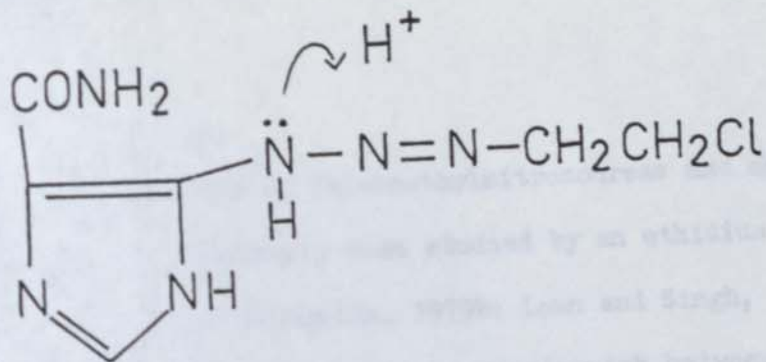
study with the isocyanate and those previously observed with bleomycin is that the strand breaks caused by the isocyanate were observed in the presence of proteinase-K whilst those of bleomycin were not. Single strand breaks caused by adriamycin only occur in the presence of proteinase-K which was suggested to indicate that these breaks are associated with protein (Zwelling et al, 1981). Although this may be the case with the isocyanates its ability to cause strand breaks in the absence of proteinase-K was not determined. However, the fact that isocyanates would be expected to preferentially bind with proteins rather than DNA (Cheng et al, 1972) may support the idea that chloroethylisocyanate derived strand breaks are protein associated. Furthermore, chloroethylisocyanate at appreciably higher concentrations than those used in this study has been shown to produce a limited number of DNA strand breaks in V79 chinese hamster cells. The production of these breaks was proposed to involve interaction of the isocyanate with some nucleases which would result in the enzymatic cleavage of DNA (Erickson et al, 1978b). The importance of these DNA strand breaks in the cytotoxicity of the isocyanates would appear minimal. Particularly as 25uM chloroethylisocyanate produces less than a 2 log cell kill of L1210 leukaemia cells (Figure 33) yet is capable of large quantities of DNA damage (Figure 37).

DNA-DNA inter strand cross linking by azolastone, MCTIC and CNU was assayed by exposing cells to 3Gy of X-ray, this has previously been estimated to produce 2.7 single strand breaks per 10^7 nucleotides (about 5000 single strand breaks per L1210 cell)(Kohn et al, 1976).

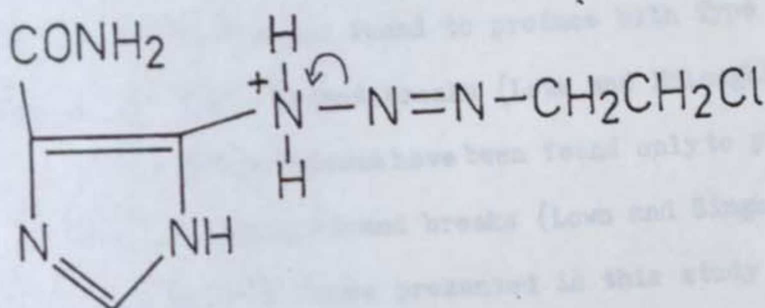
DNA-DNA interstrand cross links are defined as lesions that inhibit the increase in DNA elution normally produced by this standard X-ray dose (Kohn et al, 1976). This effect was most clearly seen with CNU (Figure 34), which produced cross linking without significant strand breakage effects. The lack of strand breaks observed with CNU (Figure 34) was another reason why this nitrosourea had been chosen in this study as strand breaks have been observed to mask the appearance of DNA-DNA interstrand cross links (Kohn, 1979).

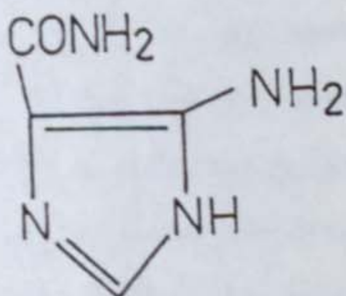
A small inhibition of the increased elution of cells exposed to 3Gy was seen with MCTIC and azolastone (Figures 35 and 36). The DNA interstrand cross link index of CNU was found to be 2 fold greater than those of azolastone and MCTIC at equitoxic concentrations (Section 4.6.4). The cross linking effect of CNU was found to increase for at least 6 - 9 hours after drug treatment. Such delayed increases in cross linking were also observed with other haloalkylnitrosoureas against a variety of cell lines (Erickson et al, 1978a).

The dissimilarity in the reaction of CNU and MCTIC with DNA as measured by alkaline elution (cf Figures 34 and 36) was a particularly surprising result. Studies on the decomposition of chloroethylnitrosoureas (Lown et al, 1979) (see Scheme III) and MCTIC (Shealy et al, 1975) (see Scheme IX) have suggested that both these compounds decompose to produce a chloroethyldiazohydroxide species. The results in this study would seem to indicate that if this is so these two compounds, although producing the same reactive species upon decomposition, react with DNA by a different mechanism.



MCTIC





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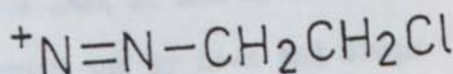
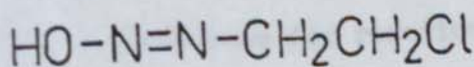
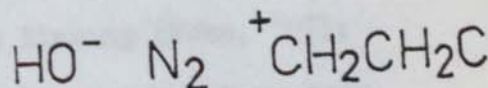




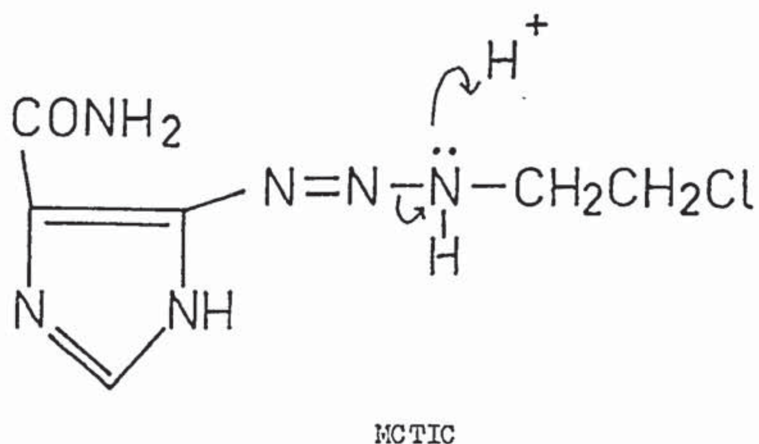
CHLOROETHYLDIAZOHYDROXIDE



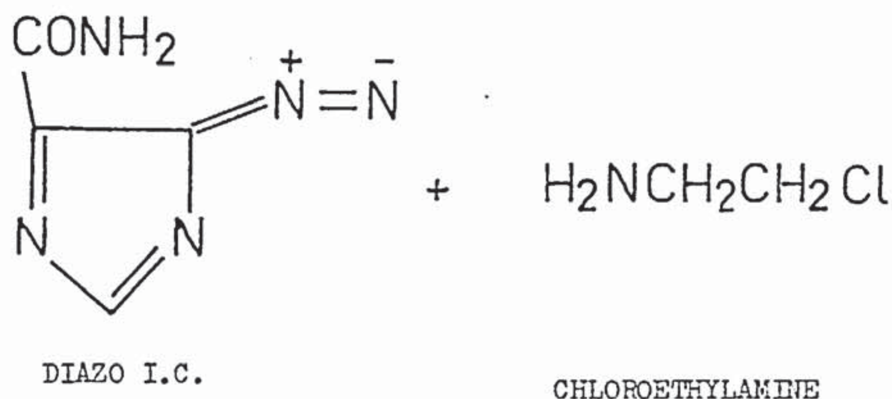
SCHEME IX – Proposed decomposition pathway of MCTIC to produce a chloroethyldiazohydroxide species (Adapted from Shealy et al, 1975)

The reaction of chloroethylnitrosoureas and chloroethyltriazenes with DNA has previously been studied by an ethidium fluorescance assay (Lown and McLaughlin, 1979b; Lown and Singh, 1982). The ethidium fluorescance assay can distinguish between two types of single strand breaks; Type I occurs relatively fast at high pH and is usually associated with alkylation of phosphate groups, and Type II which is a much slower process and is associated with alkylation of DNA bases (Lown and Mclaughlin, 1979b). Reaction of chloroethylnitrosoureas with DNA was found to produce both Type I and Type II mechanisms of single strand breaks (Lown and Mclaughlin, 1979b). Whereas chloreothyltriazenes have been found only to produce a Type II mechanism of single strand breaks (Lown and Singh, 1982). These results together with those presented in this study would suggest that CNU and MCTIC react with DNA by dissimilar mechanisms.

Further evidence obtained with the chloroethyltriazenes suggested that these compounds do not cross link DNA (Lown and Singh, 1982). One explanation for this lack of DNA interstrand cross linking observed both in this study and by Lown and Singh (1982) is that the monoalkyltriazenes have been suggested to decompose preferentially under acidic conditions (Vaughan and Stevens, 1978) and as such would not be expected to produce a chloroethyldiazohydroxide alkylating species (Scheme X). Hence the triazenes may show a preference for reaction at the more acidic phosphate sites on DNA (Lown and Singh, 1982). However, it is the chloroethylation of the DNA bases by the nitrosoureas which result in DNA-DNA interstrand cross linking (Kohn, 1977; Lown et al, 1978). Hence as the chloroethyltriazenes have been



MCTIC



DIAZO I.C. CHLOROETHYLAMINE

<u>SCHEME X</u> - Acid catalysed decomposition of MCTIC

shown to alkylate DNA bases, as indicated by their production of Type II DNA strand breaks (Lown and Singh, 1982) then one might expect them to form DNA-DNA interstrand cross links. Further work to clarify the reactions of the chloroethyltriazenes with DNA is required.

The results presented in this study show that CNU and MCTIC appear to react with DNA by a different mechanism (Figure 34 and 36) at equitoxic concentrations (Section 4.6.4). It could further be argued that this may suggest that CNU and MCTIC are cytotoxic to L1210 cells <u>in vitro</u> by a different mechanism. The similarity in the elution profiles of both azolastone and MCTIC (Figures 35 and 36) may indicate that these two compounds react with DNA in an analogous manner. Furthermore it may be suggested that their cytotoxicity may be mediated by a common mechanism. This is further supported by the fact that azolastone may decompose to produce MCTIC in aqueous conditions (Scheme VIII).

DNA-protein cross linking by azolastone and CNU was assayed by exposing the cells to 30Gy of X-ray. DNA-protein cross links are defined as lesions that inhibit the increase in DNA elution associated with the 30Gy X-ray dose. CNU again showed this effect most clearly (Figures 40 and 41) although azolastone did display some DNA-protein cross linking (Figures 42 and 43) especially after 6 hours drug free incubation. As no data was obtained for MCTIC any conclusions which may be reached regarding the difference in mechanism between CNU and azolastone remains as yet uncertain. However, it would appear that the kinetics of the formation of DNA-protein cross

links after drug removal was greatest for azolastone although the relevance of this finding is unknown.

An overall view of the alkaline elution data obtained in this study (Section 4.6.5) would suggest that at equitoxic concentrations both MCTIC and azolastone are less reactive than CNU towards DNA. If DNA is the critical target for these compounds then it would appear that unlike CNU the quantity of DNA-DNA interstrand cross links may be relatively unimportant in terms of cytotoxicity against the L1210 leukaemia *in vitro*. It is generally proposed that guanine - O^6 position is first alkylated and that the cross link is completed by a reaction with an adjacent cytosine amino group (Kohn, 1977). In the case of CNU this type of cross link may predominate but in doing so may mask a more critical kind of cross link, one that azolastone and MCTIC may produce exclusively in their limited reaction with DNA.

L1210 cells have been shown to survive concentrations of nitrogen mustard that are capable of producing several hundred cross links per cell (Ewig and Kohn, 1977). In addition the DNA interstrand cross link indices observed in this study (Section 4.6.5) are very low in comparison to those obtained in other studies (Erickson et al, 1980b). In a study of the DNA cross linking of human tumour cells treated with concentrations of CNU that produced less than a one log cell kill resulted in a DNA cross link indices of 0.080 or less (Erickson et al, 1980b). In this study the DNA interstrand cross link index of all compounds, at concentrations that produced at least a two log cell kill, were 0.064 or less (Section 4.6.5). Thus

the cells ability to recover proliferative capacity, particularly in the study of Erickson et al (1980b), after such DNA damage suggest that the quantity of cross links produced by MCTIC and azolastone is insufficient to be lethal to the cell. Indeed unless the quality of cross links is more crucial to the cells survival than the quantity, it appears that both azolastone and MCTIC may exert their cytotoxic effect through some mechanism other than DNA-DNA interstrand cross linking. This may also be true for the haloalkylnitrosoureas especially if the lethal expression of a cytotoxic lesion before repair prevents the cell from repairing other less lethal lesions, such as DNA interstrand cross linking. Thus DNA interstrand cross linking may just show the degree of reactivity of a compound to DNA and secondly the ability of such a compound to cross link DNA after an initial alkylation and not much more.

In conclusion the results presented in this thesis are the first to show that in the case of the TLX5 lymphoma the nitrosoureas, BCNU and CCNU are cross resistant with their derived isocyanates, chloroethylisocyanate and cyclohexylisocyanate, in vitro. In particular this is the case against alkylating agent insensitive murine tumours and not in cell lines that are sensitive to alkylating agents. It thus appears that in the investigations into the mechanism of action of cytotoxic agents the choice of the tumour types studied may lead to broader hypothesis concerning their mechanism of action.

Finally the new antitumour agent azolastone was found to have

a spectrum of *in vivo* antitumour activity and *in vitro* cytotoxicity similar to that of the nitrosoureas. However, from the data presented in this study, it appears that azolastone behaves more like a chloroethyltriazene, especially in their reaction towards DNA. More recent work in our laboratories further suggests that azolastone and the nitrosoureas differ in their mechanism of action. BCNU was found to inhibit DNA and RNA synthesis *in vitro* whereas azolastone at an equitoxic concentration had no effect. In addition the *in vitro* inhibition of glutathione reductase and chymotrypsin was found to be specific for the nitrosoureas and not observed with azolastone (Horgan, personal communication). Thus the mechanism of action of the nitrosoureas and azolastone would appear unrelated.

The original observations which stimulated this study were the findings that a cell line with resistance induced to a dimethyltriazene *in vivo* was cross resistant to the nitrosoureas. The cross resistance of this cell line to both the nitrosoureas and isocyanates led to the development of one of the most exciting antitumour agents discovered this decade. Azolastone is scheduled for clinical trial in man late in 1982.

APPENDIX 1

Formula of R.P.M.I. colourless media

	Mg/Litre
$Ca(NO_3)_2 \cdot 4H_2O$	100
Glucose	2000
$MgSO_4 \cdot 7H_2O$	100
KC1	400
$Na_2HPO_4 \cdot 7H_2O$	1512
NaCl	6000
L-Arginine (free base)	200
L-Asparigine	50
L-Aspartic Acid	20
L-Cystine	50
L-Glutamic Acid	20
L-Glutamine	300
Glutathione (reduced)	1
Glycine	10
L-Histidine (free base)	15
L-Hydroxyproline	20
L-Isoleucine (Allo free)	50
L-Leucine (Methionine free)	50
L-Lysine HC1	40
L-Methionine	15
L-Phenylalanine	15
L-Proline (Hydroxy-L-proline free)	20

	Mg/Litre
L-Serine	30
L-Threonine (Allo free)	20
L-Tryptophane	5
L-Tyrosine	20
L-Valine	20
Choline Cl	3
i-inositol	35
$NaHCO_3$	2000
with Hepes	25mM

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